Quantification of cDNA on GMR biosensor array towards point-of-care gene expression analysis

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1. Introduction

Many gene expression profiles have been used to detect the presence and monitor the progression of diseases, such as cardiovascular disease (Elashoff et al., 2011), tuberculosis (Maertzdorf et al., 2016), and influenza (Andres-Terre et al., 2015). These signatures measure gene regulation compared to the healthy state, and involve the simultaneous measurement of both high and low abundance transcripts. Therefore, there is a need for diagnostic technologies that can accurately quantify transcript concentration for a particular gene in real-time.

The gold standard for gene expression analysis is quantitative reverse transcription PCR (RT-qPCR), in which amplification is measured in real-time from an RNA sample of interest. However, qPCR is limited in multiplexing capabilities, as only a certain amount of filters are present to detect different fluorophore fluorescence (Dobnik et al., 2016). More recently, next generation sequencing (NGS) has become a good discovery tool to search for potential gene targets, the high cost, prevalent for high throughput gene expression analysis. While this is a good discovery tool to search for potential gene targets, the high cost, complex equipment, and time to get results render it impractical for rapid and targeted differential expression analysis for a limited number of genes (Arts et al., 2017).

We propose giant magnetoresistive (GMR) sensors as a targeted gene expression analysis platform. Previously, these devices have been shown to detect proteins, like cancer biomarkers (Gaster et al., 2009), autoantibodies (Lee et al., 2016), and common allergens (Ng et al., 2016), with high sensitivity and specificity. They have also been utilized for DNA detection (Xu et al., 2008), and have been well characterized for simultaneous mutation and methylation analysis (Rizzi et al., 2017a). GMR sensors function through localized proximity magnetic sensing. Magnetic nanoparticles can be used as tags on DNA to generate a magnetic field that is detected by the GMR sensor; in this way, binding of DNA to the sensor surface can be detected in real time (Osterfeld et al., 2008). Advantages of magnetic sensing compared to traditional optical biomolecule detection include a lower limit of detection, higher dynamic range, temperature insensitivity, and lower background levels in biological samples (Xu et al., 2008; Rizzi et al., 2017b). Importantly, because the GMR chip has 80 different sensors, the device is more robust to multiplexing than other platforms.
Gene expression analysis using GMR sensors relies on hybridization-based detection, in which GMR chips are spotted with single strand DNA (ssDNA) probes complementary to the gene of interest. Total mRNA is isolated and reverse transcribed with primers corresponding to the gene of interest to create double-stranded cDNA. Upon denaturation, the single-stranded cDNA is applied to the sensor and allowed to hybridize. In this sense, the GMR platform is quite similar to the microarray for gene expression analysis (Allison et al., 2006), but melt curve analysis can also be performed with the GMR system to further increase hybridization specificity. Moreover, the GMR system has been developed into a portable, low cost device (Choi et al., 2016), enabling clinical diagnostics at the point-of-care. To prevent cross-contamination between samples, the GMR chips are not reused; this is unproblematic because GMR chips are relatively inexpensive (each chip is roughly $2–3).

It has been shown that the GMR sensor platform has a dynamic detection range of 40 pM to 40 nM for non-amplified synthetic ssDNA (Rizzi et al., 2017b). However, there has not been extensive characterization of the sensitivity, dynamic range, and quantification accuracy of PCR-amplified DNA on the GMR for varying PCR cycle numbers. Coupled with the multiplexing capabilities of the GMR, this work is important to ensure that multiple transcripts can simultaneously be detected and quantified in a broad concentration range. In this work, we have characterized the GMR sensor platform for cDNA detection and quantification. We have demonstrated that the GMR is a strong endpoint detection technology to measure levels of cDNA, and has the potential to be applied to detect disease signatures at the point-of-care.

2. Material and methods

2.1. RNA extraction and reverse transcription

HeLa cells (line S3) were seeded into 10 cm dishes around 50–60% confluency. Afterwards, HeLa cell mRNA was extracted using the Qiagen RNeasy Mini Kit according to their protocol. cDNA was synthesized using Invitrogen’s Superscript III First Strand Synthesis System according to their protocol.

2.2. qPCR amplification

Prior to GMR detection, HeLa cDNA was qPCR-amplified (BioRad CFX96 Real-Time System) with primers spanning two GAPDH exons. We chose GAPDH expression as a test case, since it is a common reference gene for qPCR (Kozera and Rapacz, 2013). A synthetic target ssDNA was used as a standard to calibrate the detection. The sequence of the standard corresponded to the amplicon obtained from GAPDH primers, originally in a 100 μM stock diluted to a 100 nM solution. All sequences can be found in Supplementary material Table S1 (Integrated DNA Technologies). The primers had a stock concentration of 100 μM and were diluted to 10 μM prior to use. Sso Advanced Universal SYBR Grn Suprrix (Bio-rad) was used for fluorescence detection, and a master mix was created with 1:5 dilution of Supermix to primers. The total volume of each qPCR reaction was 10 μL, with 1 μL of target DNA, 7 μL of master mix, and 2 μL of DNA suspension buffer (Teknova). PCR amplification was initiated with polymerase activation and DNA denaturation at 95 °C for 30 s, followed by 40 cycles with denaturation at 95 °C for 10 s, and annealing and extension at 61 °C for 30 s. A melting ramp was performed (65–95 °C) at the end of the 40 cycles to assess primer specificity.
2.3. qPCR standard curve

A dilution series of a synthetic target ssDNA with sequence corresponding to the amplicon obtained from GAPDH primers was measured with qPCR as described above. ssDNA was diluted in a 10-fold dilution series, from 10 nM to 0.1 pM. Each subsequent concentration was amplified to saturation through a 40 cycle PCR reaction. Samples for each concentration were run in duplicate, and each intensity value on the curve was normalized to the intensity value at 40 cycles and averaged. The Cq values of the standard dilutions were extracted and plotted to create a standard curve using Bio-Rad CFX Maestro software.

2.4. GMR sensor preparation

The GMR biosensor arrays comprised of 8 × 10 sensors were fabricated as described previously (Osterfeld et al., 2008). The sensors are functionalized with amino-modified DNA probes using a surface silanization. Briefly, the surface was activated with a 15-min treatment with 15% Hydrogen Peroxide (Certified ACS, Sigma-Aldrich) in distilled water, 30-min treatment with 10% (3-Aminopropyl) triethoxysilane (Sigma Aldrich) in acetone, 30 min treatment with 5% Glutaraldehyde (Fisher Scientific) in distilled water, and a final wash with distilled water. The amino-modified DNA probes (sequences in Supplementary Table S1) were spotted (~1.5 nL) onto separate sensors of the GMR chip using a robotic arrayer (sciFlexarrayer, Scienion) according to the pattern seen in Fig. 1. Each DNA probe was diluted to 20 µM in 2x saline sodium citrate SSC (Invitrogen) from a stock solution of 20x SSC prior to spotting. A total of 32 sensors were functionalized with the probe complementary to GAPDH, 12 sensors were functionalized with a DNA sequence non-complementary to the PCR amplified product as negative control, and 12 sensors were functionalized with biotinylated DNA as positive control. The chips were stored at room temperature until use. Prior to use, the GMR chips were inserted into cartridges defining a reaction well over the sensors. The chip surface was then washed and blocked with 1% BSA in PBS as described previously (Osterfeld et al., 2008) to prevent non-specific binding.

2.5. Pre-PCR dilution series

The sensor signal vs. PCR product concentration was characterized by measuring a dilution series of PCR product of known concentration. GAPDH standard target was diluted in a 10-fold series dilution from 10 nM to 1 pM. These diluted products were qPCR-amplified in two separate reactions stopped at 15 and 18 cycles respectively. The double stranded DNA products were denatured through a modified heat and shock cooling denaturation approach as described previously (Rizzi et al., 2017b). Briefly, the samples were denatured for 10 min at 95 °C and shock-cooled for 5 min in ice to slow down re-hybridization. 120 µL of each of the denatured samples were injected on to separate chips, and allowed to hybridize to the DNA probes on the chip for 1 h at 37 °C. The chips were then washed twice with washing buffer (10 mM NaCl in Tris EDTA) to remove unbound DNA. After measuring 2 min of baseline signal, 50 µL of streptavidin magnetic nanoparticles (MNPs) (Miltenyi Biotec) were added in the sample well and the binding signal was measured until the GMR signal reached a plateau, indicating binding saturation. This was roughly between 15 and 30 min for each sample.

2.6. GMR signal acquisition

DNA hybridization causes MNPs to bind to the sensor surface that lead to a change in the measured magnetoresistive (MR) ratio of the sensor. The MR ratio was measured as described previously (Rizzi et al., 2017b). Briefly, sensors were biased with a frequency of either f = 480 Hz or 500 Hz, and an alternating magnetic field of amplitude 3 mT with frequency of f₂ = 90 Hz was supplied by an external Helmholtz coil. Upon DNA hybridization, magnetized MNPs generate a field measured at f₁ + f₂. The measured MR ratio is the ratio of signals measured at f₁ + f₂ and f₁. The DNA binding signal mentioned in 2.5 is measured in terms of ΔMR = MR − MR₀, where MR₀ represents the MR ratio before MNP addition. A National Instruments NI-6259 data acquisition card, containing an 18-bit analog to digital converter, digitizes the binding signal and interfaces with LabVIEW to generate readable results (Hall et al., 2010).

3. Results

We aim to assess GMR sensors capabilities for quantifying gene expression by detecting biotinylated GAPDH PCR products after reverse transcription and targeted amplification (Fig. 1). To develop the GMR platform for gene expression analysis, series dilutions of GAPDH synthetic DNA were qPCR-amplified to study the dependency of product concentration on the amplification parameters. Afterwards, PCR and GMR were combined to test GMR detection limits for DNA that was qPCR-amplified at varying cycle numbers, to determine the accuracy of GMR for cDNA quantification.

3.1. PCR amplification for varying initial concentrations (cᵢ) of DNA

A ten-fold dilution series of GAPDH ssDNA standard from 10 nM to 0.1 pM was qPCR-amplified for 40 cycles, along with total cDNA isolated from a HeLa cell line. The normalized intensity (I) values for each initial concentration (cᵢ) was extracted and plotted (Fig. 2A) against

![Image](Image358x119 to 504x227)

**Fig. 2.** A) 40-cycle amplification curves for both GAPDH standard ssDNA diluted down in a 10x series dilution and HeLa cDNA amplified with GAPDH primers. B) The intensity values (y-axis) from the GAPDH standards in (A) were extracted and plotted for respective cycle numbers (x-axis), and fit to sigmoid functions. Both 15 and 18 cycles gave the largest working range of detection (4 orders of magnitude).
synthetic ssDNA was concentration (pre-PCR)  respectively. Each dilution and cycle number condition was run in duplicate. The cell line DNA amplified 15 cycles gave a GMR signal of 805 (± 95) ppm from which we can calculate the initial concentration to be $c_i = 5.3 \pm 0.3$ pM that is in good agreement with qPCR estimated initial concentration of 6.0 ± 0.8 pm (Fig. 3). The dynamic range is roughly 4 orders of magnitude, and the limit of detection is 1 pM.

For 18-cycle amplification, only concentrations from 1 pM to 1 nM were fit linearly, as this was the working dynamic range, and values above and below these concentrations gave saturated GMR signal. The slope is 510 (± 30), and the intercept is 2160 (± 34), with an $R^2$ = 0.95; values in parentheses represent 95% confidence intervals (Supplementary Table S3). The cell line DNA amplified 18 cycles gave a GMR signal of 1100 (± 130) ppm from which we can calculate the initial concentration to be $c_i = 8.3 \pm 4.6$ pM that is in good agreement with qPCR estimated initial concentration of 6.0 ± 0.8 pm (Fig. 3). The dynamic range is roughly 4 orders of magnitude, and the limit of detection is 0.1 pM.

4. Discussion

The GMR platform has potential as a quantitative gene expression analysis tool. First, 15 and 18-cycle synthetic PCR products at various starting concentrations were shown to each have a dynamic range of 4 orders of magnitude on the GMR. The 15-cycle product had a limit of detection can be brought down to 0.1 pM with 18-cycle PCR amplification. More than 6 concentrations were detectable through the fluorescence signal seen in Fig. 4A (15 cycles) and 4B (18 cycles), highlighting the sensitivity of the GMR platform. Second, the qPCR standard curve showed a starting concentration of 6.0 ± 0.8 pm for the HeLa cDNA after fitting to the standard curve (Fig. 3).

6.0 ± 0.8 pm that is in good agreement with qPCR estimated initial concentration to be $c_i = 5.3 \pm 0.3$, pM and 8.3 ± 4.6 pM, respectively. This result emphasizes that the GMR can measure concentration levels with accuracy comparable to the qPCR.

The findings above demonstrate that the GMR platform can be used for endpoint detection to quantify the starting concentration of PCR products. It has previously been shown that the GMR DNA array has a dynamic detection range from 40 pM to 40 nM (Rizzi et al., 2017b). However, this range was established using a synthetic ssDNA sequence with no PCR amplification. To increase the sensitivity of endpoint detection, we have introduced PCR amplification, and have shown that the GMR limit of detection can be brought down to 0.1 pm with 18-cycle PCR amplification (Fig. 4D). PCR amplification is useful to increase GMR sensitivity to low copy numbers, which is beneficial for gene expression analysis, where many transcripts are present in low abundance.

We have shown that PCR amplification provides another degree of freedom (cycle number), which can be tuned to shift the dynamic range of GMR detection. While the low concentration transcripts are better detected on the GMR (1 pM), but provided quantitative detection at high transcript concentrations. These results suggest that 15-cycle amplification

![Fig. 3. qPCR standard curve plotted for varying concentrations of synthetic GAPDH DNA. The data were fit to a linear function, with an efficiency of 77.6%, an $R^2$ value of 0.994, a slope of −4.007, and a y intercept of −27.594. The average concentration of the HeLa cDNA was determined to be 6.0 ± 0.8 pm after fitting to the standard curve.](image-url)
on GMR is better for detection of a broader range of concentrations, whereas 18-cycle amplification on GMR might be more suited to enrich for low concentration transcripts.

Not only is the GMR system advantageous for detection of a broad range of transcript concentrations, but its multiplexing capabilities also allow for detection of many genes in parallel. This provides potential for measurement of a gene expression signature in both transcript detection and quantification.

5. Conclusions

The GMR platform provides a dynamic range of 4 orders of magnitude and a limit of detection of 1 pM and 0.1 pM respectively for 15 and 18-cycle amplified products. Cell-line DNA was shown to have a starting concentration of 6.0 ± 0.8 pM through qPCR standard curve analysis, which was subsequently confirmed with GMR analysis. For the first time, this work shows the characterization and quantification of cDNA on the GMR platform, demonstrating the prospective ability of the GMR to detect multiple mRNA transcripts expressed at different levels. Although the additional step of PCR amplification increases time for analysis, it augments the sensitivity of the GMR bioassay to lower copy numbers. Future work involves amplifying cell line DNA with different primers corresponding to a genetic signature of a particular disease; then transcript concentration can be assessed in multiplex with GMR. Ultimately, the GMR shows potential to be a useful technology in facilitating rapid, point-of-care disease diagnostics.

Acknowledgements

We would like to acknowledge Jared Nesvet for help in qPCR analysis. N.R. acknowledges support from the Stanford Graduate Fellowship. P.C. was supported by National Institutes of Health T32 training grant [2T32AR050942-06A1] and the Novo Nordisk Senior Postdoctoral Fellowship. This work was supported by National Institute of Allergy and Infectious Diseases [R01AI125197] and the Autoimmunity Center of Excellence [U19AI110491].

Disclosures

The authors declare the following competing financial interest(s): S.X.W. have related patents or patent applications assigned to Stanford University and out-licensed for potential commercialization. S.X.W. has stock or stock options in MagArray, Inc., which has licensed relevant patents from Stanford University for commercialization of GMR nano-sensor chips.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bios.2018.09.050.

References


