Electrochemical Branched-DNA Assay for Polymerase Chain Reaction-Free Detection and Quantification of Oncogenes in Messenger RNA

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Electrochemical Branched-DNA Assay for Polymerase Chain Reaction-Free Detection and Quantification of Oncogenes in Messenger RNA

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We describe a novel electrochemical branched-DNA (bDNA) assay for polymerase chain reaction (PCR)-free detection and quantification of *p185 BCR-ABL* leukemia fusion transcripts in the population of messenger ribonucleic acid (mRNA) extracted from cell lines. The bDNA amplifier carrying high loading of alkaline phosphatase (ALP) tracers was used to amplify the target signal. The targets were captured on microplate well surfaces through cooperative sandwich hybridization prior to the labeling of bDNA. The activity of captured ALP was monitored by square-wave voltammetric (SWV) analysis of the electroactive enzymatic product in the presence of 1-napthyl phosphate. The voltammetric characteristics of substrate and enzymatic product as well as the parameters of SWV analysis were systematically optimized. A detection limit of 1 fM (1×10^{-19} mol of target transcripts in 100 μL) and a 3-order-wide dynamic range of target concentration were achieved by the electrochemical bDNA assay. Such limit corresponded to ~17 fg of the *p185 BCR-ABL* fusion transcripts. The specificity and sensitivity of assay enabled direct detection of target transcripts in as little as 4.6 ng of mRNA population without PCR amplification. In combination with the use of a well-quantified standard, the electrochemical bDNA assay was capable of direct use for a PCR-free quantitative analysis of target transcripts in mRNA population. A mean transcript copy number of 62,900/ng of mRNA was determined, which was at least 50-fold higher than that of real-time quantitative PCR (qPCR). The finding was consistent with the underestimation of targets by qPCR reported earlier. In addition, the unique design based on bDNA technology increases the assay specificity as only the *p185 BCR-ABL* fusion transcripts will respond to the detection. The approach thus provides a simple, sensitive, accurate, and quantitative tool alternative to the qPCR for early disease diagnosis.

The Philadelphia chromosome (Ph) is found in ~5−25% of acute lymphoblastic leukemia (ALL) cases.[1] The Ph results from the reciprocal translocation of the long arm of chromosome 9 with the long arm of chromosome 22 producing a unique chimeric fusion gene that causes the expression of the chimeric *BCR-ABL* mRNA. There are many types of *BCR-ABL* variants. The *p185 BCR-ABL* appears in nearly 90% of pediatric cases. In Ph-positive ALL, the unique *BCR-ABL* translocation serves as a specific marker for the detection of early relapse. Quantification of Ph-positive ALL using sensitive molecular diagnostic assays would be useful. However, most of these assays were laboratories-based, labor-intensive, and time-consuming, which could delay the prognosis and decision-making for treatments. Moreover, the concentration of genetic marker is often low in the biological samples from patients with diseases in the early and remission stages. Therefore, an ultrasensitive and simple method for detecting genetic markers is clearly essential for point-of-care (POC) and early disease diagnosis.

The detection of specific ribonucleic acid (RNA) transcripts is widely used to identify the subtypes of disease and to determine the gene expression for monitoring of treatment progress. The conventional methods used to detect the expression levels of particular mRNA present in biological samples are as follows: Northern blots, ribonuclease protection analysis, microarrays, and quantitative polymerase chain reaction (qPCR). These methods are cumbersome, time-consuming, and labor-intensive. Northern blot and ribonuclease protection analysis are qualitative only and generally lack of sensitivity, which require micrograms of total RNA,[2] and therefore are not ideal for clinical diagnosis that has a limited amount of patient samples. Target amplification method such as the PCR is usually used to increase target concentration and therefore requires much less (nanograms of total RNA) starting samples.[2] The qPCR has been developed to quantify mRNA, which involves two enzymatic reactions: 1-step reverse transcription (RT) of mRNA to complementary deoxyribonucleic acid (cDNA) by the reverse transcriptase and multiple-step PCR amplification by DNA polymerase to increase the target amounts. Real-time quantification was made possible by monitoring the signal intensity generated from fluorescent dyes during PCR amplifications. The efficiency of RT reaction and PCR amplifica-

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tions could introduce bias, as any minute error introduced at the early test steps can greatly influence the final yield of the amplified products. Theoretically, such technique can detect a single copy of a gene; however, it is analytically complex and therefore mainly limited to use in research laboratories.

In the efforts to overcome the shortcoming of target amplification techniques, several signal amplification strategies have been developed in the past decades. Signal amplification accomplished by multiple labeling[3−11] is one of the most promising methods that could provide an excellent sensitivity at target concentrations as low as attomoles. The overall sensitivity usually depends on the loading of signaling molecules on the carrier platform. These labeling techniques utilized platforms such as dendrimers,[3] branched DNA (bDNA),[4] microspheres,[5,6] solid-phase carriers,[7−9] and multilayered DNA constructs[12] to carry numerous signaling molecules.

Among the signal amplification approaches, the bDNA technology rapidly became the method of choice for genetic diagnosis since 1990s.[13] In contrast to the target amplification methods, e.g., PCR, the bDNA signal amplification method quantitates target nucleic acids at physiological levels, involving a series of hybridization reactions without thermal cycling. The bDNA technology is also much more specific because the assay format requires 30 or more oligonucleotide probes to specifically bind to the target nucleic acids. The assays combine sandwich hybridization with the use of bDNA and ALP-labeled probe for signal amplification. Owing to the signal amplification effect, it allows direct detection of target gene transcription in the cell lysate samples without nucleic acids purification and PCR amplification. The bDNA technique eliminates the need for the reverse transcription of mRNA to cDNA, which in turn minimizes biases in RNA quantitative analysis. For the past decades, it has been widely used for quantification of various nucleic acid targets, particularly hepatitis virus DNA and RNA[14−16] and human immunodeficiency virus (HIV) RNA.[17−19] The bDNA technique can usually detect 10^4-10^7 copies[14−16] and in some cases below 10^3 copies of the target.[4] Recently, Panomics (Fremont, CA) has developed a new version of the bDNA technology, called QuantiGene 2.0, which increases the signal amplification from 45-fold in the previous version to 400-fold. The sensitivity of QuantiGene 2.0 can reach as low as 100 copies of RNA (10^{-22} mol) from crude lysate or plasma samples.

The ability to rapidly, sensitively, and quantitatively measure the transcriptional levels of target genes is of central importance to disease diagnosis and treatment monitoring. Conventionally, the qPCR has been one of the most common techniques used to determine the levels of target nucleic acids in the samples. In contrast to the bDNA technology, qPCR requires a sophisticated and costly system, which comprise a thermal cycler, an optical detection system, and data analysis software. The qPCR also requires complex data analysis for determining the actual copies of the target gene transcription in the samples. The vast majority of bDNA assays reported to date were performed optically by chemiluminescence[14−19] and fluorescence.[20] Electrochemical genosensing has gained considerable attention and evolved dramatically over the past two decades because it could offer an attractive alternative to optical detection. It has inherent advantages, which can be used in color or turbid media without any interference from the absorbing or fluorescing compounds in the samples. Therefore, the simple and cost-effective detection system with adequate sensitivity is ideally suitable for meeting the portability requirements of decentralized POC testing. Combining the bDNA hybridization assay and electrochemical analysis presents a new avenue to develop a simple mRNA detection and quantitative assay system, which is cost-effective and suitable for routine analysis and POC diagnosis.

To our best knowledge, there is no previous report on the electrochemical bDNA assay. The performance of genosensors is dependent on the size of target nucleic acids. Most of the development of electrochemical genosensors reported so far was demonstrated using short oligonucleotides or targets amplified by the PCR. Only few attempts[21] have been reported for the detection of the target nucleic acids from biological samples. In this work, we present a novel electrochemical bDNA assay for the direct detection and quantification of *p185 BCR-ABL* fusion transcripts in the total mRNA population extracted from positive ALL cell lines SUP-B15 and the negative cell lines HL-60. The PCR-free quantitative analysis of target gene transcripts minimized the bias, which was usually observed in the PCR-based quantitative assays. Therefore, it could offer a new and quick diagnostic test for various clinical diseases.

EXPERIMENTAL SECTION

Reagents. Alkaline phosphatase (ALP, 14 U/mg), 1-napthyl-phosphatase, phosphate-buffered saline (10 mM PBS pH 7.4), Tris-HCl buffer, bovine serum albumin, and sodium azide were purchased from Sigma (St. Louis, MO). The Oligotex Direct mRNA extraction kit, QIAquick gel extraction kit, and Qiagen plasmid mini kit were obtained from Qiagen, Inc. (Valencia, CA). BioMag Plus Streptavidin Particles was purchased from Poly-

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sciences, Inc. (Warrington, PA). The bDNA assay kit (QuantiGene 2.0 Reagent System) and the customized target-specific probe set were purchased from Panomics, Inc. (Fremont, CA). The reagent components in the kit mainly include the capture plate coated with capture probes (CPs), blocking reagent, lysis mixture, probe diluent, wash buffers, components for synthesizing the bDNA complex, and Lumigen APS-5 chemiluminescent substrate. The probe sets consisted of the capture extender primers (CEs), label extender primers (LEs), and blocker primers (BLs). The *p185 BCR-ABL*-positive human leukemic cell line SUP-B15 and negative cell line HL-60, fetal bovine serum (FBS), and Iscove's Modified Dulbecco's Medium (IMDM) were purchased from the American type Culture Collection (ATCC) (Manassas, VA). To avoid RNA degradation by RNases, all buffers or solutions were prepared with nuclease-free water (Ambion, Inc., Austin, TX), while surfaces of laboratory wares were decontaminated with RNase AWAY (Molecular BioProducts, Inc., San Diego, CA). Superscript First-strand Synthesis, Quant-iT dsDNA HS, Quant-iT dsDNA Broad Range, and Quant-iT RNA assay kits were purchased from Invitrogen (Carlsbad, CA). Power SYBR Green PCR Master Mix was obtained from Applied Biosystems (Foster City, CA). Microcon YM-30 Centrifugal filter units were obtained from Milipore (Billerica, MA). The phenol/chloroform solution was purchased from Pierce (Rockford, IL). All other chemicals and reagents are of analytical grade. All stock solutions were prepared using deionized water (18.2 MΩ·cm) purified with the Nanopure system (Kirkland, WA). All oligonucleotide pairs used for reverse transcription-polymerase chain reaction (RT-PCR) and qPCR were synthesized by Sigma-Genosys. The oligonucleotide pair of 5′-GCATGATGGAAGGGGAGGGCAAG-3′ (forward primer) and 5′-biotin-CTTGGAGTTCCAACGAGCGGCTTCA-3′ (reverse primer) was used for RT-PCR and general PCR amplification. The oligonucleotide pair of 5′-ATTGCGGAGGCGGCTATAC-3′ (forward primer) and 5′-CTCGCTGGAGGTGAGGTTCT-3′ (reverse primer) was used for the qPCR.

Instruments. Cyclic voltammetric (CV) and SWV measurements were performed with an electrochemical analyzer μAutolab type III (Eco Chemie B.V.) connected to a personal computer. Disposable screen-printed electrodes (SPEs) consisting of a carbon working electrode, a carbon counter electrode, and a silver/silver chloride reference electrode (Pine Research Instrumentation, Raleigh, NC) were used for electrochemical measurements. The SPEs were electronically connected to the analyzer. Chemiluminescent measurements were carried out with a GENios microplate reader (Tecan). The PCR and qPCR were performed with a Genius Techne PCR thermocycler (Techne Inc., Burlington, NJ) and 7900HT Fast Real-Time PCR System (Applied Biosystems), respectively. A Qubit fluorometer (Invitrogen) was used for DNA and RNA quantification. The NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA) was used to determine the purity of nucleic acids.

Electrochemical Measurements for ALP Enzymes. Stock ALP solution was prepared in 10 mM Tris-HCl (pH 8.0) and stored at 4 °C. The stock substrate solution 1-napthyl phosphate was prepared daily in Tris-HCl buffer at a final concentration of 50 mM. The working substrate solution was diluted from the stock solution with Tris-HCl buffer. The enzymatic product solution was prepared by allowing the reaction of 5 μL of ALP solution and 50 μL of substrate to proceed for 10 min unless otherwise stated. The control solution was prepared identically as for product solution by replacing ALP with Tris-HCl buffer. The CV and SWV measurements were carried out by loading a 50-μL sample solution covering the entire working area of a disposable SPE. All measurements were performed at room temperature.

Cell Cultures and mRNA Extraction. The cell lines SUP-B15 and HL-60 were used as positive and negative cell lines, respectively, for the isolation of *p185 BCR-ABL* fusion transcripts (491-bp target). The cell lines were cultured according to the ATCC's protocol. Briefly, both cell lines were cultured in IMDM medium supplemented with 20% FBS in an atmosphere of 5% CO_2 at 37 °C. Cultures were maintained by replacement of fresh medium for 2 times per week, and the cell density was maintained between 5×10^5 and 2×10^6 cells/mL. The cells were pelleted by centrifugation at 125*g* for 10 min. The mRNA in the pelleted cells was extracted with an Oligotex Direct mRNA extraction kit. The extracted mRNA was stored at −80 °C until used. The concentration of mRNA was determined by measuring the absorbance at 260 nm at pH 7.5 or the fluorescence using Quant-iT RNA assay kit. The purity was estimated by ratio between the absorbance values at 260 and 280 nm at pH 7.5.

Preparation of Sense p185 Single-Strand DNA (p185-ssDNA). *(A) Synthesis of the Biotinylated Double-Stranded DNA (dsDNA).* The total mRNA population was first reversely transcribed to cDNA using the Superscript First-Strand Synthesis kit. Subsequently, the PCR amplification of the 491-bp cDNA fragment of *p185 BCR-ABL* was carried out to produce the biotinylated dsDNA product (see Supporting Information). The following oligonucleotides were used in the PCR reaction: 5′-GCATGATGGAAGGGGAGGGCAAG-3′ (forward primer) and 5′-biotin-CTTGGAGTTCCAACGAGCGGCTTCA-3′ (reverse primer). In this reaction, the 5′-biotinylated primer in reverse direction was used to introduce the biotin at the 5′-end of antisense strand DNA fragment for the separation of sense p185-ssDNA.

(B) Purification of Sense p185-ssDNA. The sense p185-ssDNA was separated from the biotinylated PCR products by magnetic separation (see Supporting Information). The biotinylated PCR products were first immobilized on the BioMag Plus Streptavidin-coated magnetic beads. The nonbiotinylated sense p185-ssDNA was disassociated from the bead-bound antisense strands by elution with hot Tris-HCl buffer. The eluate containing pure and freely suspended sense p185-ssDNA was collected and used as the standard throughout.

(C) Sense p185-ssDNA Quantification by qPCR. In order to accurately quantify the copy number of p185-ssDNA prepared above, the plasmid DNA carrying the same *p185 BCR-ABL* fragments was prepared from *Escherichia coli* cultures. Briefly, the dsDNA prepared above was cloned into the *E. coli* expression vector pGEM-T (Promega, Madison, WI) and transferred into the *E. coli* DH5α cells for plasmid DNA production. The plasmid DNA was purified from overnight *E. coli* culture using a Qiagen Plasmid Mini Kit. Plasmid DNA concentration was measured with the Qubit fluorometer and the Quant-iT dsDNA Broad Range assay kit. The DNA purity was also estimated by absorbance ratio at 260 and 280 nm (A_{260}/A_{280}) using a NanoDrop ND-1000 spectrophotometer. In each qPCR run, the purified plasmid DNA was diluted in a 5-fold dilution series with the concentrations of 2400,

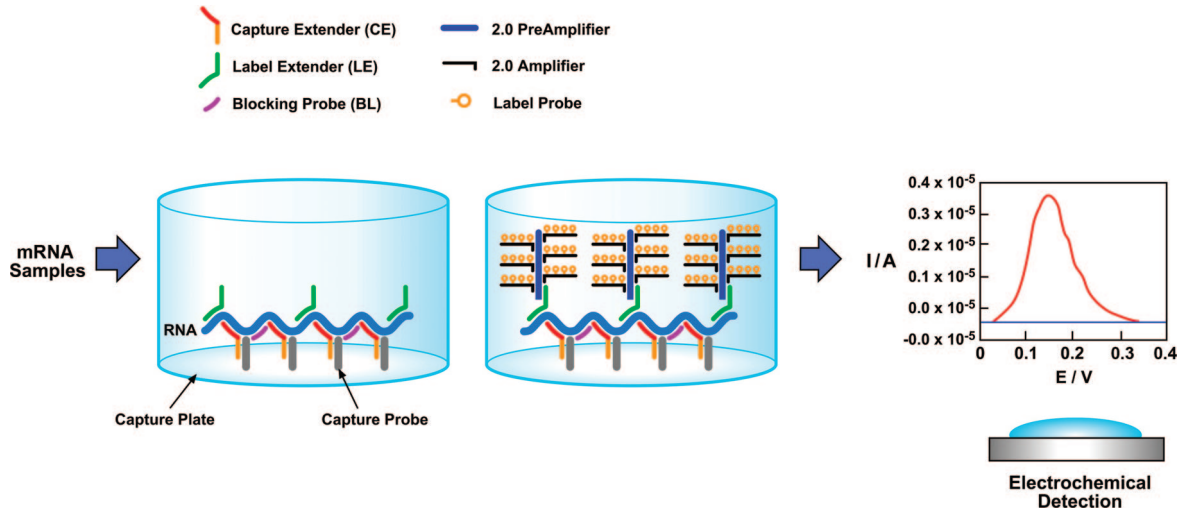

Figure 1. Schematic illustration of the branched DNA (bDNA)-amplified electrochemical detection and quantification of human leukemia *p185 BCR-ABL* mRNA fusion transcripts.

12 000, 60 000, and 300 000 copies/μL, which were used for generation of a standard curve [i.e., known copy number of plasmid DNA versus cycle threshold (C_T) value]. No template control (NTC), i.e., negative control without target DNA, was included as negative control. Each DNA samples with unknown copy numbers, i.e., the *p185 BCR-ABL* in cDNA library and the sense p185-ssDNA were consecutively diluted into three concentrations. The copy number in the DNA samples was determined on the basis of the sample C_T values against the standard curve. The oligonucleotide pair of 5′-ATTGCGGAGGCGGCTATAC-3′ (forward primer) and 5′-CTCGCTGGAGGTGAGGTTCT-3′ (reverse primer) was used for the qPCR of *p185 BCR-ABL*. See Supporting Information for the procedures of the qPCR and data analysis.

bDNA Hybridization Assay. The recommended protocols of the QuantiGene 2.0 assay kit was modified for the detection of mRNA extracted from the cell lines or the p185-ssDNA standards. Prior to hybridization, the p185-ssDNA standards and mRNA samples were denatured at 95 °C for 5 min and 65 °C for 10 min, respectively, and were immediately chilled on ice until use. Twenty microliters of the denatured target solution was mixed with lysis mixture containing blocking reagents and target-specific probe set (CEs, LEs, and BLs) to a total volume of 100 μL/well. The capture plate was incubated at 50 °C overnight (~12−16 h) in an incubator. After overnight incubation, the wells were washed three times each with of 300 μL of wash buffer. The labeling of captured targets was started with incubation 100 μL of preamplifier working solution at 50 °C for 1 h. Then, the capture wells on the plate were washed three times as described previously. The 100 μL of amplifier working solution was added into the wells and incubated at 50 °C for 1 h. After the incubation, the wells were washed three times again. Finally, 100 μL of label probe working solution was added into the wells and incubated at 50 °C for 1 h. After the wells were washed three times, the electrochemical or chemiluminescent measurement was performed. The wells upon completion of hybridization were wet with 10 mM Tris-HCl buffer (pH 8.0) while waiting for the measurements. The Tris-HCl buffer was removed and replaced with the substrate solution during the measurement. For electrochemical analysis, the wells were incubated with 50 μL of substrate solution (1 mM 1-napthyl phosphate in Tris-HCl buffer) for 30 min at room temperature. The ALP-catalyzed product solution (50 μL) was then transferred and loaded onto a SPE connected to the electrochemical analyzer for SWV analysis as described earlier. Chemiluminescent detection was performed according to the manufacturer's recommendation. The 100-μL sample of the Lumigen APS-5 chemiluminescent substrate solution was added to each well and reactions were allowed to preceed for 5 min. The luminescence was quantified in a microplate luminometer.

RESULTS AND DISCUSSION

Detection and Signal Amplification Principles. A PCR-free electrochemical detection and quantification of the human ALL oncogene, namely, the *p185 BCR-ABL* mRNA fusion transcripts (full length, 491 bp) isolated from the SUP-B15 cell lines, has been successfully demonstrated for the first time in this study. The mRNA isolated from the HL-60 cell lines was used as a negative control. The bDNA amplifiers were used to amplify the signal associated with the mRNA target rather than the DNA amplification by the PCR. All the reagents and primers involved in the bDNA assay described herein are commercially available (QuantiGene 2.0 Assay kit, Panomics). The primer sets were designed specifically to hybridize with human *p185 BCR-ABL* transcripts, covering the region from 180 to 491 bp, in which CEs hybridized with BCR domain and LEs hybridized with ABL domain. Such design increases the specificity of the assay as only the *p185 BCR-ABL* fusion transcripts will respond to the detection.

The principle of the electrochemical bDNA assay is illustrated in Figure 1. Recognition of the mRNA target relies on cooperative hybridization among the three groups of primers, i.e., CEs, LEs, and BLs. This probe set was designed specifically to hybridize to human *p185 BCR-ABL* mRNA. Upon addition of RNA samples to the wells precoated with the CPs, the *p185 BCR-ABL* mRNA was captured on the well surfaces of a 96-well microtiter plate through the hybridization of CEs with the precoated CPs. The CEs were designed in such a way that a portion of the primer hybridized with the CPs while another portion hybridized with a specific region of the *p185 BCR-ABL* mRNA. Multiple CEs ensured the

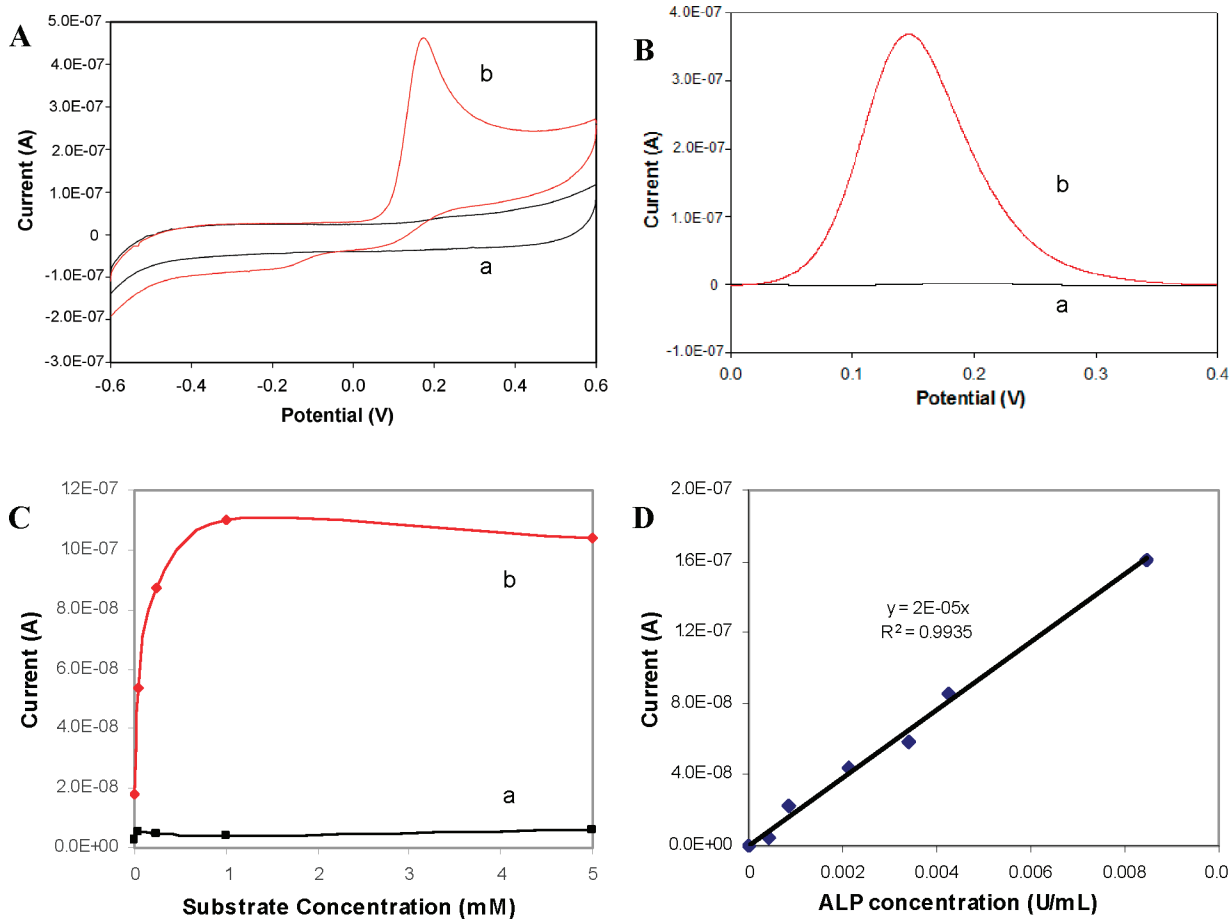

Figure 2. (A) CV and (B) SWV voltammograms of 1-napthyl phosphate substrate solution in the absence (curves a) and presence (curves b) of ALP using the SPE in which curves a = 2.5 mM substrate and curves b = 0.025 U/mL ALP in 2.5 mM substrate. Scanning conditions for CV: potential scanning, 0.1 V/s; SWV, step potential, 0.004 V; amplitude, 0.025 V frequency, 25 Hz. (C) Optimization of substrate concentration ranged from 0.01 to 5 mM. Curves a and b represent substrate solution in the absence and presence of 0.1 U/mL ALP, respectively. (D) The linear plot of SWV detection of ALP. All enzymatic reactions were allowed to proceed for 10 min.

hybridization specificity as only the target strands were captured and anchored to the well surface. Likewise, a portion of the LEs was hybridized with the mRNA target while another portion hybridized with a bDNA amplifier. A group of BLs was used to block exposed portions (i.e., unoccupied by either CEs or LEs) to avoid nonspecific hybridization and to increase the stability of the hybrids. The bDNA amplifier comprised a set of nucleotide-derived preamplifiers, amplifiers, and ALP-labeled probes. Following the hybridization of target, the target-bound hybrids were tagged with the bDNA amplifiers via specific hybridization of preamplifiers to LE sites. Each bDNA amplifier carries ~400 ALP enzymes as reporting molecules. There were a total of four bDNA amplifiers designed to couple to each mRNA target enabling a 1600-fold of signal amplification. The ALP activities corresponding to the amount of mRNA target were quantified by SWV measuring the electroactive enzymatic product in the presence of 1-napthyl phosphate substrate solution. The enzymatic reaction was allowed to proceed in the well prior to the transfer to a disposable SPE electrode for electrochemical analysis.

Electrochemical Measurements of ALP on the SPE. In the current work, ALP enzymes were used as the signal reporting molecules for electrochemical quantification of RNA targets. 1-napthyl phosphate in Tris-HCl buffer was used as the electrochemical substrate for the ALP assay. The electroactive product formed by ALP enzymatic reaction can be oxidized electrochemically, and the current responses were monitored at the SPE by voltammetric detection.

In this study, the voltammetric characteristics of 1-napthyl phosphate substrate and its enzymatic product at the SPE were investigated by CV in the potential range of +0.6 to −0.6 V at a sweep rate of 0.1 V/s in Tris-HCl buffer. Figure 2A shows the typical voltammograms of the substrate solution (curve a) and the substrate solution after 10 min of enzyme catalytic reaction (curve b). A well-defined anodic oxidation peak was observed with peak potential at +0.15 V in the presence of ALP enzymes in the substrate solution (curve b) attributed to the electrooxidation of enzymatic product. No redox peaks were observed in the blank substrate solution (curve a). Likewise, a typical SWV voltammogram in Figure 2B indicates the oxidative current response of the enzymatic product (curve b) compared to the null response of blank substrate (curve a). The favorable SWV peak current of enzymatic product was used to determine the amount of ALP enzymes, which is correlated to the mRNA targets.

The analytical performance of the SWV analysis of 1-napthyl phosphate substrate and its ALP-catalyzed product, namely, the accumulation effect, substrate concentration, level of background current, and the enzymatic reaction time, were evaluated and optimized. The accumulation effect of enzymatic products on the

SWV analysis was not significant (result not shown). An accumulation step was not employed in the subsequent SWV measurements. It is well-known that the rate of enzymatic reaction increases with the substrate concentration at a constant amount of enzyme. Figure 2C shows the influence of substrate concentrations on the current responses of products in the presence of 0.1 U/mL ALP enzymes. The product responses were obtained by end-point assay with a 10-min enzymatic reaction time. The current responses of product solution (curve a) increased dramatically with the increase of substrate concentration from 0.01 to 0.25 mM and increased gradually from 0.25 to 1 mM. The responses were saturated at concentrations beyond 1 mM. In contrast, the blank substrate solution (curve b) exhibited a negligible current response in spite of the increasing substrate concentration from 0.01 to 5 mM. Substrate solution containing 1 mM 1-napthyl phosphate in Tris-HCl buffer was used subsequently. Figure 2D displays the linear plot of the current responses versus ALP concentrations performed under the optimized conditions. A detection limit of 0.000 17 U/mL (corresponds to 87 fM or 4.8×10^{-15} mol of ALP in 55 μL of total assay solution) was estimated on the basis of 3 $\times$ standard deviation (SD) of determination of the zero standards, i.e., blank substrate solution in connection with the 10-min enzymatic reaction.

bDNA Hybridization Assay of p185-ssDNA Standards. In recent years, the bDNA has been explored as an alternative to the qPCR for quantitative analysis of genetic materials. In both cases, it is critical to choose the right control gene and to prepare a standard for reliable quantitative or semiquantitative analysis of the target genes. Conventionally, the qPCR assays use the plasmid DNA containing the target gene or housekeeping gene as standard or internal control standard, respectively. The preparation of such standards is tedious and complex. Due to the inherent hybridization principle, the quantitative assay of RNA targets achieved by the bDNA and other sandwich hybridization assays required ssDNA as the standard rather than the dsDNA. Therefore, the first strand cDNA transcribed from the target mRNA had been used as standards.[21] However, the use of first strand cDNA bearing antisense sequence of RNA requires a different set of primers, which in turn increases the cost and complexity of the preparation and assays. In this study, ssDNA bearing the same sequence and length of the RNA target (i.e., p185-ssDNA) was synthesized and used for the standard, which obviated the need for additional sets of primers. The p185-ssDNA standard was purified from the double-stranded PCR products (detailed in the Experimental Section). The DNA sequencing analysis confirmed that the PCR product amplified from the cDNA library was identical (result not shown) to the target gene. The quality of this standard was evaluated by agarose gel electrophoresis. As illustrated in Figure 3, lane 2 was the dsDNA PCR product of target gene amplified from the cDNA library. Lane 3 was the p185-ssDNA isolated from dsDNA PCR product via biotinylated purification, where dsDNA of target gene was not detected on the agarose gel. Due to the ssDNA fragment, the gel fractionation pattern was distinct from that of dsDNA fragment.

The p185-ssDNA derived from the target mRNA template was used as standards to evaluate the performance of the electrochemical bDNA assay for *p185 BCR-ABL* gene. Figure 4A displays the typical electrochemical response with increasing concentrations of standard. Well-defined voltammetric peaks were observed with low concentrations of standard. The peak currents increased with the increase of standard concentrations. A negligible signal was observed in the negative control sample (in the absence of p185-ssDNA). The resulting calibration plot of the currents versus standard concentrations (Figure 4B) is linear over the range of $2.2 \times 10^5 - 1.6 \times 10^8$ copies of targets and is suitable for quantitative analysis. The sample loading beyond the upper limit was saturated (data not shown). The 3 log linear range is comparable to the performance achieved by luminescence detection (>3.5 log as claimed by the manufacturer). A detection limit of 6.1×10^4 copies was achieved based on the basis of 3 $\times$ SD of determination of the zero standards. This detection limit corresponds to 1 fM (1×10^{-19} mol of target nucleic acids in the 100-μL sample), or ~17 fg, which is comparable to that reported earlier for detecting the breast cancer susceptibility gene (tumor protein p53, 1182 bp) with a detection limit of 0.5 fM.[21] To our best knowledge, this is the lowest reported amount of full length genetic targets detected by electrochemical bDNA assay. The detection limit of bDNA assay could vary dependent on the length of target nucleic acids. A longer target of nucleic acid enables more binding of bDNA amplifiers through the LEs. This implies that the selection and preparation of ssDNA standard of similar or identical length and sequence is crucial for accurate quantitative bDNA assay. The high sensitivity and specificity exhibited by our proposed assay were with good reproducibility. A series of six measurements of 6×10^6 copies of standard ssDNA yielded reproducible signals with a relative standard deviation of 6.9%.

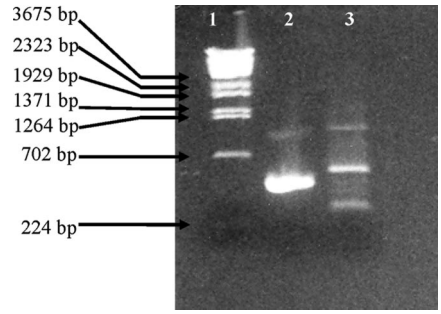

Figure 3. Agarose gel electrophoretic image of the double-stranded (lane 2) and single-stranded DNA (lane 3) for the *p185 BCR-ABL* gene. Gel electrophoresis was run at 8 V/cm in 1$\times$ TBE buffer on a 1% agarose gel. Lane 1 is the B*stE*II λ DNA marker.

Detection of *p185 BCR-ABL* Fusion Transcripts (mRNA) Extracted from SUP-B15 Cell Line. Figure 5A shows the PCR-free electrochemical detection of mRNA targets and the excellent specificity of our proposed assay. In this study, mRNA targets were extracted from the SUP-B15 cell line whereas those obtained from the HL-60 cell line were used as a negative control. The current responses increased unambiguously with increasing concentrations of the mRNA target (from 4.6 to 53.8 ng). The resulting calibration (Figure 5B) indicates that the assay responds linearly with the amount of mRNA target. In contrast, we observed the null response to the negative control mRNA samples in spite of the increasing amount added to the assay. Similarly, a null response was observed in the blank control (0 ng of mRNA). Such performance is attributed to the effective blocking by the blocking reagents and BLs. Furthermore, the cooperative hybridization of both CEs and LEs ensured the specific recognition to the fusion

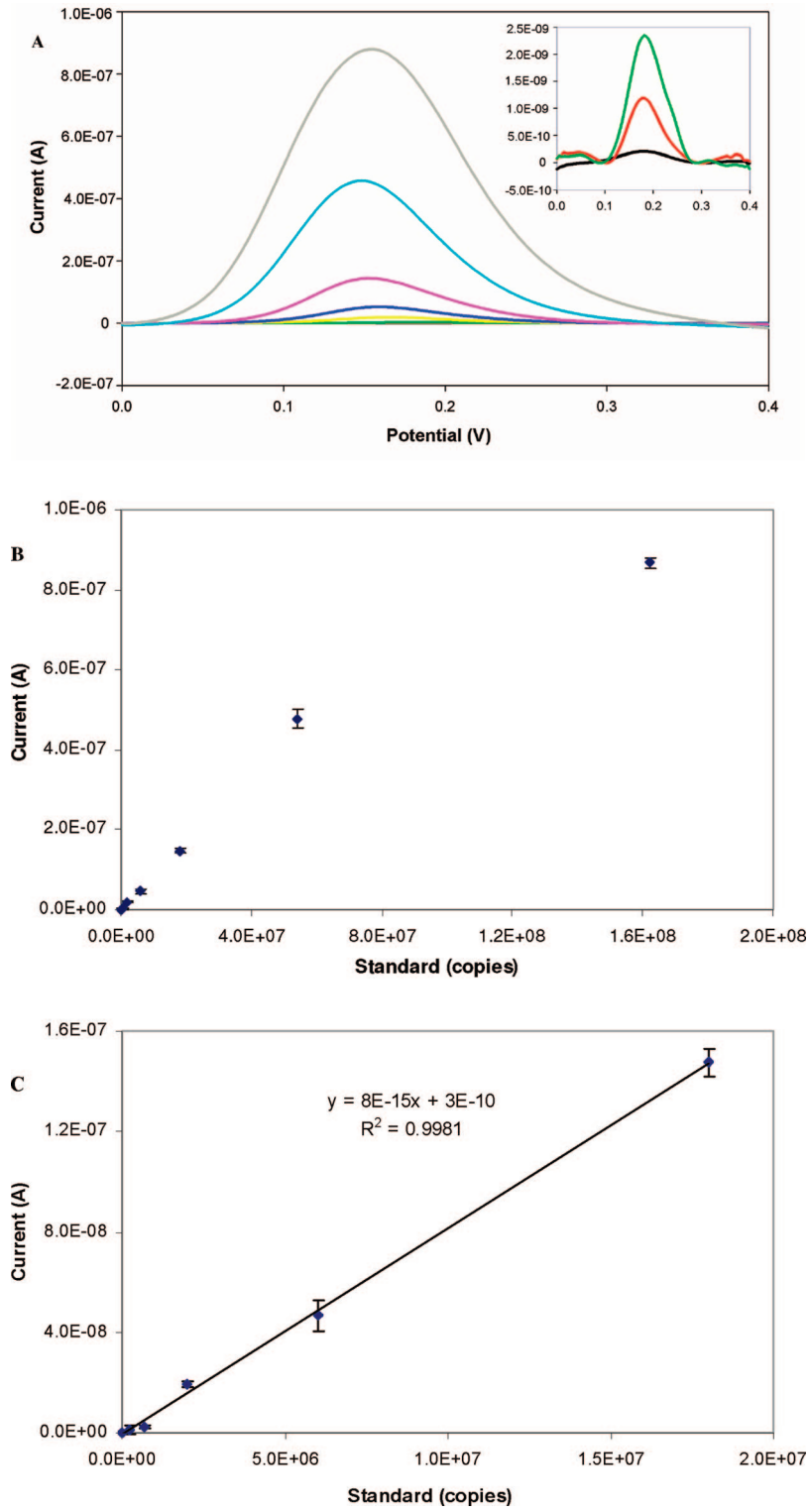

Figure 4. (A) Typical SWV responses with increasing standard concentrations. From bottom to top: 0, 2.2 × 10^5, 6.7 × 10^5, 2.0 × 10^6, 6.0 × 10^6, 1.8 × 10^7, 5.4 × 10^7, and 1.6 × 10^8 copies. The inset shows the enlarged voltammograms of 0 − 6.7 × 10^5 copies. (B) The resulting calibration plot with the lower left portion being enlarged as in (C). SWV measurements were performed with 1 mM substrate and 30-min enzymatic reaction. Other conditions of SWV analysis and bDNA hybridization assay were detailed in the Experimental Section. Error bars are based on standard deviation with $n = 2$.

transcripts containing both *p185 BCL-ABL* sequences, which in turn eliminated the binding of any other sequences. A detection limit of ~4 ng of total mRNA population was achieved based on the basis of 3 × SD of determination of the zero standards. A comparative study was carried out to evaluate the performance of the electrochemical assay as compared to that of conventional chemiluminescent detection. Figure 6 presents the chemiluminescent responses of the mRNA target performed under the

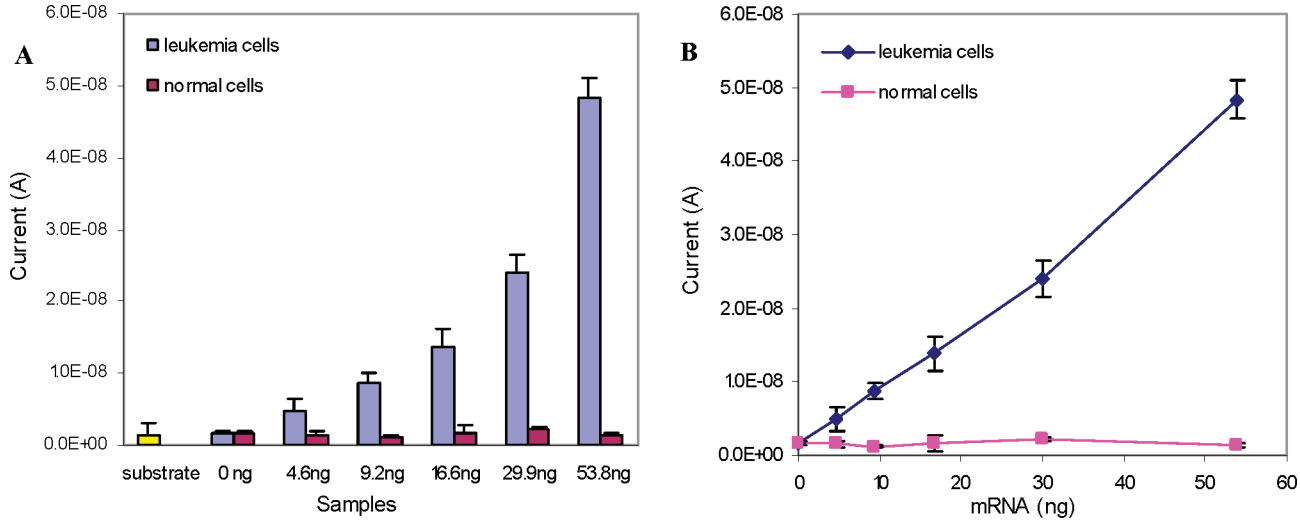

Figure 5. (A) Direct electrochemical measurement of *p185 BCR-ABL* mRNA fusion transcripts. The mRNA samples were extracted from the positive leukemia cell line SUP-B15 and negative cell line HL-60. (B) The corresponding linear response and null response of SWV to the mRNA samples extracted from the positive and negative cell lines, respectively. Conditions were the same as described in Figure 4. The data point with 0 ng represents the control sample. Error bars are based on standard deviation with $n = 2$.

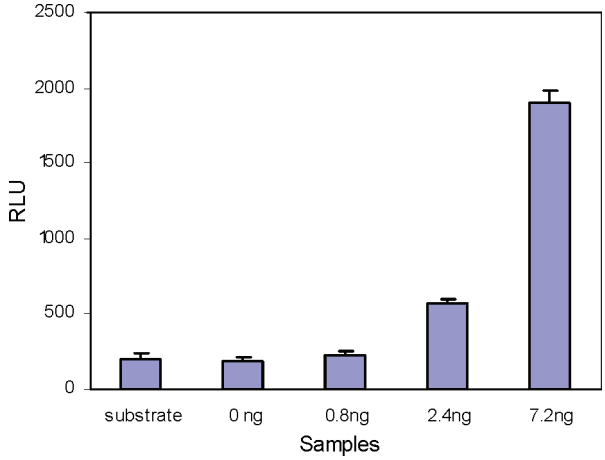

Figure 6. Chemiluminescent measurement of the *p185 BCR-ABL* mRNA fusion transcripts. The data point with 0 ng represents the control sample. The luminescent detection was performed using the Lumingen APS-5 substrate by following the manufacturer's instruction in the bDNA assay kit. Error bars are based on standard deviation with $n = 2$.

identical conditions. The assay achieved a detection limit of ~2.4 ng of total mRNA population, which is in the same order of magnitude achieved by our electrochemical analysis. Our assay unambiguously detects the target gene from nanograms of mRNA samples, which is an amount of at least 200-fold less than that required for Northern blot analysis (0.5−20 μg of mRNA).[22] Such an amount is also comparable to 5−50 ng of mRNA required for a two-step RT-PCR analysis by using Superscript First-Strand Synthesis kit (Invitrogen's recommendation: 50−500 ng of mRNA for RT reaction followed by the PCR using 10% of the resulting RT reaction product).[23] The current assay could be applied for gene expression analysis by incorporating the housekeeping gene for normalizations. In gene expression studies, it has been a challenge to discriminate the gene expression that differs less than 2-fold. Our approach overcomes such limitation, which was evidenced by the well-resolved current responses between 2-fold differences in the mRNA target (Figure 5B).

Quantitative mRNA Assay: Electrochemical bDNA Assay versus Fluorescent qPCR. An mRNA population may contain thousands of genes. A specific gene may comprise only 0.01−3% of the entire mRNA population.[24] In this study, the actual copies of the target gene in the mRNA population were determined by using an electrochemical bDNA assay in comparison to that of a conventional fluorescent qPCR.

Most of the genetic analyses reported to date are semiquantitative in which the gene expression is quantified in terms of the fold of change relative to that of a housekeeping gene. Unlike the semiquantitative method, the absolute quantification assay depends on knowing the absolute quantities of the nucleic acid standards. Collins et al. proposed a method to generate a trustworthy standard for a bDNA assay.[25] We demonstrated herein a standard curve method for absolute quantification of the target gene in the mRNA population. The p185-ssDNA bearing an identical sequence of the target gene was prepared from the biotinylated double-strand PCR product and used as a standard in the electrochemical bDNA assay. This p185-ssDNA standard was quantified by spectrophotometry and qPCR. Both techniques provided similar quantities in which absorbance value was ~37% higher than that of the qPCR. The value obtained by the qPCR was used in the subsequent tests.

Figure 7 shows the electrochemical bDNA assay for absolute quantitative analysis of the *p185 BCR-ABL* mRNA. The mRNA samples were diluted in nuclease-free water to three different concentrations with each run in triplicate. The corresponding average current responses were correlated to those of p185-ssDNA standards. Our assay determined that the mRNA sample contained an average of 62 900 copies of *p185 BCR-ABL*/ng of mRNA.

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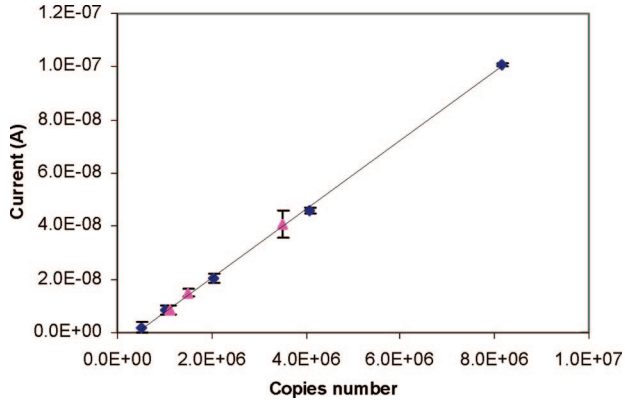

Figure 7. Electrochemical bDNA assay for quantification of target genes in the mRNA samples. The unknown quantities of the target genes on three different amounts of mRNA sample (▲) were determined using a set of 5 points of p185-ssDNA standards (◆). Conditions were the same as described in Figure 4. Error bars are based on standard deviation with $n = 3$.

Considering the fact that the expression level of a specific gene could vary between batches of culture, the same batch of mRNA was reverse transcribed to cDNA followed by the qPCR (see Supporting Information for details), which yielded an average of 1240 copies/ng of mRNA template. The amount of target gene contained in the mRNA sample determined by our proposed assay was at least 50-fold higher than that of the qPCR analysis. This result is consistent with the finding reported earlier,[17,19] which suggests that the qPCR method might underestimate the actual amount of target gene due to the drawbacks inherent in the RT reaction (i.e., incomplete reverse transcription from mRNA to cDNA) and bias introduced during the thermal cycles. The underestimation of target content in patient samples could lead to false negative diagnosis and delays in the treatment. The current assay demonstrated a reliable alternative to the qPCR method for clinical diagnosis.

CONCLUSIONS

We have demonstrated a novel electrochemical bDNA assay for simple, sensitive, accurate, and quantitative detection of genetic marker in the mRNA population without RT and PCR amplification. The use of bDNA (QuantiGene 2.0) for signal amplification showed a 10^3-fold improvement in assay sensitivity, which enables the detection of full-length *p185 BCR-ABL* transcripts at femtomolar levels from as little as nanograms of total mRNA population. The electrochemical analysis is consistently comparable to the chemiluminescent detection and can be readily integrated for the development of POC systems. The electrochemical responses were unambiguously resolved for less than 2-fold difference in gene expression level. In combination with the use of a well-quantified standard, the proposed assay indicated that qPCR could underestimate the target gene by at least 50-fold. Our approach has addressed the shortcomings of the qPCR analysis by the reduction of the assay complexity and minimization of assay bias caused by nonideal RT efficiency. The newly developed assay provides a new approach for disease diagnosis.

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SUPPORTING INFORMATION AVAILABLE

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

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Supplementary Information

Electrochemical Branched-DNA Assay for PCR-free Detection and Quantification of Oncogenes in Messenger RNA

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Supplementary Experimental Section

EXPERIMENTAL SECTION

Preparation of sense p185 single-strand DNA (p185-ssDNA).

(A) Synthesis of the biotinylated double-strand DNA (dsDNA). The total mRNA population was first reversely transcribed to cDNA using the Superscript First-strand Synthesis kit following the manufacturer's protocol. Briefly, a 10μL-mRNA/primer mixture [1 mM of deoxyribonucleotide triphosphate (dNTP) mix, 0.5 μg of Oligo(dT), 100 ng of mRNA template] was heated at 65 ^{o}C for 5 min and immediately cooled on ice for 10 min. The reverse transcription (RT) reaction mixture, which contains the denatured RNA/primer mix, 1×RT buffer, 5 mM magnesium chloride, 10 mM dithiothreitol, 40 units of RNaseOUT^{TM} Recombinant RNase Inhibitor and 50 units of SuperScript^{TM} II Reverse Transcriptase at a final volume of 20-μL, was incubated at 42 ^{o}C for 50 min. The reaction was terminated by heating at 70 ^{o}C for 15 min and then immediately chilled on ice for at least 10 min. 1 μL (4 units/μL) of the RNase H was added into the final RT reaction mixture and incubated for 30 min at 37 ^{o}C to remove the mRNA before proceeding to amplification of the target cDNA. The oligonucleotide pair of 5'-GCATGATGGAAGGGGAGGGCAAG-3' (forward primer) and 5'-biotin-CTTGGAGTTCCAACGAGCGGCTTCA-3' (reverse primer) was used for the amplification of 491 bp cDNA fragment of *p185 BCR-ABL*. The 5'-biotinylated primer in reverse direction was used to introduce the biotin at the 5'-end of antisense strand DNA fragment for the separation of sense strand DNA. The PCR was performed on a Genius Techne PCR thermocycler with a final volume of 50 μL consisting 2 μL of cDNA, 2.5 units of EX Taq^{TM} DNA polymerase, 1× PCR buffer, 0.2 mM dNTP, and 1 μM of each reverse and forward primers. The reaction was first incubated at 94 ^{o}C for 2 min. Then, it was carried out for 30 cycles at the following conditions: 94 ^{o}C for 30 seconds, 62 ^{o}C for 30 seconds, and 72 ^{o}C for 45 seconds and followed by a final extension step at 72 ^{o}C for 2.5 min. After the PCR amplification, the biotinylated PCR products were purified by agarose gel separation and the agarose digestion using β-agarase I. The β-agarase I was removed by phenol:chloroform extraction. The sample was also desalted and concentrated using Microcon YM-30 centrifugal filter unit to improve the biotinylated purification of sense p185-ssDNA.

(B). Purification of sense p185-ssDNA. The recommended procedure of the BioMag® Plus Streptavidin was modified to capture the biotinylated PCR products to the streptavidin-coated magnetic beads. The amount of the biotinylated dsDNA PCR product was first determined by Quant-iT dsDNA HS assay kit. The biotinylated PCR product was coupled on the magnetic beads at a ratio of 35 pmoles dsDNA per mg beads in the coupling/wash buffer (10 mM PBS, pH 7.4, 1 % BSA, 0.1 % sodium azide and 1 mM sodium ethylenediamine tetraacetic acid. The coupling reaction was carried out at 4 ^{o}C overnight with gentle mixing and subsequently washed three times with ice-cold coupling/wash buffer. The sense p185-ssDNA without biotinylation was separated by suspending the dsDNA-bound beads in 10 mM Tris-HCl buffer (pH 8.0, preheated to 95 ^{o}C) and heating at 95 ^{o}C for 5 min. The beads carrying the biotinylated antisense single-strand DNA (ssDNA) were magnetically separated immediately. The supernatant containing the sense p185-ssDNA was collected and concentrated using Microcon YM-30 centrifugal filter by centrifugation at 15000×g. The concentrated sample was analyzed by agarose gel electrophoresis and quantified by qPCR. This sense p185-ssDNA sample was used as the standard throughout.

(C). The qPCR and data analysis for the sense p185-ssDNA and cDNA samples. The qPCR and data analysis were performed in the 7900HT Fast Real-time PCR System using Power SYBR® Green PCR Master Mix. The procedures for the qPCR and data analysis were mainly adapted from the manufacturer's manuals. The oligonucleotide pair of 5'-ATTGCGGAGGCGGCTATAC-3' (forward primer) and 5'-CTCGCTGGAGGTGAGGTTCT-3' (reverse primer) was designed by the instrument's software, synthesized and used for the amplification of *p185 BCR-ABL*. The 2× Power SYBR® Green PCR Master Mix contained SYBR® Green I dye, AmpliTag Gold® DNA polymerase LD, dNTPs with dUTP/dTT blend, passive reference ROX™ dye and optimized buffer components. Each PCR reaction mixture with a final volume of 50 μL consisted 25 μL of 2× Power SYBR® Green PCR Master Mix, 0.5 μL of each 5 μM reverse and forward primers, 10 μL of DNA template (i.e. the plasmid DNA standard, p185-ssDNA or cDNA samples) and 14 μL of deionized water. The PCR reactions for each type of DNA template were performed in quadruplicate. The thermal profile was 50 ^{o}C for 2 minutes (activation of Uracil-DNA Glycosylase), 95 ^{o}C for 10 minutes (AmpliTag Gold® DNA polymerase activation and denaturation of template DNA), followed by 40 cycles of 95 ^{o}C for 15 seconds and 60 ^{o}C for 1 minute.

The amplification plots showing the fluorescence intensity of each reaction plotted against PCR cycles were generated for each run. The threshold line was set in the exponential phase of the amplification. Cycle threshold (C_T) values were determined for each reaction. Data was collected and viewed using the software and graphics programs provided with the 7900HT Fast Real-time PCR System. Calibration curve [i.e. known copy number of plasmid DNA versus cycle threshold (C_T) value] was plotted automatically by the instrument software. The copy number in the samples (p185-ssDNA or cDNA) was determined on the basis of the sample C_T values against the standard curves established by the plasmid DNA with known copy number.