A Catalytic DNA Probe with Stem-loop Motif for Human T47D Breast Cancer Cells

Fei GAO,*1,*2 Feng LIU,*2† Jing ZHENG,*2 MeiYun ZENG,*4 and Yuyang JIANG*1,*2,*3†

*1 Department of Chemistry, Tsinghua University, Beijing 100084, P. R. China
*2 The Ministry-Province Jointly Constructed Base for State Key Lab-Shenzhen Key Laboratory of Chemical Biology, the Graduate School at Shenzhen, Tsinghua University, Shenzhen, Guangdong, 518055, P. R. China
*3 Department of Pharmacology and Pharmaceutical Sciences, School of Medicine, Tsinghua University, Beijing 100084, P. R. China
*4 Shenzhen Kivita Innovative Drug Discovery Institute, Shenzhen, Guangdong 518055, P. R. China

In vitro selection methods allow for isolation of DNAzymes (catalytic DNAs) from random DNA pools. Here we describe a fluorogenic DNAzyme, LYF5, isolated using a double-random selection approach: a random DNA pool was selected against a complex molecular mixture derived from a breast cancer cell line, T47D. LYF5 specifically indicates the T47D breast cancer cell line with high sensitivity. After sequence optimization, the second-generation DNAzyme, 2G-LYF5, exhibited an approximately 2-fold higher cleavage percentage. Finally, we have determined that the intramolecular stem-loop motif plays a crucial role in 2G-LYF5 activity. Our findings underscore the capability of single-stranded DNA molecules to perform highly sophisticated functions that are amenable to the development of diagnostic tests for early identification of breast cancer.

Keywords DNAzyme, double-random selection, stem-loop, T47D, breast cancer, early identification

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Introduction

Cancer is one of the most common threats to the health of human beings. Worldwide, breast cancer is the most commonly diagnosed cancer in women, accounting for a quarter of all types of cancers. In 2012 it accounted for 1.68 million cases and 577000 deaths.1 Of even greater concern, the incidence of breast cancer is increasing, especially in the developing world, due to economic development and adoption of western lifestyles.2 For the classification of malignant tumors, the tumor node metastasis (TNM) staging system is the most commonly used staging system for breast cancer.3 Clinically, each unique TNM stage responds to a specific treatment and prognosis; for example, early stage breast cancers can be completely resected using surgery and have high 5-year patient survival rates.4 Several early detection methods exist, such as mammography and magnetic resonance imaging (MRI).5,6 However, in developing countries, the majority of breast cancer cases are diagnosed in very late stages because of the lack of early screening, inadequate diagnosis, inadequate treatment, and other issues.7 Therefore, development of a simple and rapid detection method is necessary to improve breast cancer outcomes and survival worldwide.

Living cells possess the distinct ability to grow under adequate nutritional conditions and can exchange complex molecules with their extracellular environment.8 Therefore, a simple, rapid test that takes advantage of the extracellular secretion mixture (ESM) of breast cancer cells would serve as the basis for an optimal detection method for these cells. Because ESMs could be considered to be distinctive cocktails, each ESM could be detected using a specific probe without performing complex purification procedures. Furthermore, probe binding could facilitate identification of a possibly specific diagnostic breast cancer biomarker.

These considerations have prompted us to develop a novel detection method based on catalytic DNA molecules (DNAzymes), which are a special class of single-stranded functional nucleic acids that have catalytic abilities.9,10 DNAzymes can be artificially identified from a random-sequence DNA library by in vitro selection11–14 and have been increasingly applied as molecular tools for various applications.15–18 One powerful type of DNAzyme is an RNA-cleaving fluorogenic DNAzyme (RFD). This special DNAzyme is designed to cleave a chimeric DNA/RNA substrate that contains a single ribonucleotide as the cleavage site, which is flanked by a pair of nucleotides modified with a fluorophore (F) and a quencher (Q). These features provide a convenient way to link the DNAzyme’s cleavage activity to a physical fluorophore-quencher separation event, and consequently obtain an increase in the fluorescence signal.19,20 So far, several of these reporter DNAzymes have been isolated and characterized,21,22 and some of them have been used to develop simple fluorescent or colorimetric biosensor assays.23,24
Although DNAzymes that directly detect the ESM of a given living cell have been applied to identification of pathogenic bacteria,25-28 their use in detecting eukaryotic cancer cells has not yet been reported. Detection of eukaryotic ESM is more of a challenge because eukaryotic cells have much slower growth rates and lower ESM concentrations than do prokaryotic cells. Therefore, selection of a special DNAzyme that can distinguish between a specific cancer cell line and normal cells poses special challenges.

This work is based on the hypothesis that a characteristic component of each extracellular secretion mixture collected from a given cell line culture can be identified using a DNAzyme (Fig. S1a, Supporting Information). Therefore, in this work we developed a method to separate specially-modified RFDs that can respond directly to the ESM produced by a model breast carcinoma cell T47D after double-random in vitro selection. Our results demonstrate that RFDs can be selected from a DNA pool to fluorescently indicate the ESM that is produced by a specific cancer cell type, and that such probes can be used to develop a simple and rapid test to detect this cell line. It is also probable that in the future such fluorogenic probes could be applied to early detection, diagnosis, and prediction of the response of cancer cells to therapy.

Experimental

Reagents and chemicals

T4 DNA ligase, T4 polynucleotide kinase (PNK), Taq DNA polymerase, dNTPs, and ATP were obtained from Takara Biotechnology Co., Ltd. (Dalian, China). Ampicillin, N,N,N′,N′-tetramethylethylendiamine (TEMED), and a 40% solution of acrylamide/bis-acrylamide (29:1) were obtained from Sangon Biotech (Shanghai, China). pGEM-T Vector Systems I was obtained from Promega (USA) and contained a T4 cloning vector (pGEM-T), 2× rapid ligation buffer, and T4 DNA ligase. Complete protease inhibitor cocktail tablets were obtained from Roche Ltd. (Switzerland). L-15 medium was obtained from Gibco (USA). Dulbecco’s modified Eagle’s medium (DMEM), Iscove’s modified Dubcco’s medium (IMDM), RPMI-1640, and fetal bovine serum (FBS) were obtained from Hyclone (USA). Reduced serum modification of Eagle’s minimum essential medium (Opti-MEM®) was obtained from Life Technologies (USA). All other chemicals were purchased from Sigma-Aldrich (USA) and used without further purification. E. coli JM109 is routinely maintained in our laboratory. Filters of 0.22 μm and molecular sizing columns were obtained from Merck Millipore (Germany). The water used in this study was RNase-free double deionized (ddH2O) and autoclaved. The 2× selection buffer contained 100 mM HEPES (pH 7.5), 400 mM NaCl, and 10 mM MgCl₂. All solutions were freshly prepared before use. All oligonucleotides were synthesized by Takara Biotechnology Co., Ltd. (Dalian, China).

Cell lines and cell culture conditions

All cell lines were obtained from the cell bank at the Shanghai Institutes for Biological Sciences. T47D (human breast carcinoma), U251 (human glioma), and A375 (human melanoma) cell lines were cultured in DMEM medium supplemented with 10% FBS. The MDA-MB-231 (human breast carcinoma cell line) was cultured in L-15 medium supplemented with 10% FBS at 37°C in 100% air. Cell lines used in this work included HCC1937 (human breast carcinoma), BT-549 (human breast carcinoma), BT-474 (human breast carcinoma), MCF-10A (human mammary epithelial), HCC827 (human non-small cell lung cancer), NCI-H460 (human lung large cell carcinoma), K562 (malignant myeloid), COLO 205 (colon carcinoma), HeLa (human cervical carcinoma), and L02 (human normal liver cells) that were cultured in RPMI-1640 supplemented with 10% FBS. All cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ and 95% air, except for MDA-MB-231 cells (described above).

Sample preparation

Each cell line in exponential growth phase was cultured in its optimal medium, washed twice with PBS, and sub-cultured in Opti-MEM® with 5% FBS. The default cell concentration used for culture was 1×10⁶ cells/mL (CPM). After 36 h, the crude supernatant of each cell line was collected, supplemented with a complete protease inhibitor cocktail (Roche), filtered twice through a 0.22 μm filter, aliquoted into 1.5 mL microcentrifuge tubes, and stored at –20°C.

In vitro selection procedure

Procedures for in vitro selection were similar to that described in a previous study.29 Prior to selection, three different ESM samples were collected: ESM from the T47D cell line (ESM-TD), ESM from the MCF-10A cell line, and the ESM from the HeLa cell line. The ESM samples were prepared by removing cells after 36 h of culture in Opti-MEM®. To efficiently exclude candidate DNA molecules responding to a wide variety of ESM, the extracellular secretion mixture for the negative control (ESM-NC) was prepared by mixing ESMs from MCF-10A and from HeLa cells in a ratio of 1:1. The DNA random library was incubated in a selection buffer (SB) consisting of 50 mM HEPES, pH 7.5, 200 mM NaCl, and 5 mM MgCl₂ for 5 h in order to remove all self-cleaving DNAzymes. In this initial negative step probably separated out the vast majority of non-specific DNAzymes, as exhibited after subsequent purification of uncleaved DNA molecules using 12% denaturing polyacrylamide gel electrophoresis (dPAGE). Next, we incubated these purified uncleaved DNA molecules with ESM-TD in SB for 30 min as a positive selection step for isolating DNAzymes specific for ESM-TD. After this selection step, the 5’ cleavage fragment containing fluorophore was separated from the 3’ fragment to remove quenching of the fluorescent dye, and the fluorescing fragment was subsequently easily detected by fluorescence imaging. Due to this feature, 5’ fluorescent cleavage DNA sequences were purified by 12% dPAGE, amplified by polymerase chain reaction (PCR), and used for the next cycle of selective amplification. After four rounds of selective amplification, we altered the counter selection procedure by incubating DNA molecules with ESM-NC. This was intended to ensure that the final viable DNAzyme would be highly specific for ESM-TD.

Sequencing of the selected DNAs

After 16 rounds of selection, the cleaved DNA molecules were amplified by PCR. The amplification thermocycling steps were as follows: 94°C for 1 min; 15 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 45 s; 72°C for 10 min. PCR products were ligated with PGM-T vector at 4°C overnight. About 2 ng ligation fragments were transformed into 100 μL E. coli JM109 competent cells. Positive clones were selected and sequenced by Invitrogen (Guangzhou, China). The oligonucleotides sequence of LY5 is shown as follows (from 5’): CACGGATCCT GACAGTGCAA GGGGACGGGA CGGGGCGATA TTCTTAGTGGG AAGTGGCAGC TCGTCCGAC TCTTCTAGC FRQGGTTGCA TCAAGA.
The oligonucleotides sequence of 2G-LYF5 are shown as follows (from 5') CTATGTTGGGA AGTGGAATCT TCTGTGCRFQ GGTGCGATCA AGA.

Activity assay of DNA molecules

The DNAzyme LYF5 and the other mutants of LYF5 were produced by T4 DNA ligase-mediated ligation of the FS substrate to the relevant synthetic DNA oligonucleotide using the same method as described for in vitro selection. Following purification using 12% dPAGE, all of the ligated DNAs were pooled into a 10 μM stock and stored at -20°C.

The dPAGE-based analysis was carried out as follows. Reaction mixtures were made of 24 μL of a given ESM (e.g. ESM from T47D cell line), 25 μL of 2×SB and 1 μL of 10 μM relevant construct. The negative control consisted of 24 μL of ESM-NC, 25 μL of 2×SB, and 1 μL of 10 μM relevant construct. Each mixture was incubated at 37°C for 30 min and the reactions were quenched by adding 50 μL of a mixture containing 100 mM EDTA and 8 M urea. After ethanol precipitation, the DNA molecules were analyzed by 12% dPAGE. The fluorescent DNA bands were analyzed using a fluorimager (Bio-Rad, ChemiDocXRS) with an excitation at 488 nm and emission at 520 nm.

The fluorescence-based analysis of the activity of each construct was also performed using the fluorimager with the same excitation and emission wavelengths described above. To set up the reaction mixture, 24 μL of the given ESM-TD (cultured from 10^12 T47D cells/mL), 25 μL of 2×SB, and 1 μL of 10 μM of the relevant construct were mixed in a 96-well assay plate (Costar, USA), and incubated at 37°C. The negative control consisted of 24 μL of a given ESM-NC, 25 μL of 2×SB, and 1 μL of the 10 μM relevant construct. After a 30 min incubation, fluorescence intensities were determined.

Characterization of LYF5 target resistance to protease, RNase inhibitors, and heat treatment of ESM-TD

For ESM-TD pretreatment with proteinase K, 1 μL proteinase K stock was mixed with 23 μL of ESM-TD and 25 μL of 2×SB and the mixture was incubated at 50°C for 1 h. For treatment of ESM-TD with trypsin, the reaction was carried out in a similar way except at 37°C. The third aliquot of ESM-TD was incubated at 90°C for 10 min. Following these treatments, 1 μL of 10 μM ESM-TD stock was added to each sample and further incubated at 37°C for 30 min. Each reaction was then quenched and the reaction mixtures analyzed by 12% dPAGE using the same procedures described above.

In order to analyze the ESM-TD to confirm the specificity of LYF5, three 24 μL aliquots of ESM-TD were mixed with 25 μL of 2×SB and the mixtures were treated with 1 μL RNasin (40 U/L), 1 μL ribonuclease inhibitor (40 U/L), or 0.5 μL ribonuclease vanadyl complexes (200 mM). These treated ESM-TD samples were then incubated with 1 μL 10 μM LYF5 and incubated at 37°C for 30 min. Each reaction was then quenched and the reaction mixtures analyzed using a dPAGE-based cleavage assay.

To determine the molecular weight of the potential protein target, the ESM-TD was described as prepared above and six aliquots were taken. Five aliquots were individually passed through a membrane-based molecular sizing centrifugal column with a molecular weight (Mw) cutoff of 3 KDa, 10 KDa, 30 KDa, 50 KDa, and 100 KDa, (Merck Millipore, Germany). The Mw of the sixth aliquot was above 100 KDa. These samples were then used to induce the activity of LYF5 in the same way as described above for the dPAGE-based cleavage assay.

Results and Discussion

Isolation and identification of an optimal RFD candidate from a random DNA pool by in vitro selection

As described in the Experimental section, RFD isolation was achieved by using the single-stranded DNA library (containing strands of 40 random nucleotides) and this library was subjected to a double-random selection strategy (Fig. S1b, Supporting Information). Briefly, in vitro selection was used to select RFD candidates from a DNA library against the ESM produced by cell line T47D, a classical cell line used for the study of breast cancer in vitro.

Detailed selection procedures are described in the Supporting Information. After 16 iterations (described in Fig. 1(a)), a strong cleavage activity depending on the ESM from the T47D cell line (ESM-TD) was established, which was monitored by fluorescence imaging: a >30% cleavage result was obtained using ESM-TD in 30 min compared to <2% cleavage with ESM-NC in SB. The final cleaved DNA pools were amplified, cloned, and sequenced.

Analysis of the sequenced DNA pools from round 16 indicated that they could be categorized into six families based on their primary sequence homology. Each family’s dominant DNA molecules are described in Fig. 1(b). The activities of these dominant DNA molecules were examined by fluorescence analyses in 96-well plates (Fig. 1(c)). Obviously, the 5th family’s dominant DNA molecule showed the highest fluorescence intensity when compared with the other families’ DNA molecules and also exhibited distinct fluorescence intensity differences between ESM-TD and ESM-NC. Therefore, these results suggest that this DNA molecule is the most promising candidate for detection of the T47D cell line with high activity and specificity. This molecule was chosen for further study and was named LYF5 (named after one of the earliest designers of an RNA-cleaving fluorogenic DNAzyme, Li Y. F.; the “5” designates the 5th dominant DNA molecule).

The sequence of LYF5 (5’CACGGATCTT GACAAGTCA A GGGAGGAGA C CGGGGCGAT A TCTTAGTCG G AAAGCCGCAGC TCCGTCCGAC TCTTCTCAG C FRQQGTTGCA TCAAGA) is shown in Fig. 1(c).

LYF5 responds to ESM-TD with high specificity

To further confirm that the cleavage activity of LYF5 was highly dependent on ESM-TD, we subsequently investigated the response of LYF5 to ESMs produced by breast cell lines and other human cell lines (including MCF-10A and HeLa, previously used during counter selection). The cell lines for this experiment are described in the Experimental section. Equal concentrations of each cell type were initially cultured in Opti-MEM® for 36 h. The ESMs were then prepared and used to induce the cleavage of LYF5 in a 30 min reaction. The dPAGE results are shown in Fig. 2(a). All of the ESMs from these randomly selected cell lines were completely unable to activate LYF5’s cleavage activity, except for ESM-TD, suggesting that LYF5 is indeed highly specific for T47D and has a strong ability to discriminate between ESMs from various cell lines.

Although a single ribonucleotide present within the DNAzyme is probably not a good substrate for ribonucleases, we wished to examine whether the observed cleavage activity of LYF5 might be due to possible ribonucleases (RNases) involved in ESM-TD. To guard against potential RNase contamination, all of the cleavage reactions were carried out in buffers made with nuclease-free water. In addition, we examined LYF5’s cleavage activity in the presence of different RNase inhibitors, including...
RNasin (1 U/μL), ribonuclease inhibitor (1 U/μL), and vanadyl complexes (20 mM). Figure 2(b) shows that the cleavage activity did not change in the presence of the inhibitors. Therefore, this result further demonstrated the specificity of the LYF5 DNAzyme response to ESM-TD.

LYF5 responds to ESM-TD with high sensitivity

With the demonstration of the high specificity of LYF5, the next step was to investigate the detection sensitivity of this DNAzyme using different inspection methods. In order to accomplish this, we first prepared a variety of ESM-TD solutions cultured with T47D cells using different cell concentrations ranging from 10⁶ to 10⁶ cells/mL (CPM) for 36 h. Then we incubated the DNAzyme, LYF5, with different ESM-TD solutions and checked the signal response by 12% dPAGE. The image observed using a fluorescence imager is shown in Fig. 2(c). The result shows that the LYF5 signal response to a series of ESM-TD is clearly observed with equal or greater than 500 CPM. To confirm the CPM-dependent signal correlated with amounts of specifically cleaved fragments of LYF5, the samples were also tested with the initial library. The result in Fig. 2(d) definitely shows that as the CPM decreased, the cleaved bands of the initial library were not observed in the 12% dPAGE. The results suggest that the cleavage activity of LYF5 is indeed highly dependent on the CPM of T47D. Furthermore, the lowest limit of detection is 500 CPM using dPAGE-fluorescence analysis.

Structure optimization by nucleotide truncation and 2D structural simulation

Although the LYF5 DNAzyme exhibited outstanding ESM-TD target detection in SB, LYF5 is a relatively large DNA molecule consisting of 96 nucleotides. Previous studies in Li’s laboratory had confirmed that both ends of primer binding sites in a selective DNAzyme are non-functional sequences.30 To determine whether the primary structure within the non-substrate sequences of the DNAzyme could be minimized, we successively synthesized a series of DNA molecules with variable truncations from the 5′ end, 3′ end, and both ends (Fig. 3(a)). These truncated mutants were examined for RNA-cleavage activity by 12% dPAGE, and the results are summarized in the embedded graph in Fig. 3(b) (activities shown are relative to the activity of LYF5, set to 100%). The result showed that primer binding sites could be simply deleted from both ends of LYF5 without significantly reducing its catalytic activity. Interestingly, several truncated mutants were more effective than the wild-type DNAzyme. However, if the truncations from the 5′ end and 3′ end extend into a core sequence region, we found that the
cleavage activities suddenly disappeared for LYF5-5 and LYF5-7. The disappearance of the truncated DNAzyme’s activity strongly suggested that core nucleotides are missing in LYF5-5 and LYF5-7. Based on this result, we subsequently designed a truncated mutant with deletions from both sides. The truncation of 53 nucleotides from both sides produced a 43-nt enzyme (denoted as 2G-LYF5) that had significantly improved catalytic activity, with 1.8-fold higher activity than that of LYF5 under our experimental conditions. The primary sequence of 2G-LYF5 is shown in Fig. 3(c).

To confirm whether the residual sequence (5’-CTTAG TGGGA AGTGG) in non-substrate sequences of 2G-LYF5 is essential to the catalytic activity of DNA molecules, a series of single-nucleotide mutants was designed with changes in these nucleotides. In Fig. 4, we found that the relative activities of these mutants decreased as compared to that of 2G-LYF5. The results suggest that this block of 15 reserved nucleotides in 2G-LYF5 is indeed a critical motif for its cleavage activity. We consequently chose this sequence-optimized DNAzyme, 2G-LYF5, for further optimization studies.

In order to further investigate the DNAzyme’s higher-level structure, a simulation of a secondary structure for 2G-LYF5 derived using comparative sequence analysis is shown in Fig. 5. In this model, we found there is a typical intramolecular hairpin structure. In order to determine if the higher-level structure was essential to the catalytic activity of 2G-LYF5, we designed another series of mutant DNA molecules to test the importance of the stem-loop structure. Figure 5 shows the data from a set of catalytic activity experiments, where the activity of each mutant DNA is compared with 2G-LYF5. Interestingly, the catalytic capability of 2G-LYF5 was completely lost with the shortening of the number of base pairs in the stem, while it
gradually decreased with lengthening of the number of stem base-pairs. This effect is probably due to the total disruption of the hairpin structure when the hydrogen bonds of base pairs are reduced to fewer than three base-pairs. The results indicate that both the loop and stem of the hairpin are very conservative structures needed for catalytic activity.

Investigation of ESM-TD targets detected by LYF5

To investigate the nature of the targets (proteins or small molecules) in the ESM-TD that activate LYF5, we treated ESM-TD with two broad-spectrum serine proteases: trypsin and proteinase K. ESM-TD treated with either protease was not able to activate LYF5. We also determined the targets were not heat-resistant by incubating ESM-TD at 90°C for 5 min (Fig. S2a, Supporting Information). The disappearance of the cleavage activity in the presence of protease-treated or heated ESM-TD strongly suggests that the responsive target is most likely some kind of protein.
In order to determine the possible molecular weight of the target, we used various molecular sizing columns to separate and size the targets. First, ESM-TD was passed through centrifugal columns with molecular weight cutoffs of 3 KDa, 10 KDa, 30 KDa, 50 KDa, and 100 KDa. Although the filtrates from these columns did not induce the cleavage of LYF5, the ESM-TD fraction containing molecules larger than the 100 KDa MW cutoff possessed cleavage activity (Fig. S2b). The results indicate that the target involved in ESM-TD has a molecular weight above 100 KDa. Our results extend the scope of depended targets to macromolecular organic species derived from eukaryotic cells. The identification of the possible protein target (or targets) is beyond the scope of this report and will be pursued in a future study.

Conclusions

In this study, we employed an in vitro selection technique to derive T47D responsive RNA-cleaving fluorogenic DNAzymes (RFDs) by applying a double-random selection approach, where a random-sequence single-stranded DNA library was selected against a complex molecular library of candidate targets from a human breast carcinoma cell, T47D. To ensure that the final evolved RFDs have high specificity for T47D, the initially evolving DNA library was subjected to a counterselection step, where a similar molecular library of candidate targets from control cell lines (MCF-10A and HeLa) were used to enhance the efficiency of evolution. Using this design scheme, we isolated an RFD designated LYF5 that not only is highly T47D-selective, but also has high sensitivity to the ESM of T47D, with detection limits as low as 500 CPM.

More importantly, after sequence optimization of the second-generation DNAzyme probe, the 43-nt 2G-LYF5 had a much smaller molecular weight and higher cleavage capability, approximately 2-fold higher, than LYF5. After single-nucleotide truncation of catalytic sequences (CTTAG TGGGA AGTGG) of 2G-LYF5, it was found that each nucleotide present in CTTAG TGGGA AG is highly conserved. Similar to most of the famous RNA-cleaving DNAzymes, such as 8-17 and DEC 22-18,31 in the catalytic region of 2G-LYF5, these nucleotides can also form a simple but critical intramolecular motif: a 12-nt hairpin structure with a 3 base-pair conserved stem and a constant 8-nt loop. Preliminary experiments to identify its possible target have revealed that the biological target is probably a protein (or proteins) with a molecular weight greater than 100 KDa.

In summary, our study demonstrates that employing an extracellular molecular library of eukaryotic cells is a highly efficient method for developing a specific DNAzyme probe by bypassing labor-intensive and time-intensive target identification procedures. Although our method successfully allowed us to detect cancer cells using one cancer cell line, T47D, we believe that this methodology could easily be implemented for use with other cancer cells, such as hepatoma carcinoma cells or subtypes of breast cancer cells. Furthermore, our findings illustrate the capability of single-stranded DNA to perform highly sophisticated functions that are amenable to the development of diagnostic tests for early detection and rapid identification of
breast cancer cells and should be particularly helpful to cancer patients in developing countries.

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Supporting Information

Figure S1 Schematic of hypothetical reaction and double-random selection is depicted. Figure S2 Methodology for investigation of potential targets are depicted. Details of double-random selection are also described in the Experimental section. This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

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