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Application of Exonuclease III-Aided Target Recycling in Flow Cytometry: DNA Detection Sensitivity Enhanced by Orders of Magnitude

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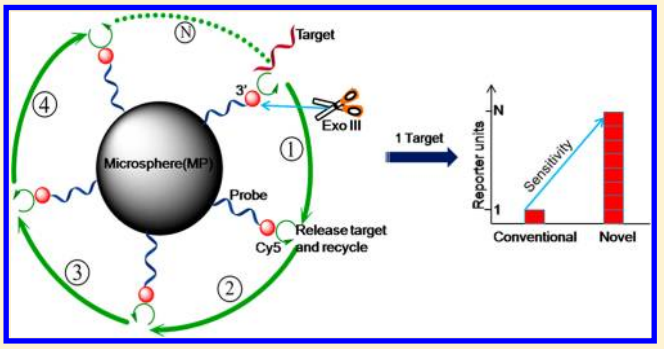
Supporting Information

ABSTRACT: DNA-functionalized microspheres in conjugation with flow cytometry detection are widely used for high-throughput nucleic acid assays. Although such assays are rapid and capable of simultaneous analysis of multiple nucleic acid analytes in a single test, the intrinsic limitation in sensitivity remains challenging. Here we report a simple, highly sensitive, and reproducible method based on Exonuclease III-aided target recycling technique applied for DNA quantification in flow cytometry. By loading a high density of Cy5-labeled probe DNA on microspheres (15 μm), we achieved hitherto unreported DNA detection limit of 3.2 pM in flow cytometry bead assay, enhancing the sensitivity by a factor of over 56.8 compared to the conventional direct hybridization bead assay. Furthermore, we evaluated multiplexing capability by simultaneous detections of two target DNAs with FAM and Cy5 reporter conjugated probes. Therefore, the novel Exonuclease III-amplified flow cytometry bead assay has great potential for the rapid, sensitive, and accurate detection and quantification of nucleic acids in clinical diagnosis and biomedical research.



Flow cytometry based nucleic acid bead assays have become increasingly important due to their flexibility and capability for the design of high-throughput multiplexing assays. Either the reporter dyes or microsphere carriers can be encoded with varieties of optical identities, which will meet the high demand of parallel analysis of multiple nucleic acid targets and molecular signatures of individual patients or environmental samples. Microspheres were first introduced as a solid support for the attachment of DNA in 1987, and the surface DNA hybridization was found to be comparable to that of a homogeneous solution reaction.[1] Since 1998, the Luminex multiplexing bead technique (also known as suspension arrays)[2] has led to the flourishing development of multiplexed DNA analysis in the identification of environmental microorganisms and infectious pathogens.[3−5] The arrays employ direct DNA hybridization on the matrix of beads,[6] each carrying one type of capture DNA probe, specific to each target DNA. Suspension arrays directly hybridize biotinylated PCR-amplified products of target DNA and then use streptavidin-phycoerythrin (SA-PE) or similar streptavidin-conjugated fluorescence dyes as a reporter. However, the analytical sensitivity and dynamic range of such multiplex assay strategies are currently remaining challenging.[7,8]

Exonuclease III (Exo III) is a 3′ to 5′ phosphatase, which will digest phosphodiester bonds from the 3′ blunt end or recessed end of double-stranded DNA. This feature of selective nucleotides digestion has been recently utilized to "recycle" target molecules, which will enhance the detectable signal level by hundreds of times compared to conventional methods, thus leading to polymerase chain reaction (PCR)-like sensitivity.[9] Exo III-based amplification has found wide applications in electrochemistry,[10] surface plasmon resonance (SPR),[11] and fluorescence-based DNA detection.[12−15]

In this study, we couple the Exo III-aided target recycling technique to a flow cytometry based DNA bead assay. The Exo III technique can recycle the low concentration target DNA analytes, which effectively amplifies the detectable signal limit and detects the analytes in a large analytical range, and thus the sensitivity of the DNA bead assay in flow cytometry has been dramatically enhanced. On the other hand, the flow cytometry technique maintains its advantages of flexibility, high throughput, and multiplexing compatibility through the suspension array assays.[16,17] Compared to other well-explored Exo III-aided DNA detection platforms, the Exo III-aided flow cytometry assay also permits the preparation of uniform probe-loaded microspheres (beads), which makes it possible to achieve reproducible assay results with minimal washing steps

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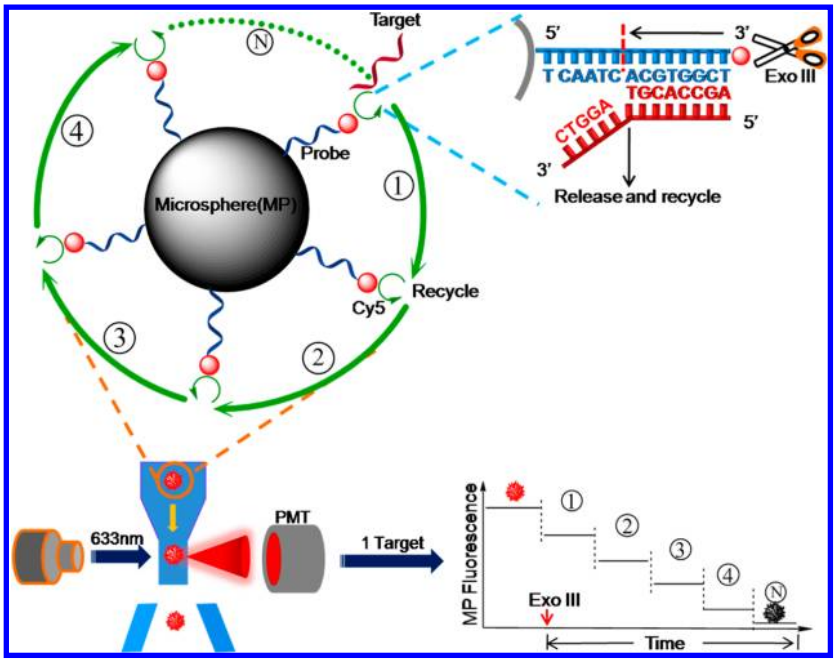

Figure 1. Schematic representation of Exo III based target recycling coupled to amplified flow cytometry DNA bead assay. Each round of target recycling could remove one Cy5 molecule from the microsphere surface, and the drop in fluorescence intensity of individual microsphere (functionalized by Cy5 probe) is proportional to the concentration of target DNA. Cy5 fluorescence intensity was monitored by flow cytometry. This is illustrated as the graph in bottom right, showing the gradually decreasing fluorescence on the microsphere from the activation of target recycling by Exo III.

for rapid and low-cost potentials.[18,19] Moreover, due to the easy control of surface probe-DNA densities on the beads, a large analytical range from pM to nM can be achieved. The beads also enable a multiplexing capability through the wide variety of available encoding techniques to simultaneously detect even 10^2−10^4 analytes.[20−22] Therefore, this superior DNA bead assay will result in a reproducible, separation-free, highly sensitive, high-throughput, and multiplexed DNA assay platform.

EXPERIMENTAL SECTION

Materials. The 15-μm carboxyl polymer microspheres (PC07N/8783) and 8-μm carboxyl magnetic microspheres (UMC4N/10487) were purchased from Bangs Laboratories. The 6-μm carboxyl polymer microspheres (cat. no. 17141) were purchased from Polysciences, Inc. Exonulease III was purchased from New England Biolabs (NEB). 2-(*N*-Morpholino)ethanesulfonic acid (MES) and *N*′-ethylcarbodiimide hydrochloride (EDC) were purchased from Sigma. All oligonucleotides were obtained from Integrated DNA Technologies.

Preparation of Probe DNA Functionalized Microspheres. The EDC method was used to conjugate the amine-modified Cy5 probe DNA onto the surface of the carboxyl microsphere. First, 5 μL of 10% solids of 15-μm microspheres was suspended in 50 μL of 50 mM MES buffer (pH = 6). Then varying amounts of probe DNA molecules were added into the solution. After brief mixing of the microsphere and probe mixture, 1 mg EDC was added to initiate the covalent reaction, and the total volume of the mixture was adjusted to 200 μL. Then the mixture was incubated on a vortex mixer for 2 h in a dark environment at room temperature. Finally, the Cy5 probe DNA functionalized microspheres were washed 3 times (9000 rpm for 5 min) and stored in MES buffer for further analysis. The same protocol was applied to Cy5 probe functionalized 8-μm and 6-μm carboxyl microspheres and FAM probe functionalized 15-μm microspheres.

Hemocytometer Counting of Microspheres. A Nikon TS100 microscope was employed to determine the concentration of microspheres. Before reacting with the target DNA, the number of functionalized microspheres was counted by hemocytometer (BLAUBRAND). The result was used to calculate the required concentrations of microspheres for further experiments.

Exo III Amplified DNA Bead Assay. The Exo III-amplified DNA detection was performed as follows: 100 U Exo III, 2000 probe functionalized microspheres (PD-High, PD-Medium and Cy5 6-μm microspheres) were added to react with varying concentrations of target DNA within a solution of a final volume of 100 μL using NEB buffer. The mixtures were incubated for 2 h on Eppendorf thermomixer (37 °C and 800 rpm). The same procedure was applied to perform the specificity evaluation except the concentration of all mismatch and perfect match target DNAs at 50 nM. The fluorescence intensities of microspheres were analyzed by a customized Cytek DxP6 flow cytometer, equipped with 25 mW 639 nm and 15mW 488 nm lasers. Flow cytometer settings: FSC Gain = 0.1×, SSC PMT = 65 V, BluFL1 PMT (FAM) = 215 V, RedFL1 PMT (Cy5) = 393 V.

Kinetics Study. The kinetics study of Exo III-aided DNA bead assay was carried out under the following conditions: 200 U Exo III, 4000 PD-High, PD-Medium microspheres, or Cy5 5.75-μm microspheres, 156 nM target DNA, and total volume in 200 μL of NEB buffer. Twenty-microliter samples were taken sequentially to be analyzed by flow cytometer at every 20 min in the period of 2 h.

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Multiplexed DNA Analysis. The multiplexed detection was performed in total volume of 100 μL of NEB buffer, containing 100 U Exo III, 1500 PD-High microsphere and 1500 FAM-Probe 2-Microsphere, and four combinations of target DNAs: (A) 0 nM T1 + 0 nM T2, (B) 50 nM T1 + 0 nM T2, (C) 0 nM T1 + 50 nM T2, (D) 50 nM T1 + 50 nM T2. Before running in the flow cytometer, 40 μL of 0.01 M NaOH was used to adjust the pH of solution to avoid the inefficiency of excitation of FAM dye.

RESULTS AND DISCUSSIONS

Assay Principle of Exo III-Amplified DNA Bead Assay. The schematic diagram of Figure 1 illustrates the principle of our novel technique for bead assays. By virtue of the ability to mediate 3′ blunt-end cleavage of double-stranded DNA, Exo III can selectively digest fluorescence reporter conjugated probes on a microsphere in a 3′ to 5′ direction, so that the target DNA will be released to hybridize with a secondary probe DNA on the microsphere. Because one target DNA can be recycled hundreds of times to repeatedly initiate the removal of fluorescence reporter, this Exo III amplifying technique can improve sensitivity by a factor greater than 1 order of magnitude. Taking advantage of quantifying the signal strengths of individual microsphere,[23] flow cytometry can detect the signal of extremely low concentrations of target DNA after Exo III amplification, thereby making the Exo III-based sensitive DNA detection and flow cytometry based rapid bead assays perfectly compatible with each other.

In order to evaluate the efficiency and robustness of the Exo III-amplified DNA bead assay, we first selected Cy5 as a fluorescence reporter and 15-μm (diameter) carboxyl polystyrene microspheres as the probe DNA carrier. Cy5 is a cyanine dye widely used in real time PCR[24] and microarray applications[25] due to its brightness (high extinction coefficient) and flexible capability to label nucleic acids. We chose slightly larger microspheres as carriers, because the high density of carboxyl groups on their larger surface areas permits the attachment of thousands of probe DNAs. To demonstrate the effectiveness of probe DNA−microsphere conjugation, we precisely controlled the loading density of probe DNAs on a single microsphere by varying the initial concentration of Cy5 conjugated DNA probe (5′-/5AmMC12/TGTGTGTGCCAG-ATTGAGGTCTTC/3Cy5Sp/-3′). The chemically covalent immobilization of an amine-modified probe onto the carboxyl microsphere surface was activated by ethylcarbodiimide (EDC).[26] As shown in Figure 2 and Supplementary Figure S1, the fluorescence intensity of the microsphere (Red FL1 channel) gradually increased as the amount of probe increased from 2 pmol to 125 pmol and then reached a plateau of saturation with higher probe concentrations (250−500 pmol). The mean intensity (3.35) of the unlabeled microsphere was more than 3 orders of magnitude lower than that of the labeled microsphere populations. These results confirmed that the Cy5 probe DNA was successfully conjugated onto the microsphere.

Assay Kinetics. To investigate the reaction kinetics of the Exo III-based bead assay, the intensities of two typical populations of microspheres (refer to Figure 2, PD-High and PD-Medium) in the presence of 156 nM target DNA were monitored in real time. This was carried out by sequentially sampling the reacted microspheres with an interval of every 20 min followed by flow cytometry quantification of the fluorescence signal strength of reacted microspheres. The drop in signal fluorescence intensity was calculated by comparing the mean signal strength of reacted microsphere populations to the mean intensity of initial microspheres. The absolute change in the mean intensity along reaction time has been plotted as Figure 3. The reaction was active in the first 20 min and quickly reached completion by the time of 30 min, and the intensity of reacted microspheres after 1 h then became stable. Therefore, for reliable quantitation, a 2-h reaction time was used in the following experiments.

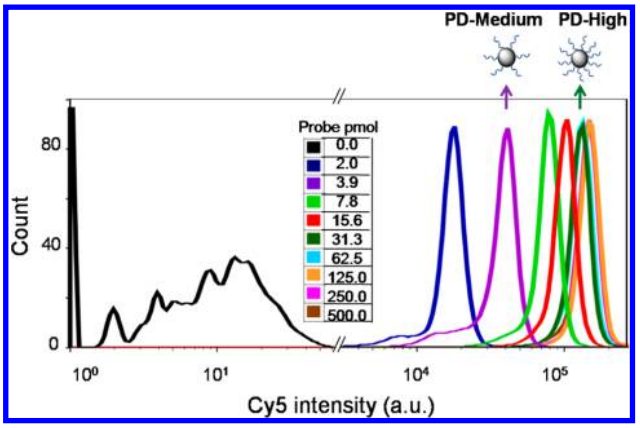

Figure 2. Flow cytometric monitoring of the fluorescence intensities of carboxyl microspheres upon addition of varying amounts of Cy5-labeled probe DNA (0, 2.0, 3.9, 7.8, 15.6, 31.3, 62.5, 125, 250, and 500 pmol). The fluorescence intensity of the microsphere gradually increased (2−125 pmol) and then reached a plateau (250−500 pmol). Probe Density-Medium (PD-Medium) and Probe Density-High (PD-High). The assay was performed in the solution containing 1 mg EDC and 5 μL of 15 μm microspheres (10% solids).

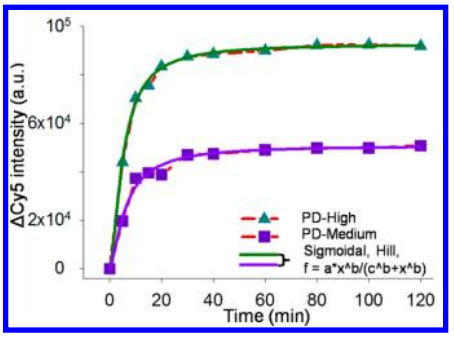

Figure 3. Kinetics study of the Exo III-amplified DNA bead assay. *Y*-axis value represents the drop of Cy5 fluorescence intensities of microspheres. The solid colored lines were fitted to a Hill, 3-parameter sigmoidal equation by SigmaPlot software. Experiments conditions: 200 U Exo III, 4000 PD-High or PD-Medium microspheres, 156 nM target DNA, and total volume in 200 μL of NEB buffer. Twenty-microliter samples were taken to collect data at each time point.

DNA Detection Sensitivity. In order to explore the optimum factors to achieve a higher sensitivity with a large analytical range, incremental concentrations of target DNA 1(T1, 5′-GAA GAC CTC AAT CTG TGT GGC-3′), ranging from 38 pM to 10 μM, were analyzed by the PD-High and PD-Medium microspheres, respectively. After 2-h reactions, the mean fluorescence intensities of each population of reacted microspheres were measured by flow cytometry, and the absolute drop in intensity versus the incremental concentrations of target DNAs have been plotted as Figure 4. The trends of these reactions for both PD-High and PD-Medium microspheres follow a sigmoid increase, but the PD-High

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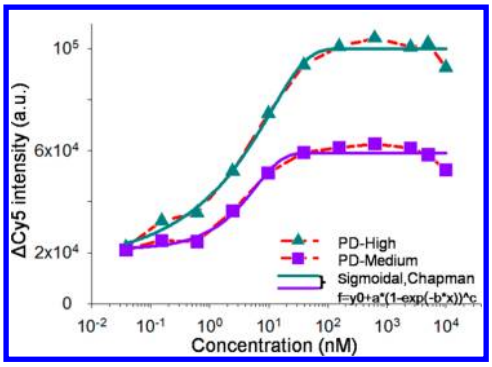

Figure 4. Change of fluorescence intensity of Cy5 probe DNA funtionalized microspheres as a function of target DNA concentration (10 μM, 5 μM, 2.5 μM, 625 nM, 156 nM, 39 nM, 9.8 nM, 2.4 nM, 610 pM, 152 pM, and 38 pM). The solid colored lines were fitted to a Chapman, 4-parameter sigmoidal equation by SigmaPlot software. Experiments conditions: 100 U Exo III, 2000 PD-High or PD-Medium microspheres, target DNA concentrations, and total volume in 100 μL of NEB buffer. Incubation time: 2 h.

microspheres exhibited a larger dynamic concentration range (from 38 pM to 625 nM) compared to the PD-Medium microspheres (from 610 pM to 39 nM). Interestingly, in the presence of excess target DNA, the produced signals showed a slight drop, presumably due to homologous DNA strand competition, which is consistent with the retarded hybridization phenomena reported by Galau et al.[27]

Moreover, in order to evaluate the limit of detection (LoD) of the novel Exo III-amplified bead assay, the established protocol according to ref 28 was employed, described as $f(\mathrm{LoD}) = (\Delta S_1 + \Delta S_2 + \ldots + \Delta S_n)/n + 2 \times \mathrm{SD}$, where f is the sigmoidal curve function (Figure 4), which stands for the signal decrease in mean intensity (ΔS_n) of a microsphere population at trail n, and SD is the standard deviation over n trails of experiments. In this work, four trails ($n = 4$) have been carried out by testing both PD-High and PD-Medium microspheres in the presence of Exo III, but in the absence of target DNA. The slight signal decreases in mean intensities of both populations were analyzed by flow cytometry and fitted into the above formula, resulting in the corresponding LoDs for both PD-High and PD-Medium microspheres. The PD-High microspheres achieved the lowest LoD of 3.2 pM, which was 10 times more sensitive than the PD-Medium microspheres (37.1 pM). Therefore we conclude that high-density probe microspheres offer a larger analytical range and permit more sensitive detection of low abundance nucleic acids. Both the large analytical range and low LoD achieved by PD-High microspheres eliminates the potential concerns of steric hindrance reported in previous literature.[29] The LoD of 3.2 pM achieved by PD-High microspheres provides evidence that the Exo III-aided target recycling technique can significantly improve the sensitivity of flow cytometry based DNA bead assays by a factor of over 56.8 compared to the conventional hybridization DNA bead assay.[7] The slight decrease (~1/15) of mean fluorescence intensity of Cy5-functionalized microspheres in the absence of target DNA might be ascribed to hairpin formation of the probe, which could allow Exo III to nonspecifically remove the Cy5 reporter from microspheres. Nevertheless, it might be easily overcome by hairpin-free probe design.

Assay Specificity. To further evaluate the specificity of the Exo III-amplified bead assay, we extended our experiments by reacting both PD-High and PD-Medium functionalized microspheres with 1-base and 3-base mismatch and nonmatch DNA sequences (shown in Figure 5A). Each type of reaction was tested with four replicates, and the assay specificity was described as the drops in normalized fluorescence intensity compared to the perfect match target DNA (PD-High and PD-Medium results shown in Figure 5B and C). The specificity decreased consistently as the number of mismatch bases increased (dropped by 22%, 38%, and 91% for 1-base mismatch, 3-base mismatch, and nonmatch groups, respectively). The PD-Medium bead population resulted in slightly lower specificity. This is because the lower probe density may have lower steric hindrance and electrostatic repulsion, which may provide more chance to interact with mismatch sequences.[30,31] Moreover, the specificity of the Exo III-aided bead assay can be further improved by utilizing smaller microspheres (6 μm) (Supplementary Figure S2). Compared to the normalized signal of the perfectly matched target, the specificities were calculated in 1-base mismatch group as 35% drop (6 μm) versus 22% drop (15 μm) and in the 3-base mismatch group as 60% drop (6 μm) versus 38% drop (15

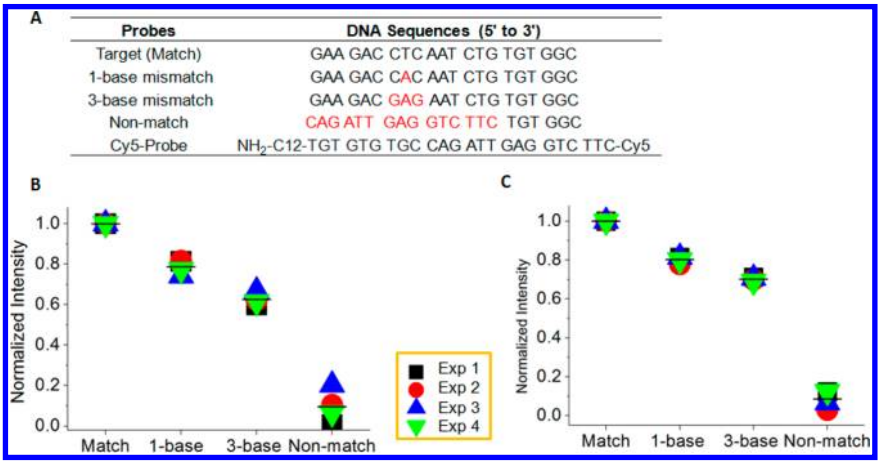

Figure 5. Specificity of the Exo III amplified DNA bead assay. (A) Sequences of target, 1-base and 3-base mismatch, and nonmatch target DNA. The bases marked in red represent the mismatch positions. (B, C) Scatter plot showing the normalized intensity for each group respectively; all data were normalized to the perfect match target signal. Different colors in each group represent four repeated experiments (Exp) data. (B) PD-High microspheres. (C) PD-Medium microspheres.

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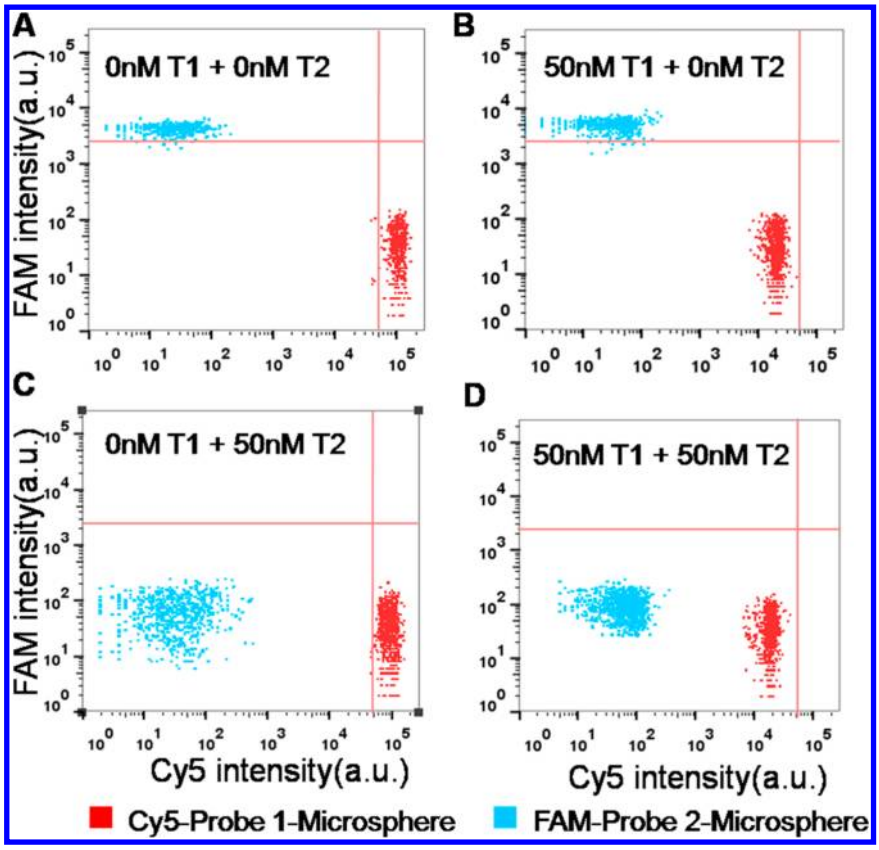

Figure 6. Multiplexed analysis of Target DNA-1 (T1) and Target DNA-2 (T2) using Cy5-Probe 1-Microsphere (PD-High group) and FAM-Probe 2-Microsphere and bead panel. FAM (Ex 488 nm, PMT 530/30), Cy5 (Ex 639 nm, PMT 661/16). Experiments conditions: 100 U of Exo III, 1500 Cy5-Probe 1 and FAM-Probe 2-Microspheres, (A) 0 nM T1 + 0 nM T2, (B) 50 nM T1 + 0 nM T2, (C) 0 nM T1 + 50 nM T2, (D) 50 nM T1 + 50 nM T2, and total volume in 100 μL of NEB buffer. Incubation time: 2 h.

μm). This may be caused by the small sized microsphere having a higher disassociate rate between mismatch targets and capture probes. These results suggest that the Exo III-amplified bead assay could be applied for detection of single-nucleotide polymorphisms (SNPs).

Stability and Other Factors of Microspheres. The performance of this assay was further comprehensively characterized by functionalized-microsphere stability (Supplementary Figure S3), different sizes of microspheres (Supplementary Figure S4), and the number of reaction microspheres (Supplementary Figure S5). Supplementary Figure S3 shows that the fluorescence intensities were stable at above 92% of initial value (over 6 days), suggesting the functionalized microspheres have long shelf life. Supplementary Figure S4 suggests larger sized (15 μm) microspheres have better contrast than smaller microspheres (8 μm) (81.9% vs 49.8%) in the presence of the same concentration of target DNA (50 nM). Supplementary Figure S5 suggests the smaller number of reaction microspheres resulted in higher sensitivity, probably because there would be more target DNA reacting with probe DNAs on each of the reduced number of microspheres.[32]

Multiplexed Capability. To verify that our technique is compatible for multiplexing, we prepared a second type of functionalized microspheres by conjugating with another probe, DNA-FAM[33] (5′-/5AmMC12/TGTGTGTGCAGCATTG-AGTTGGAG/36-FAM/-3′), designed to hybridize with target DNA-2 (T2, 5′-CTCCAACTCAATGCTTGTGGC-3′). In the presence of different combinations of T1 and T2 (0 or 50 nM), two types of functionalized microspheres can simultaneously detect two DNA targets without cross-talk between the DNA analytes (shown in Figure 6 and Supplementary Figure S6). These results suggest that the Exo III-amplified DNA bead assay can be successfully multiplexed.

In conclusion, this work discovered a new platform technology for a high-speed, multiplexed DNA assay with remarkably enhanced sensitivity. We successfully applied an Exo III-aided target recycling technique coupled with a flow cytometric bead assay, with an improved sensitivity by a factor of over 56.8 compared to that of a conventional flow cytometric nucleic acid assay. This provides a high-throughput protocol with a PCR-like sensitivity, an excellent analytical range, and flexibility in the design of multiplexed assays, as well as minimizing the washing and sample preparation steps. Therefore, we propose that this Exo III-amplified flow cytometric nucleic acid bead assay has the potential to become a powerful tool in microbial detection, gene expression profile analysis,[34] single-nucleotide polymorphism (SNP) analysis,[35,36] DNA biomarker detection, and drug discovery areas.[37]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Wolf, S. F.; Haines, L.; Fisch, J.; Kremsky, J. N.; Dougherty, J. P.; Jacobs, K. *Nucleic Acids Res.* **1987**, *15*, 2911.
(2) Smith, P. L.; WalkerPeach, C. R.; Fulton, R. J.; DuBois, D. B. *Clin. Chem.* **1998**, *44*, 2054.
(3) Defoort, J. P.; Martin, M.; Casano, B.; Prato, S.; Camilla, C.; Fert, V. *J. Clin. Microbiol.* **2000**, *38*, 1066.
(4) Spiro, A.; Lowe, M. *Appl. Environ. Microbiol.* **2002**, *68*, 1010.
(5) Freeman, R.; Liu, X.; Willner, I. *J. Am. Chem. Soc.* **2011**, *133*, 11597.
(6) Dunbar, S. A. *Clin. Chim. Acta* **2006**, *363*, 71.
(7) Spiro, A.; Lowe, M.; Brown, D. *Appl. Environ. Microbiol.* **2000**, *66*, 4258.
(8) Tai, V.; Burton, R. S.; Palenik, B. *Mar. Ecol.: Prog. Ser.* **2011**, *426*, 133.
(9) Zuo, X.; Xia, F.; Xiao, Y.; Plaxco, K. W. *J. Am. Chem. Soc.* **2010**, *132*, 1816.
(10) Fan, Q.; Zhao, J.; Li, H.; Zhu, L.; Li, G. *Biosens. Bioelectron.* **2012**, *33*, 211.
(11) Lee, H. J.; Li, Y.; Wark, A. W.; Corn, R. M. *Anal. Chem.* **2005**, *77*, 5096.
(12) Freeman, R.; Liu, X.; Willner, I. *Nano Lett.* **2011**, *11*, 4456.
(13) Liu, X. Q.; Aizen, R.; Freeman, R.; Yehezkeli, O.; Willner, I. *ACS Nano* **2012**, *6*, 3553.
(14) Zhang, M.; Guan, Y. M.; Ye, B. C. *Chem. Commun.* **2011**, *47*, 3478.
(15) Zhao, C. Q.; Wu, L.; Ren, J. S.; Qu, X. G. *Chem. Commun.* **2011**, *47*, 5461.
(16) Nolan, J. P.; Sklar, L. A. *Trends Biotechnol.* **2002**, *20*, 9.
(17) Nolan, J. P.; Mandy, F. F. *Cell. Mol. Biol.* **2001**, *47*, 1241.
(18) Kettman, J. R.; Davies, T.; Chandler, D.; Oliver, K. G.; Fulton, R. J. *Cytometry* **1998**, *33*, 234.
(19) Kellar, K. L.; Iannone, M. A. *Exp. Hematol.* **2002**, *30*, 1227.
(20) Dunbar, S. A.; Jacobson, J. W. *Cytometry* **2000**, *42*, 317.
(21) Dunbar, S. A.; Vander Zee, C. A.; Oliver, K. G.; Karem, K. L.; Jacobson, J. W. *J. Microbiol. Methods* **2003**, *53*, 245.
(22) Dunbar, S. A. *Clin. Chim. Acta* **2006**, *363*, 71.
(23) Ortaldo, J. R.; Sharrow, S. O.; Timonen, T.; Herberman, R. B. *J. Immunol.* **1981**, *127*, 2401.
(24) Vandesompele, J.; De Preter, K.; Pattyn, F.; Poppe, B.; Van Roy, N.; De Paepe, A.; Speleman, F. *Genome Biol.* **2002**, *3*, RESEARCH0034.
(25) Schena, M.; Shalon, D.; Heller, R.; Chai, A.; Brown, P. O.; Davis, R. W. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 10614.
(26) Lu, J.; Martin, J.; Lu, Y.; Zhao, J.; Yuan, J.; Ostrowski, M.; Paulsen, I.; Piper, J. A.; Jin, D. *Anal. Chem.* **2012**, *84*, 9674.
(27) Galau, G. A.; Smith, M. J.; Britten, R. J.; Davidson, E. H. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 2306.
(28) Armbruster, D. A.; Tillman, M. D.; Hubbs, L. M. *Clin. Chem.* **1994**, *40*, 1233.
(29) Peterson, A. W.; Heaton, R. J.; Georgiadis, R. M. *Nucleic Acids Res.* **2001**, *29*, 5163.
(30) McCarthy, B. J.; Church, R. B. *Annu. Rev. Biochem.* **1970**, *39*, 131.
(31) Bajaj, M. G. Ph.D. Thesis, Massachusetts Institute of Technology, 2005.
(32) Connolly, A. R.; Palanisamy, R.; Trau, M. *J. Biotechnol.* **2010**, *145*, 17.
(33) You, Y.; Tataurov, A. V.; Owczarzy, R. *Biopolymers* **2011**, *95*, 472.
(34) Yang, L.; Tran, D. K.; Wang, X. *Genome Res.* **2001**, *11*, 1888.
(35) Armstrong, B.; Stewart, M.; Mazumder, A. *Cytometry* **2000**, *40*, 102.
(36) Bortolin, S.; Black, M.; Modi, H.; Boszko, I.; Kobler, D.; Fieldhouse, D.; Lopes, E.; Lacroix, J. M.; Grimwood, R.; Wells, P.; Janeczko, R.; Zastawny, R. *Clin. Chem.* **2004**, *50*, 2028.
(37) Meza, M. B. *Drug Discovery Today* **2000**, 38.