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Ratiometric Fluorescence Coding for Multiplex Nucleic Acid Amplification Testing

Ye Zhang[†], Liben Chen[‡], Kuangwen Hsieh[‡], and Tza-Huei Wang^{*,†,‡,§}

[†] Department of Biomedical Engineering, Johns Hopkins University, Baltimore, Maryland 21218, United States

[‡] Department of Mechanical Engineering, Johns Hopkins University, Baltimore, Maryland 21218, United States

[§] Institute for NanoBioTechnology, Johns Hopkins University, Baltimore, Maryland 21218, United States

Abstract

Although nucleic acid amplification testing (NAAT) has become the cornerstone for molecular diagnosis of diseases, expanding the multiplexed detection capacity of NAAT remains an important objective. To this end, encoding each nucleic acid target with a specific fluorescently labeled probe has been the most mature approach for multiplexed detection. Unfortunately, the number of targets that can be differentiated via this one-target-one-fluorophore multiplexed detection approach is restricted by spectral overlaps between fluorophores. In response, we present herein a new multiplexed detection approach termed ratiometric fluorescence coding, in which we encode each nucleic acid target with a specific ratio between two standard fluorophores. In ratiometric fluorescence coding, we employ the padlock probe chemistry to encode each nucleic acid target with a specific number of binding sites for two probes labeled with different fluorophores. Coupling the padlock probes with either rolling circle amplification (RCA) or hyperbranched rolling circle amplification (HRC), we transform each nucleic acid target into a specific template that allows hybridization with the fluorescently labeled probes at predesigned ratios, thereby achieving multiplexed detection. For demonstration, we detected DNA targets from six infectious diseases and demonstrated the potential for further expanding the multiplexing capability of our approach. With further development, ratiometric fluorescence coding has the potential to enable highly multiplexed detection of nucleic acid targets and facilitate molecular diagnosis of diseases.

Graphical Abstract:

*Corresponding Author thwang@jhu.edu. Tel: (410)516-7086. Fax: (410) 516-7254.

The authors declare no competing financial interest.

ASSOCIATED CONTENT

Supporting Information

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Additional information includes Table S1 (nucleic acid sequences used in the study), Figure S1 (gel electrophoresis result), and Table S2 (approximation of maximum number of fluorescence ratios for further expanding multiplexing capacity) (PDF)

Nucleic acid amplification testing (NAAT) has emerged as a popular technique for the diagnosis of many diseases including cancer,^{1,2} genetic disorders,³ mitochondrial disorders,⁴ and infectious diseases.^{5,6} Indeed, NAAT methods such as real-time polymerase chain reaction (PCR),^{7,8} ligase chain reaction (LCR),^{9,10} and nucleic acid sequence-based amplification (NASBA)^{11,12} have been widely employed for nucleic acid detection because of their high sensitivity, specificity, and rapid turn-around-time. In addition, a number of different approaches can be coupled with NAAT to achieve multiplexed detection of multiple targets in a single assay, which is a particularly useful capability for clinical diagnosis of diseases.^{5,13} Among these approaches, which range from differentiating the melting temperatures of NAAT amplicons^{14,15} to assigning targets with probes that have distinct lengths,^{16–19} the most commonly employed strategy has been using a target-specific, fluorescently labeled oligonucleotide probe for each target nucleic acid sequence. While this “one-color-one-target” approach is straightforward and effectively leverages the mature fluorescence detection infrastructure associated with NAAT methods, it unfortunately has a limited capacity of multiplexing because spectral overlaps between fluorophores restrict the number of fluorophores that can be used within a single assay. As such, there remains a need for fluorescence-based approaches that extend beyond the one-color-one-target restriction and expand the multiplexing capacity of NAAT.

A natural and promising extension to the one-color-one-target approach is using a unique combination of multiple fluorophores to encode each target sequence. A handful of strategies for demonstrating this approach have been reported. For example, 15-plex detection using only 4 fluorophores has been achieved through a strategy called multicolor combinatorial probe coding (MCPC).^{20,21} To achieve 15-plex detection via MCPC, however, 32 different fluorescently labeled probes were synthesized, which escalated the cost of this strategy. Moreover, the probe design principle in MCPC capped its multiplexing capacity to 15 when using four fluorophores, and as spectral overlaps between fluorophores make it difficult to incorporate additional fluorophores, MCPC had likely reached its maximum multiplexing capacity. Another strategy, which was coined binary-scheme mathematical theory, employed each fluorescence color as a “digit” in the binary code and assigned each target sequence with a unique binary code of at least two colors/digits.²² Unfortunately, even when using four fluorophores, this strategy still only achieved 4-plex detection. Therefore, despite the potential, effective strategies for leveraging combinations of multiple fluorophores to achieve multiplexed NAAT remain underdeveloped to date.

Herein, we describe ratiometric fluorescence coding, our new strategy for expanding the multiplexed detection capacity of NAATs by encoding each nucleic acid target with a specific fluorescence ratio. Specifically, in ratiometric fluorescence coding, we transform each target into a specific DNA template that can hybridize with multiple fluorescently labeled probes with distinct colors at a predesigned, unique ratio. In doing so, we can specifically detect each target while obviating the restriction of spectral overlaps between fluorophores. Moreover, since only the ratios of fluorescent probe hybridization sites in DNA templates are designed to vary but the sequences of the hybridization sites remain the same, our ratiometric fluorescence coding can utilize the same set of fluorescently labeled probes to detect multiple targets. For demonstrating the concept of ratiometric fluorescence coding, we utilized padlock probe chemistry²³ for probe design and coupled with either rolling circle amplification (RCA) or hyperbranched rolling circle amplification (HRCA)^{24–26} for DNA template generation, which allows hybridization with fluorescently labeled probes. Using our RCA and HRCA assays and only two fluorescently labeled probes, we demonstrate the detection of the gene sequences from up to six infectious diseases, including four sexually transmitted diseases (STDs) and two urinary tract infections (UTIs), with distinct and highly resolved fluorescence ratios, illustrating that ratiometric fluorescence coding offers ready means for expanding the multiplexed detection capacity of NAATs.

EXPERIMENTAL SECTION

Materials and Reagents.

All DNA oligonucleotides including synthetic targets, padlock probes, and primers were purchased from Integrated DNA Technologies, Inc. (Coralville, IA) and both fluorescently labeled peptide nucleic acid (PNA) probes were purchased from PNA Bio (Newbury Park, CA). Reagents for ligation, RCA, and HRCA reactions, including 9°N™ DNA ligase, 10× 9°N DNA ligase reaction buffer, T4 gene-32 protein, phi29 DNA polymerase, Exonuclease I (*E. coli*), Exonuclease III (*E. coli*), 10× phi29 DNA polymerase reaction buffer, Bst DNA polymerase, and 10× Bst polymerase reaction buffer were purchased from New England BioLabs, Inc. (Ipswich, MA). Deoxyribonucleotide triphosphate (dNTP) was purchased from Invitrogen Corp. (Carlsbad, CA). Formamide, sodium chloride (NaCl), EDTA (pH 8.0), bovine serum albumin (BSA), betaine, and H₂O (PCR grade) were purchased from Sigma-Aldrich Corp. (St. Louis, MO).

Nucleic Acid Sequences.

Padlock probes targeting gene sequences in *Proteus mirabilis* (PM, 238:275 from AF240693) *Human immunodeficiency virus 1* (HIV-1, 258:305 from KC966998), *Neisseria gonorrhoeae* (NG, 362:411 from X52364), *Chlamydia trachomatis* (CT, 50:98 from JX648604), *Treponema pallidum* (TP, 801:846 from KC966998), and *Escherichia coli* (EC, 189:230 from AY447194) were designed in-house. All padlock probe and synthetic target sequences are given in Table S1. The sequence of the RCA primer is 5'-CTAAAGCTGAGACATGACGAGTC, and the sequence of the additional primer for performing HRCA is 5'-TCAGAACTCACCTGTAG. The sequences of the red and the

green PNA probes are [5' AlexaF647] TCAGAACTCACCTGTTAG and [5' AlexaF488] CCCTAACCCCTAACCTAA, respectively.

Padlock Probe Hybridization and Ligation.

Our ratiometric fluorescence coding assays began with padlock probe hybridization and ligation, which were achieved in a single-tube reaction. The 25- μL reaction mixture contained 45 nM of each linear padlock probe, synthetic DNA targets (at different concentrations), 0.4 units/ μL 9°N™ DNA ligase, and 1 $\times$ 9N DNA ligase reaction buffer (10 mM Tris-HCl, 600 μM ATP, 2.5 mM MgCl₂, 2.5 mM dithiothreitol, 0.1% Triton X-100, pH 7.5 at 25 °C). Padlock probe hybridization and ligation was performed at 60 °C for 1 h. For subsequent HRCA reaction, only 100 pM of each padlock probe were ligated in the presence of synthetic DNA targets or pathogen genomic DNA to reduce nonspecific amplification and improve signal-to-noise-ratio.

RCA and HRCA Reactions.

RCA or HRCA were performed immediately after ligation of padlock probes to generate DNA templates that allow hybridization with fluorescently labeled probes. For RCA, 2 μL of the ligation product was mixed with 800 nM rolling-circle primer, 400 μM dNTPs, 10 U/ μL phi29 DNA polymerase, and 1 $\times$ phi29 DNA polymerase reaction buffer (50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 200 $\mu\text{g}/\text{mL}$ acetylated BSA) to a final volume of 25 μL . Of note, due to its high strand-displacing activity, phi29 DNA polymerase was added last to the reaction mixture, which was then immediately incubated at 31 °C for 1 h to perform RCA, followed by 70 °C for 10 min to inactivate the polymerase and stop the reaction.

For HRCA, 25 μL of the ligation product was first treated with 10 units of Exonuclease I and 50 units of Exonuclease III at 37 °C for 1 h to remove extra linear padlock probes that were not circularized, followed by a brief incubation at 85 °C for 10 min to inactivate the enzymes. Then 4 μL of this enzyme-treated ligation product was mixed with 400 nM of each of the two rolling-circle primers, 400 μM dNTPs, 40 ng/ mL T4 gene-32 protein, 10 U/ μL Bst DNA polymerase, and 1 $\times$ Bst polymerase reaction buffer (20 mM Tris-HCl (pH 8.8), 10 mM KCl, 2.7 mM MgSO₄, 5% v/v DMSO, 0.1% Triton $\times$ 100) to a final volume of 25 μL . The reaction mixture was incubated at 60 °C for 1 h to perform HRCA, followed by 85 °C for 10 min to inactivate the polymerase and stop the reaction.

Fluorescent PNA Hybridization and Detection.

A total of 10 μL of RCA or HRCA product was mixed with 500 nM AlexaF488-labeled PNA probe and 500 nM AlexaF647-labeled PNA probe in a PNA hybridization buffer (40% formamide, 10 mM NaCl, and 50 mM Tris-HCl (pH 8)), heated at 85 °C for 5 min, and then incubated at room temperature in the dark for 2 h to allow hybridization. Nonhybridized, excess fluorescent PNA probes were removed by using S400-HR microspin columns (GE Healthcare). Each purified sample was pipetted into a well of a 384-well plate (Corning Inc.) and read out on a Typhoon image scanner (GE Healthcare) in both the red channel (peak excitation at 633 nm with 670 nm band-pass emission filter) and the green channel (peak excitation at 488 nm with 526 nm short-pass emission filter) at a photomultiplier tube (PMT) voltage of either 600 or 650 V. Of note, for each experiment, the PMT voltage was kept the

same between the red channel and the green channel (i.e., both at 600 V or both at 650 V). Between experiments, however, the PMT voltage was sometimes adjusted between either at 600 or at 650 V.

Data Analysis.

Red and green fluorescence intensities of each sample measured by the Typhoon image scanner were quantified by ImageQuantTL software (GE Healthcare). The R/G ratio was calculated via dividing the red fluorescence intensity by the green fluorescence intensity.

RESULTS AND DISCUSSION

Design of Padlock Probes for Ratiometric Fluorescence Coding.

Our ratiometric fluorescence coding encodes each target of interest with a specific fluorescence color ratio. This is achieved by transforming each target to a specific DNA template sequence with a predesigned and unique ratio of binding sites for multiple fluorescently labeled probes with distinct colors. As such, upon hybridization with fluorescent probes, the template-probe complex displays the specific ratiometric fluorescence code whose intensity of each fluorescence color can be measured to decode and identify the target (Figure 1A). Importantly, in ratiometric fluorescence coding, the number of maximum target-specific fluorescence ratios (i.e., the multiplexing capacity) for a given number of fluorescence colors is governed by a polynomial relationship with the number of fluorescent probe binding sites (S) that can be designed on a DNA template. In the case of two fluorescence colors, the maximum achievable fluorescence ratios are $\frac{(S^2 + 4S - 1)}{4}$ for an odd number of S and $\frac{(S^2 + 3S + 3)}{4}$ for an even number of S , respectively. For example, with only a red fluorophore (e.g., AlexaF647) and a green fluorophore (e.g., AlexaF488), we can achieve up to 7 fluorescence ratios if we design 4 fluorescent probe binding sites ($S = 4$) on each DNA template, and up to 11 ratios if we design 5 binding sites ($S = 5$) on each DNA template. Moreover, in ratiometric fluorescence coding, only the numbers of fluorescence probe binding sites in the DNA templates are designed to vary but the sequences of the fluorescence probe binding sites remain the same. As such, the same fluorescently labeled probes can be used for detecting multiple targets. These features allow our ratiometric fluorescence coding to expand the multiplexing capacity when compared with the traditional one-color-one-target approach and multifluorophore strategies without requiring a large number of fluorescently labeled probes.

Padlock probe chemistry provides an effective means for achieving ratiometric fluorescence coding due to its flexibility for probe design. In this work, we design each padlock probe to contain a target-specific hybridization region, a uniquely predesigned ratio of binding sites for the red and the green fluorescent probes, and general primer sites (Figure 1B). In the presence of its specific DNA target, the initially linear padlock probe is first hybridized to the target and then ligated to become a circular probe, from which DNA templates can be generated via various amplification techniques to amplify the number of fluorescent probe binding sites at the predesigned fluorescent probe ratio encoded in each padlock probe.

^{24,25,27} Following this design principle, we designed six circularizable padlock probes to detect 6 common infectious diseases (4 STDs and 2 UTIs) in this work. The target-specific regions of these 6 padlock probes target conserved sequences from *Proteus mirabilis* (PM), *Human immunodeficiency virus 1* (HIV1), *Neisseria gonorrhoeae* (NG), *Chlamydia trachomatis* (CT), *Treponema pallidum* (TP), and *Escherichia coli* (EC). Moreover, we incorporated the binding sites for the red and the green fluorescent probes at specific red to green (R/G) ratios into these padlock probes: 0:1 for PM, 1:2 for HIV-1, 1:1 for NG, 2:1 for CT, 3:1 for TP and 1:0 for EC, respectively (Figure 1C).

We have also coupled padlock probe chemistry with molecular amplification techniques, such as RCA and HRCA, to amplify the fluorescence signals in ratiometric fluorescence coding. More specifically, we begin by challenging the infectious disease-related DNA targets to the mixture of our six target-specific, linear padlock probes. In the presence of a DNA target, the corresponding linear padlock probe is hybridized and ligated to form a closed padlock probe. Then RCA reaction amplifies along the circular padlock probe to generate DNA templates with hundreds of tandem repeats that serve as hybridization sites for fluorescent probes. In this work, we employed AlexaF488- and AlexaF647- peptide nucleic acid (PNA) as the hybridization probes, because its electro-neutral polypeptide backbones enable high hybridization efficiency with the DNA templates.^{28,29} In addition, the dissociation rate of PNA/DNA hybrid is significantly slower than that of DNA duplexes, thus allowing the removal of nonhybridized, excess PNA from the reaction without reducing the fluorescence signals of the template-probe complexes due to dissociation of hybridized fluorescent probes. After fluorescent PNA probe hybridization and purification, the red and the green fluorescence intensities of the template-probe complexes are measured, and the resultant R/G fluorescence ratio is used to identify the DNA target (Figure 1D).

Ratiometric Fluorescence Detection with RCA.

Using our RCA-based ratiometric fluorescence coding assay, we first demonstrate the detection of six infectious disease-related gene sequences with distinct fluorescence ratios. To do so, we used six synthetic oligonucleotides derived from conserved gene sequences of PM, HIV-1, NG, CT, TP, and EC as the targets, challenged each of them to our mix of six padlock probes and measured the red and the green fluorescence intensities of each sample at the end of the RCA reaction and probe hybridization. Fluorescent images of the six samples reveal that the six targets indeed yielded fluorescence ratios that were noticeably different from each other (Figure 2A). Moreover, the measured R/G ratio of each target closely matched its designed R/G ratio. For example, for the PM sample, the corresponding padlock probe of which is inserted with only green fluorescent probe binding sites, we observed only green fluorescence and no red fluorescence was detected, resulting in a measured R/G ratio of 0. While for the HIV-1 sample we observed weak red fluorescence and strong green fluorescence and measured a R/G ratio of ~0.5. In contrast, for the TP sample, we observed strong red fluorescence and weak green fluorescence, and measured a R/G ratio of ~3.0. Whereas for the EC sample, the padlock probe of which contains only red fluorescent probe sequence, we only observed fluorescence in the red channel and thus the R/G ratio was measured to be infinity. These fluorescence detection results clearly indicate

that our RCA-based ratiometric fluorescence coding assay can achieve multiplexed detection of nucleic acid targets based on distinct R/G ratios.

Our RCA-based ratiometric assay detects the six infectious disease-related gene sequences with high reproducibility. Here, we replicated our assay for PM, HIV-1, NG, CT, TP, and EC synthetic targets in four separate experiments and plotted the average R/G ratios with error bars based on the four independent measurements (Figure 2B). The close match between the measured R/G ratios and the predesigned R/G ratios provides strong support for the design principle of ratiometric fluorescence coding. In addition, the small error bars of measured ratios indicate that each target-specific ratio is indeed significantly different from each other and that our assay is highly reproducible. Taken together, these results also suggest that additional R/G ratios can be incorporated into our RCA-based assay by inserting additional binding sites in the padlock probes, which can expand the multiplexed detection capacity of the assay.

Ratiometric Fluorescence Detection with HRCA.

Toward improving the sensitivity of multiplexed detection via our ratiometric fluorescence coding method, we show the compatibility between our method and HRCA. Compared with the linear amplification mechanism in RCA, HRCA employs a second primer to achieve exponential amplification of double-stranded DNA (dsDNA) templates (Figure S1) for fluorescent probes.^{30,31} Our PNA probe plays a critical role here, because strong binding affinity between PNA and DNA ensures our PNA probes can hybridize to the DNA templates even in the presence of complementary DNA strands. To verify the HRCA-based assay, we mixed our four padlock probes with each of the HIV-1, NG, CT, and TP synthetic targets and detected the red and the green fluorescence intensities of each sample at the end of the HRCA reaction and probe hybridization. Similar to the results from RCA reactions, fluorescent images of these four HRCA samples also show fluorescence ratios that were noticeably distinct (Figure 3A). For example, we again observed relatively weak red fluorescence and strong green fluorescence for the HIV-1 sample, yielding a measured R/G ratio of ~ 0.8 . On the other hand, strong red fluorescence and weak green fluorescence could be observed from the CT sample and the TP sample, with measured R/G ratios of ~ 3.4 and ~ 5.2 , respectively. We similarly replicated the experiments four times and plotted the measured R/G ratios with errors bars (Figure 3B). The results indicated high reproducibility of our HRCA-based assay for detecting STD-related gene sequences based on distinct R/G fluorescence ratios.

Having verified robust multiplex detection through both our HRCA-based and RCA-based ratiometric fluorescence coding assays, we note that the measured R/G ratio for each HRCA sample was higher than that for its RCA counterpart. The difference in measured R/G ratios may be caused by the difference in self-quenching of neighboring Alexa Fluoro 647 molecules between the HRCA and the RCA samples.^{32,33} Specifically, in RCA, the template-probe complex that is formed when thousands of probes hybridize onto long single-stranded DNA templates could fold into a spherical conformation in solution.^{34,35} This conformation confines the Alexa Fluoro 647 molecules, and their proximity causes them to quench each other, thus reducing the red fluorescence intensity in RCA samples. In

contrast, double strand DNA templates produced from HRCA were only hundreds of base pairs in length (Figure S1), which allowed much fewer fluorescent probes to hybridize onto each template and reduced the potential for self-quenching of neighboring Alexa Fluoro 647 molecules. As a result, the intensities of red fluorescence in HRCA samples were elevated, resulting in higher R/G ratios.

Finally, we examine the sensitivity of our HRCA-based ratiometric fluorescence coding assay and its ability to detect genomic DNA. Here, we titrated our four STD synthetic targets to 1 pmol, 1 fmol, 1 amol, to 1 zmol, tested them with our HRCA-based assay, and measured their red and green fluorescence intensities. For each target, as the input concentration lowered, both the red and the green fluorescence intensities expectedly reduced as a result of lower concentration of dsDNA templates generated from lower concentration of target, though all four targets were still detected at 1 zmol (Figure 4A). More importantly, for each target, even as the input concentration decreased by 9 orders of magnitude, the measured R/G ratios remained fairly consistent (Figure 4B). For example, the R/G ratios for NG synthetic targets remained at ~2 for the 4 input target concentrations. The consistency of measured R/G ratios for the 4 targets across a wide range of input concentrations further illustrates the robustness of our ratiometric fluorescence coding method. Finally, we also used our HRCA-based assay to detect 200 ng and 2 ng of NG genomic DNA (extracted from *Neisseria gonorrhoeae* Strain FA1090, ATCC). We observed strong red and green fluorescence in the sample with 200 ng of NG genomic DNA sample and noticeably weaker fluorescence in the sample with 2 ng of NG genomic DNA (Figure 4C, left). Moreover, the measured R/G ratios in both genomic DNA samples were comparable to that of the NG synthetic target (Figure 4C, right). These results therefore suggest that our HRCA-based ratiometric fluorescence coding assay can also detect STD genomic DNA based on its predesigned fluorescence ratio.

CONCLUSION

In conclusion, we have developed a new ratiometric fluorescence coding concept that can be employed to significantly expand multiplexing capacity of NAAT by assigning a specific fluorescence color ratio for coding each target. Unlike common one-color-one-target scheme, we produce a target-specific DNA template with a predesigned ratio of binding sites for distinctly colored fluorescent probes. Our concept has been validated using padlock probe chemistry coupled with RCA or HRCA reactions. With our custom-designed padlock probes targeting infectious disease-related genes, we have demonstrated our ratiometric fluorescence coding method for multiplexed detection of 6 nucleic acid targets using only two distinctly colored fluorescent probes, as well as its potential for further expanding the multiplexing capacity. In addition, our HRCA-based assay has been utilized to detect genomic DNA from a specific STD.

There are several areas to improve our method for highly multiplex and sensitive detection of nucleic acids. First, the multiplexing capacity of our method can be readily expanded by using more fluorescence colors and designing more fluorescent probe binding sites. For example, using 3 colors and 5 binding sites ($S = 5$), the maximum number of ratio can reach 40 (Table S2). Since the commercially available phosphorylated single-stranded DNA is

limited to 200 bp from Integrated DNA Technologies, Inc. (Coralville, IA), decreasing the lengths of fluorescent probes can be an alternative approach to provide more available probe binding sites on a padlock probe. Especially using high affinity DNA analogues, such as PNA, as fluorescent probes, the probe length can be decreased to 12-mer or even shorter,^{36,37} which will allow more than 10 binding sites on a single padlock probe. In addition, while the current interaction of ratiometric fluorescence coding has been demonstrated for identifying one potential target out of multiple possible candidates, we can extend its utility to simultaneously detecting multiple targets in a single reaction by coupling it with single molecule detection method.^{38,39} The molecule-by-molecule measurement scheme in single molecule detection system can measure fluorescently labeled template-probe complexes one at a time in both red and green channel simultaneously, thus allows differentiation of template-probe complexes from different targets based on their distinct R/G ratios, which can be utilized for simultaneous detection of multiple targets in a single reaction. In addition to RCA and HRCA reactions, we also envision that strategies for coupling our ratiometric fluorescence coding concept with other nucleic acid amplification techniques such as LCR and PCR can be readily developed. Therefore, given the good performance of our ratiometric fluorescence coding method and the means to improve it, we believe our method has the potential to perform large-scale multiplexed detection of nucleic acids upon further development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Overview of ratiometric fluorescence coding for multiplexed nucleic acid detection. (A) Our ratiometric fluorescence coding concept utilizes a combination of fluorophore colors and their fluorescence intensity levels to achieve multiplexed detection. For example, upon hybridization of fluorescent probes with two colors (red and green) onto a single DNA template with a specific ratio, the template-probe complex can be decoded by resolving the intensity level of each color and display the specific red to green (R/G) fluorescence ratio. (B) The internal part of our padlock probe consists of one or two general primer sequences common for all the padlock probes and several red and green fluorescent probe sequences, which have a unique R/G ratio for each padlock probe. In the presence of corresponding target, padlock probe is ligated to a closed circle for subsequent amplification. (C) Six padlock probes with different R/G ratios were designed for infectious disease detection. Six circularizable padlock probes encoded as R/G ratio of 0:1, 1:2, 1:1, 2:1, 3:1, and 1:0 are designed for targeting gene sequences in *Proteus mirabilis* (PM), *Human immunodeficiency virus 1* (HIV-1), *Neisseria gonorrhoeae* (NG), *Chlamydia trachomatis* (CT), *Treponema pallidum* (TP), and *Escherichia coli* (EC) respectively. (D) RCA-based ratiometric fluorescence assay starts with the mixture of our six target-specific padlock probes and infectious disease-related DNA target. When binding to a specific target, the phosphorylated 5' end and the 3' end of the padlock probe are brought into proximity that leads to formation of a circularized probe via ligation. Subsequently, RCA reaction is performed to generate long ssDNA with thousands of tandem repeats complementary to the padlock probe sequences, serving as binding sites for fluorophores-labeled PNA probes. Upon

hybridization with PNA-AF647 (red) and PNA-AF488 (green), the fluorophores-labeled amplification products are scanned in both green and red channels and the measured R/G fluorescence ratios can be used to identify DNA targets in the initial input.

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Figure 2.

(A) RCA-based ratiometric fluorescence coding assay was validated via detecting six infectious disease-related genes. With PM, HIV-1, NG, CT, TP, and EC synthetic targets and H₂O control, the resultant fluorophores-labeled RCA products were scanned under both red (top row) and green (bottom row) channels. From left to right in each channel, the fluorescent spots represent fluorophores-labeled RCA products resultant from PM, HIV-1, NG, CT, TP, and EC synthetic targets and H₂O control, respectively. Moreover, measured R/G ratio for each sample is close to the predesigned ratio. The spots of fluorophores-labeled PM amplification products present fluorescence only in green channel with a measured R/G ratio of 0. The spots of the HIV-1 sample show weak fluorescence in red channel and strong fluorescence in green channel with a measured R/G ratio of 0.48. The spots of NG sample show nearly equivalent fluorescence intensities in red and green channel with a measured R/G ratio of 1.20. In contrast, the spots of CT and TP samples show strong fluorescence in red channel and weak fluorescence in green channel with measured R/G ratios of 2.21 and 3.06, respectively. The spots of the EC sample show fluorescence only in the red channel with a measured R/G ratio of $+\infty$. The spot of the H₂O control sample shows no fluorescence signal in either red or green channel. (B) Our assay was replicated in four independent experiments for detecting the six infectious disease-related synthetic targets. The small error bars of measured R/G ratios indicate a robust reproducibility.

Figure 3.

(A) HRCA-based ratiometric fluorescence coding assay was verified using our four padlock probes and STD synthetic targets. In the presence of HIV-1, NG, CT, and TP synthetic targets, HRCA generates dsDNA amplification products that are hybridized with red and green fluorescent PNA probes. The resultant fluorophores-labeled HRCA products were scanned under Typhoon image scanner in both red (top row) and green (bottom row) channels. From left to right in each channel, the fluorescent spots represent fluorophores-labeled HRCA products of HIV-1, NG, CT, TP, and H₂O control samples, respectively. The spots of fluorophores-labeled HIV-1 amplification products show weak fluorescence in red channel and strong fluorescence in green channel with measured R/G ratios of 0.86. While the spots of NG, CT, and TP samples show strong fluorescence in red channel and weak fluorescence in green channel with measured R/G ratios of 1.93, 3.43, and 5.20, respectively. The spot of H₂O control sample shows no fluorescence signal in either red or green channel. (B) Our HRCA-based assay was also replicated in four separated experiments. Each measured target-specific R/G ratio is significantly different from each other and their small error bars indicate high reproducibility of our HRCA-based assay.

Figure 4.

(A) Sensitivity of our HRCA-based ratiometric fluorescence coding method was verified using a gradient input of each STD synthetic targets ranging from 1 pmol, 1 fmol, 1 amol, to 1 zmol. The fluorophore-labeled HRCA samples were scanned under Typhoon scanner in both red (left panel) and green (right panel) channels. The synthetic target inputs are HIV-1, NG, CT, and TP from left to right in each row, and target input concentrations are 1 pmol, 1 fmol, 1 amol, and 1 zmol from top to bottom in each column. The fluorescence intensities in both red and green channels were observed to decline as target input decreasing due to the

deduction of dsDNA templates generated through HRCA. (B) For each target, the measured R/G ratios remained fairly consistent across the input concentrations, further indicating the robustness of our method. (C) Our HRCA-based assay was performed to detect 200 ng and 2 ng of NG genomic DNA. The resultant fluorophores-labeled HRCA samples were scanned under in both red and green channels, and R/G fluorescence ratios were measured (left). R/G ratios of two NG genomic DNA samples were measured to be comparable to that of NG synthetic target using our HRCA-based assay (right).