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A Universal Polymerase Chain Reaction Developer

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Abstract: *The versatility of PCR, the gold standard for amplification of DNA targets, is hampered by the laborious, multi-step detection based on gel electrophoresis. We propose a one-step, one-tube method for the rapid (5 min) naked-eye detection of PCR products, based on controlled aggregation of gold nanoparticles. Our method is universal, instrument-free, and ultra-sensitive, as it could detect as low as 0.01 zeptomoles of HIV template DNA in an excess of interfering human genomic DNA.*

The use of nanomaterials and their combination with biotechnology tools is leading to several breakthroughs in molecular biology and diagnostics. A series of outstanding achievements have been reported in recent years, such as gold nanoparticle (AuNP)-mediated boosting of the sensitivity of molecular diagnostics and intracellular imaging,[1a,b,c] development of ultra-sensitive assays for proteins,[2a,b,c] quantification of miRNA molecules by the naked eye,[3] or graphene-based efficient capture of circulating tumor cells.[4] Moreover, AuNPs have proven to be extremely useful for the specific detection of DNA point mutations.[5a,b] In this framework of nascent technologies, however, PCR still represents the gold standard for amplification and detection of nucleic acids, thanks to its versatility, low cost, and universality. In fact, beyond some important evolutions (including quantitative and digital PCR), qualitative PCR alone currently accounts for millions of reactions each year. Nevertheless, despite several innovations in instrumental and enzymatic performances, PCR necessarily requires the laborious and time-consuming gel electrophoresis-based detection, which involves a multi-step procedure (gel casting, electrophoretic run, gel staining, and gel visualization), and needs skilled personnel. On the other hand, fluorescence-based detection methods offer the possibility to quantify the target, but require instrumental readout and expensive reagents, both avoidable drawbacks when only a qualitative result is required. In this work, we propose a universal tool, based on the controlled aggregation of AuNPs, to "develop" PCR by the naked eye in a few minutes, with no need for any instrumentation. This approach is versatile and low-cost, and could be extremely useful for a wide range of applications, from molecular biology, to clinical tests, quality controls, and point of care diagnostics.

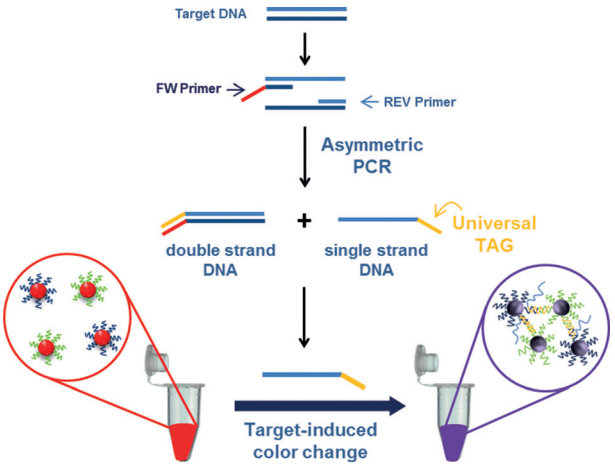

Figure 1. Assay principle. Asymmetric PCR is performed using a standard reverse primer (light blue) and a forward primer including a sequence complementary to a universal tag (in red). The forward primer is depleted during the amplification, thus single-stranded products, containing the universal tag sequence (yellow), accumulate in the late phase of the amplification. Two set of AuNPs are functionalized with oligonucleotide probes, each complementary to a portion of the universal tag. Upon addition of a sample containing the amplified target, AuNPs will aggregate and color-shift owing to the hybridization of the AuNPs probes with the universal tag.

Figure 1 illustrates the assay principle. The target DNA is first amplified through asymmetric PCR to generate a readily hybridizable single strand product for efficient detection, avoiding sample denaturation steps after amplification. Notably, our assay is universal, thanks to the design involving a fixed sequence attached to the forward primer that generates, during amplification, a universal tag attached to the single-stranded PCR amplicon. This means that the production of the universal tag is strictly dependent on the target detection, but it is independent of its sequence. Since the naked-eye detection of the target is then based on the tag-induced aggregation of DNA-functionalized AuNPs (with consequent red-to-violet color change), our assay strategy renders the detection of any target universal.[6]

In more detail, asymmetric PCR is performed using a set of primers specific to the target of interest, in uneven ratios. The forward primer is the limiting reagent and is depleted during the amplification. Thus, in the late phase of amplification, a single-stranded product, containing the universal tag sequence, accumulates in the reaction. In this way, it is not necessary to denature the sample to allow the subsequent hybridization-based detection. The universal tag, complementary to the fixed sequence on the forward primer, is not present in the starting reaction mixture, since it is synthesized during the amplification only in the presence of the target, while unspecific side products are not generated. The

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detection of these asymmetric PCR products is then performed by a colorimetric method in a few minutes and in a one-step, one-tube reaction, without the use of any instrumentation or expensive fluorescent reagents. The working principle is based on the well-known property of AuNPs, functionalized with appropriate probe oligonucleotides, to cross-link in the presence of a complementary target, leading to a visible color-shift. This strategy, demonstrated in 1996 by Mirkin's group[7] and later employed in several elegant proof-of-principle detection schemes,[8] has, to date, suffered from some technical limitations for the practical detection of PCR products in clinics, such as the lack of universality, as a distinct set of target-specific functionalized AuNPs should to be prepared for each detection, or the need for denaturation of the double-stranded PCR product to allow efficient hybridization with AuNPs. In our case, after amplification, the detection is directly performed by adding a small aliquot (1–2 μL) of the amplified sample to the universal AuNPs probe suspension at room temperature. This elicits, in the presence of the target, a clear and rapid color change of the solution owing to the aggregation of two types of DNA-functionalized AuNPs, each bearing a fixed sequence complementary to half of the universal tag. In the absence of the target in the original sample, no universal tag is produced during the PCR amplification, and consequently no color change is observed. Importantly, this smart assay can be routinely performed at ambient conditions and was shown to work very efficiently, while maintaining excellent detection sensitivity and specificity. Indeed, even if nonspecific by-products were present in the PCR reaction, these would not interfere with the colorimetric detection, because in our method there are three crucial conditions for an amplicon to cause AuNPs crosslinking: i) it must contain the universal tag; ii) it has to be single-stranded, that is, amplified by the action of both primers in an asymmetric manner; and iii) it must have a size very close to the intended target, as larger amplicons would interfere with AuNPs aggregation, owing to steric hindrance effects.

The PCR developer was tested with three different proof-of-concept targets, representative of three different real-world diagnostic applications in which rapid and qualitative detection of a nucleic acid target is relevant.

First, the PCR developer was applied to the detection of a generic target in human genomic DNA. The model target chosen in this case was β-actin. The starting material for the amplification was 1 μL of a genomic DNA obtained by standard spin-column extraction from a cell culture lysate (about 100 ng μL^{-1}). The forward primer bore a common 5′ sequence, complementary to the universal tag recognized by the set of DNA-functionalized AuNPs (Table 1). The design of this universal sequence was checked by BLAST to avoid nonspecific amplifications, and did not match any sequence in the human genomic DNA. Moreover, secondary structures and self-pairing were excluded. After asymmetric amplification, as a control, a native polyacrylamide gel confirmed the presence of the desired single strand amplification product, together with its double strand counterpart, and the absence of unspecific products (Figure 2a). Then, our naked-eye detection assay was tested directly by adding 2 μL of each amplified sample to the AuNPs developer solution, and a clear color change was observed in 5 minutes (Figure 2a). Owing to the high sensitivity of the AuNPs-based detection, raw, unprocessed PCR samples could be analyzed as such, without the need to purify the single-stranded product from the reaction. Indeed, double-stranded PCR products, albeit present in a large proportion, did not interfere with the detection. Genomic DNA targets suitable for this rapid and low-cost assay include congenital chromosomal rearrangements and DNA mutations, such as deletion and insertion, as a qualitative ON/OFF detection is sufficient for diagnostics purposes.

Table 1: Sequences of the primers and probes used in this study (the universal tag is underlined).

	Primer/Probe	Sequence
β-actin	Forward	ACATCAGAGTTTCCAGCACAATGAAGATCA
	Reverse	AGGAAAGACACCCACCT
eGFP	Forward	ACATCAGAGTTTAAGGCTACGTCCAGGAG
	Reverse	TTGAAGAAGATGGTGCG
HIV	Forward	ACATCAGAGTTTCTAGGTGTGAATATCAAGC
	Reverse	CTACCTTGTTATGTCCT
AuNPs	probe A	ATCATCATACATCA
	probe B	GAGTTTACCAAGTA

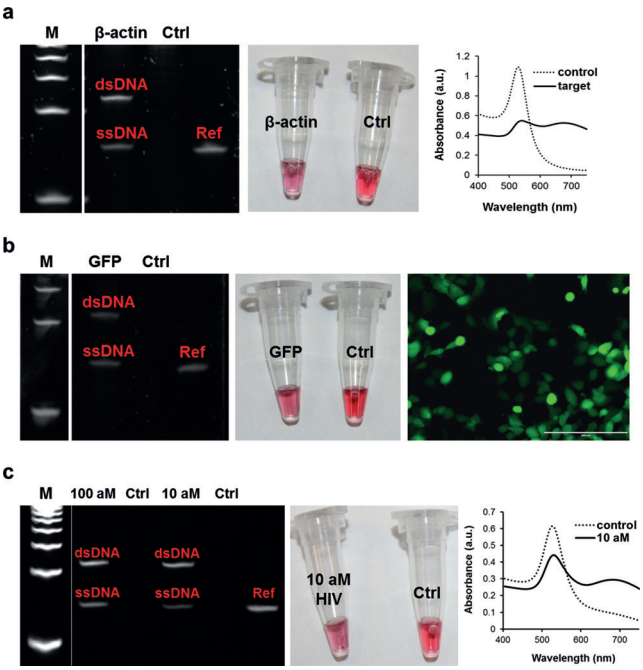

Figure 2. Application of the PCR developer to the detection of three different targets. In all cases, native polyacrylamide gel electrophoresis (PAGE; left) confirmed the presence of double-stranded (dsDNA) and single-stranded (ssDNA) amplification products. Reference (Ref) is a synthetic ssDNA with the same sequence of the expected single-stranded amplification product. All panels, middle: representative photographs showing naked-eye colorimetric detection of each target. a) Detection of β-actin (right panel: representative UV/Vis spectra of AuNPs in the presence/absence of the target). b) Detection of GFP transgene (right panel: fluorescence microscopy image of MDA-MB-231/GFP cell culture). c) Detection of HIV (right panel, representative UV/Vis absorbance spectra of AuNPs in the presence/absence of 10 aM of HIV target).

The second application was the detection of the green fluorescent protein (GFP). In research laboratories where transgenic animals or plants are generated, a large number of samples have to be routinely screened in order to identify those where incorporation of the transgene is successful. A common marker of transgenes is GFP.[9] To demonstrate the applicability of our method to the rapid screening of transgenes, we have applied it to the detection of GFP in a virally transduced cell line. To this aim, we have extracted genomic DNA from a MDA-MB-231/GFP cell line and, as a control, from an HT29 cell line. We performed asymmetric PCR with a GFP specific primer set (Table 1), and colorimetric detection, similar to the method described above for β-actin. Specific amplification of a GFP gene fragment was observed only in MDA-MB-231/GFP genomic DNA and not in controls containing HT29 genomic DNA (Figure 2b). Consistently, colorimetric detection turned positive (red-to-violet color shift) only for positive PCR samples, demonstrating that this assay can be applied to hasten the screening work and cut costs in the development of transgenic animals and plants.

A third application where qualitative analysis is relevant is the detection of infectious agents/diseases. This finds utility both in clinical diagnostics, especially in emergency units, when fast time-to-results might be critical, but also in quality control along food production lines or distribution chains, for the rapid identification of food-borne pathogens. To exemplify this kind of application, we selected the detection of HIV. Notably in this case, sensitivity is a crucial issue, so we tested the efficacy of our naked-eye method to detect very low concentrations of viral DNA, mixed with a high amount of interfering genomic material. We managed to amplify and detect colorimetrically as little as 10^{-2} zeptomoles of viral DNA (corresponding to 10 aM), just through visual inspection, in a sample containing 100 fM of human genomic DNA (Figure 2c). This simulates a standard concentration of DNA extracted from a blood sample, infected with around 5000 copies ml^{-1} of HIV (a clinically relevant viral load, according to the World Health Organization 2010 criterion for switching to second-line treatment http://apps.who.int/iris/bitstream/10665/44379/1/9789241599764_eng.pdf). In this test, the colorimetric shift, indicative of the amplification of the HIV genome fragment, was clearly observable in the positive sample after 8 minutes (a slightly higher time, owing to the extremely low target concentrations). A detailed analysis of the sensitivity of our colorimetric assay is reported in Figure S2 (Supporting Information), showing a limit of detection (LOD) of 2 aM. This concentration corresponds to 1.2 copies of viral DNA per μL of sample, indicating that our naked-eye method can be used to amplify and detect down to a few copies of target DNA.

It is worth mentioning that, due to the use of universal AuNP probes, any target can be analyzed with the same mixture of DNA-functionalized AuNPs, provided that the universal tag has been generated during the amplification. For this reason, and because no processing steps are needed for the detection apart from adding the PCR product to the premixed AuNPs solution, this optimized AuNPs mixture works as a true "PCR developer". Moreover, the universality of the assay facilitates its application in multi-wells formats to simultaneously detect several different targets and/or different patients. Notably, when several samples have to be analyzed, while a standard thermocycler usually can accommodate up to 96 samples simultaneously, gel electrophoresis becomes a bottleneck to parallelization, as only 10–20 samples can usually be analyzed at the same time in a standard agarose gel, unlike the high multiplexing capabilities of our assay. Time and parallelization issues are particularly important in many situations, such as in clinical diagnosis and point-of-care applications, as well as in emergency units in remote areas or in field-hospitals, where a large number of samples have to be processed for qualitative screening purposes. Moreover, the universal tag sequence has been designed to be optimal in terms of nanoparticle aggregation and induction of a large color shift, while simultaneously avoiding specificity issues, being unrelated to target. It is well-known, in fact, that the color-shift of AuNPs occurs when their inter-particle distance (d) is lower than their size, and exponentially increases as a function of 1/d.[10] Thus, the use of the universal tag in our assay not only guarantees the universality of the assay, but also acts as a very efficient NPs plasmon coupler, owing to its short sequence (12 bps, corresponding to ca. 4 nm). This is another important advantage with respect to the use of PCR amplicons as the AuNPs cross-linker, as these have to be significantly longer (typically >40 bps, ca. 14 nm), to guarantee specific PCR reactions, thus impairing the overall sensitivity of the assay. In this case, the design of our short tag enables a ≥ 3-fold increase in the fractional plasmonic shift, occurring upon target detection, as compared to classical PCR amplicons.[10] On the other hand, the use of shorter sequences is unfeasible because it would approach the limit for room temperature hybridization and consequent particle aggregation.

In conclusion, we demonstrated a low-cost, fast, and universal method to replace gel electrophoresis post-processing of standard PCR reactions. This simple and instrument-free method can be used even by untrained personnel at ambient conditions, and, if coupled to miniaturized thermal cyclers, could be of high importance in point-of-care diagnostics, as it could allow performing fast screenings in settings with simplified laboratory facilities, and permitting on-field analysis of food and water. This system enables highly multiplexed analyses and overcomes the technical limitations of current technologies, and can be exploited in a wide range of practical applications, as exemplified by the three assays demonstrated in this paper.

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