SUPPORTING INFORMATION

Colorimetric Detection of Human Papilloma Virus by Double Isothermal Amplification

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Experimental Section

Materials

The chimeric RNA-DNA oligonucleotide (COs) (Table 1) was purchased from Thermo Scientific, while the target and control sequences (Table 1) from Integrated DNA Technologies. All oligonucleotides were used without further purification. The oligonucleotides concentration was determined by measuring the absorbance at 260 nm, with molar extinction coefficients determined using the nearest-neighbor approximation.

Streptavidin-functionalized magnetic microparticles (MMPs) with a diameter of 2.8 µm (Dynabeads M-280) were purchased from Invitrogen. Recombinant E. Coli RNase H (1.5 U/µL,) was purchased from Promega. ABTS was obtained as the di-ammonium salt from Sigma-Aldrich, and stock solutions were prepared in Sigma-Aldrich DNAse/RNAsae-free water. ABTS stock solution concentration was measured spectroscopically using as a molar extinction coefficient $\varepsilon_{340} = 3.66 \times 10^4 \text{M}^{-1}\text{cm}^{-1}$. Then, the obtained ABTS solution was stored at -20 °C until use.

Hemin was purchased from Sigma-Aldrich and used without further purification. Hemin stock solutions were prepared in dimethylsulfoxide (DMSO) and stored frozen at -20 °C, repaired from light. Diluted stock solution was prepared in folding buffer just before use. As hemin tends to form dimers or larger aggregates in water solution, all buffers contained 0.05 % w/v of triton X-100, as this low concentration of detergent was found to be optimal for the disaggregation of hemin, as well
as for its optimal peroxidative activity. Hemin concentration was determined using standard spectroscopic methods and molar extinction coefficient $\varepsilon_{398} = 8.0 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$.

All DNase/RNase-free reagents used for the preparation of the folding buffer (10 mM Tris-HCl, 200 mM NaCl, 20 mM KCl, 0.05 % Triton X-100, pH 7.4), reaction buffer (50 mM Tris-HCl, 200 mM NaCl, 20 mM KCl, 8 mM MgCl$_2$, 0.05 % Triton X-100, pH 8.2) and ABTS buffer (50 mM Tris-HCl, 200 mM NaCl, 20 mM KCl, 0.05 % Triton X-100, pH 8.2) were purchased from Sigma-Aldrich.

*Preparation of hemin-DNAzyme complex and immobilization onto MMPs.*

The COs were heated at 95 °C for 5 minutes and then slowly cooled at room temperature. This temperature was maintained for 1 h to allow the correct folding of the DNA sequence in the G-quadruplex conformation. Then hemin was added at a concentration ratio of 2:1 with respect to DNAzyme and incubated for 1 h at room temperature to allow the formation of catalytically active hemin-G-quadruplex complexes.

Conjugation of hemin-complexed biotinylated COs to streptavidin-coated MMPs was carried out according to the directions of the manufacturer. Briefly, 10 µL of MMPs stock (10 mg/mL) were washed three times with folding buffer to remove the preservative storage buffer and resuspended in 16.8 µL of folding buffer. Then, 3.2 µL of previously prepared COs complexes were added to the MMPs and the resultant mixture was incubated at room temperature for 30 min to allow conjugation.

To ensure maximum loading on MMPs, an excess of hemin-complexed biotinylated COs was used (40 pmol), corresponding to twice the binding capacity the MMPs. After conjugation, the MMPs were washed 3 times to remove the unbound hemin-complexed biotinylated COs, of which twice with folding buffer and once with reaction buffer and resuspended in reaction buffer to a final volume of 10 µL.
RNase H-assisted target recycling

MMPs, modified with COs complexes, were incubated in the presence of different concentrations of the target DNA (in the range 1000 nM to 0.01 nM) and RNase H (30 U/mL) at 37 °C for 1 hour in reaction buffer (final volume 20 µL). After cooling the vial on ice for 5 min, the MMPs were removed using a magnet and the supernatant, containing the released DNAzyme-Hemin complexes, was collected and used for the kinetic measurement.

Quantification of recycling efficiency

Samples were prepared as described in the previous paragraphs, with the exception that, in the last step, the DNAzyme content in the collected supernatants was quantified using the Quant-iT™ OliGreen kit (Invitrogen), in order to know the number of DNAzyme molecules released per each target molecule. We calculated that, at the lower concentrations tested, target recycling amplifies the signal by a factor of about $10^3$ (i.e., a single target molecule causes the release of ca. 1000 DNAzyme molecules). However, at the highest concentrations tested, recycling is not possible, as the amount of target molecules (1 µM) approaches that of the immobilized DNAzyme molecules, so only one molecule of DNAzyme can be released by each target. Therefore, recycling, and so signal amplification, is more evident at lower concentrations. In addition, also the second amplification step is more efficient at lower DNAzyme concentration (producing a further amplification of ca. $10^3$), so the combination of these two factors leads to an overall “flattening” of the final signal. However, the important point is sensitivity at low concentrations that are of clinical relevance, and our assay provides a total amplification efficiency $>10^5$, which is comparable to that of a traditional PCR.

Kinetic measurements

Reaction kinetics were followed by monitoring the appearance of the ABTS radical cation at 414 nm for 5 min using a UV-vis spectrophotometer (Thermo Scientific, NanoDrop 2000).
Reactions were initiated by the addition of sample containing the DNAzyme-Hemin complex, released by RNase H cleavage, to 100 µL of ABTS buffer, containing 4 mM ABTS and 2 mM H$_2$O$_2$. 
Figure S1. Color readouts in samples containing lower concentration of target DNA (left: 0.1 nM) compared to the control. The naked-eye limit of detection is 0.1 nM.
Figure S2. Specificity of the method towards the HPV16 target. Three HPV strains with different degrees of divergence from the HPV16 target were used as a control. The sequences with a higher degree of divergence (HPV18 and HPV11) generated an absorbance signal not distinguishable from the background. For HPV31, which showed the highest degree of similarity to HPV16 (only 3 nucleotides difference), the temperature of the assay was adjusted to 54 °C, in order to avoid unspecific signal. These data demonstrate also the robustness of the method, which returned the same performance (for the specific detection of HPV16) in a broad temperature range.
Figure S3. Selectivity of the assay in presence of high concentration of interfering DNA. Samples containing different amounts of HPV16 were assayed together with a mixture of HPV18 and HPV11 (the latters at a fixed total concentration of 1 µM). The signals obtained were dependent only on the concentration of HPV16 and followed the same profile of the samples containing HPV16 alone.
Figure S4. Stability of the COs-MMPs conjugates. After prolonged storage at 4 °C (15 days), the DNAzyme retains its enzymatic activity, as indicated by comparison, at time 0 and after 15 days of storage, of the absorbance signal at 1000 nM of target concentration. Moreover, the background value (0 nM target) is also unvaried after storage (data not shown), indicating that the RNA portion of the probes is not degraded and thus no target-independent release of the DNAzyme has occurred during storage.

References