Supporting information

Controllable Autocatalytic Cleavage-Mediated Fluorescence Recovery for Homogeneous Sensing of Alkyladenine DNA Glycosylase from Human Cancer Cells

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Optimization of reaction time of T7 exo-directed autocatalytic recycling amplification

The reaction time of T7 exo-assisted autocatalytic recycling signal amplification affects the amplification efficiency of whole CESA system, and thus it was optimized in this research. As shown in Figure S1, 5 μL of hAAG-catalyzed excision products were added to 20 μL of amplification reaction system including 250 nM HP2, 700 nM signal probes, 15 U of T7 exo, and 2 μL of 10× NEBuffer 4, and then incubated at 25 °C in the dark for different time. With the reaction time varying from 0 to 60 min, the fluorescence intensity increases rapidly from 0 to 50 min and gradually reaches the plateau above 60 min. Therefore, 60 min is selected to be the optimal amplification time.

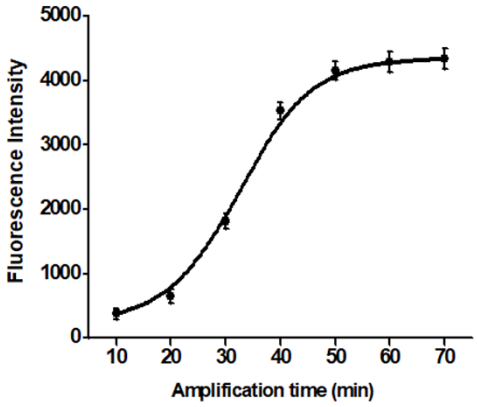

Figure S1. Variance of fluorescence intensity with different amplification reaction time. The 0.1 U/μL hAAG, 0.3 U/μL APE1, 100 nM HP1, 250 HP2, 700 nM signal probe, and 15 U of T7 exo were used in the experiments, respectively. Error bars show the standard deviation of three independent experiments.

Optimization of HP2 concentration

HP2 is a critical factor which can be hybridized by trigger 1 originally caged in HP1 to induce T7 exo-catalyzed the first recycling cleavage to release the trigger 2 that can be hybridized by signal probe to induce T7 exo-catalyzed the second recycling cleavage to eventually liberate the

fluorophore molecules from the FAM-BHQ1 FRET pair. Thus, the concentration of HP2 is essential to the improved sensitivity of this proposed strategy. As shown in Figure S2, with the increasing concentration of HP2, the fluorescence intensity enhances correspondingly and reaches the maximum value at the concentration of 250 nM. Thus, the optimal concentration of HP2 is determined to be 250 nM.

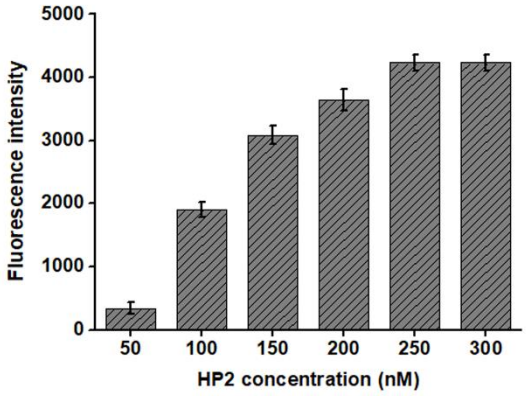

Figure S2. Variance of fluorescence intensity with different concentrations of HP2. The 0.1 U/μL hAAG, 0.3 U/μL APE 1, 100 nM HP1, 700 nM signal probe, 15 U of T7 exo, and the amplification time of 60 min were used in the experiments, respectively. Error bars show the standard deviation of three independent experiments.

Optimization of signal probe concentration

The concentration of signal probe is directly related to the detection sensitivity of the proposed strategy. To achieve higher sensitivity, the optimization of signal probe concentration is necessary. As shown in Figure S3, the fluorescence intensity increases gradually with the increasing concentration of signal probes and reaches the plateau at the concentration of 700 nM. Therefore, 700 nM signal probe is used in subsequent researches.

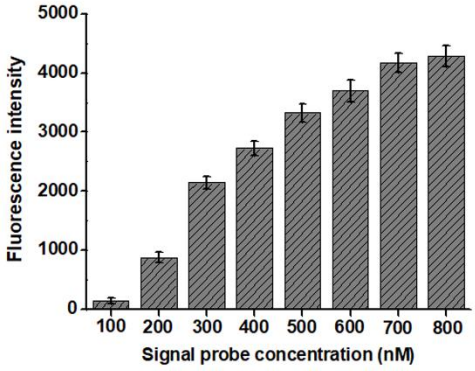

Figure S3. Variance of fluorescence intensity with different concentrations of signal probe. The 0.1 U/μL hAAG, 0.3 U/μL APE 1, 100 nM HP1, 250 nM HP2, 15 U of T7 exo, and the amplification time of 60 min were used in the experiments, respectively. Error bars show the standard deviation of three independent experiments.

Optimization of the amount of T7 exo

T7 exo is the "heart" of the proposed CESA system, which is responsible for the running of DNA alkylation-actuating autocatalytic recycling signal amplification, and thus its amount should be optimized in this research. As shown in Figure S4, with the amount of T7 exo increasing from 2 to 15 U, the fluorescence intensity improves significantly, followed by slight decrease beyond 15 U. Therefore, 15 U of T7 exo is used in the subsequent researches.

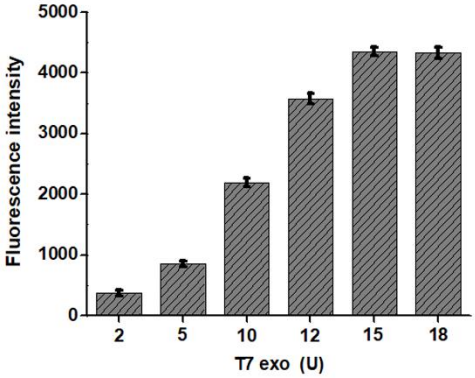

Figure S4. Variance of fluorescence intensity with different amounts of T7 exo. The 0.1 U/μL hAAG, 0.3 U/μL APE1, 100 nM HP1, 250 nM HP2, 700 nM signal probe, and the amplification

time of 60 min were used in the experiments, respectively. Error bars show the standard deviation of three independent experiments.

Cellular hAAG analysis with the western blotting and ELISA

To evaluate the expression level of hAAG enzyme in different parts of human cells, we used the western blotting (Figure S5) and enzyme-linked immunosorbent assay (ELISA) (Figure S6) to analyze the protein extracts from cytoplasm, nucleus, and whole cell extracts, respectively. As shown in Figure S5A, we investigated the level of hAAG enzyme in cytoplasm, nucleus, and whole cell by using western blotting and rabbit anti-hAAG polyclonal antibody. Distinct bands (~33 KDa) are observed in the presence of nucleus (Figure S5A, lane 2) and whole cell extracts (Figure S5A, lane 3), respectively, while only a weak band is observed in the presence of cytoplasm extract (Figure S5A, lane 1), indicating that the expression of hAAG in nucleus is much higher than that in cytoplasm, consistent with the value measured in the presence of internal reference protein (i.e., histone H3). Moreover, the intensities of above bands were semi-quantified by densitometry. As shown in Figure S5B, with the internal reference protein (i.e., actin) as the control, the values of relative intensities in response to nucleus (Figure S5B, red column) and whole cell extracts (Figure S5B, green column) can be obtained, and the level of hAAG protein in nucleus and whole cell extracts is calculated to be 5.9- and 6.3-fold higher than that in cytoplasm extract (Figure S5B, blue column), respectively, indicating that despite the presence of hAAG enzyme in cytoplasm, it mainly locates in nucleus, consistent with the previous reports [1-3]. The difference of hAAG enzyme level in cytoplasm and nucleus can be ascribed to: (1) hAAG is responsible for the repair of alkylative and oxidative damages in genomic DNA, and thus it mainly locates in the nucleus of human cells [3, 4]; (2) the mitochondria in cytoplasm contains a few

genome DNA, probably resulting in the presence of a small amount of DNA repair enzymes (e.g., hAAG) [5, 6].

In addition, the ELISA was performed to verify this issue using an ELISA kit (ZK-H2553) (ZIKER Bio, Shenzhen, China). As shown in Figure S6A, obvious white-to-yellow color change is observed in the presence of nucleus and whole cell extracts, respectively. In contrast, almost no visible color change is observed in the presence of cytoplasm extract, consistent with the control with only lysis buffer. Furthermore, the corresponding optical densities (O.D.) were quantified at 450 nm using a SpectraMax i3× multi-mode microplate reader (Molecular Devices, San. Jose, CA, USA). As shown in Figure S6B, only low O.D. is detected in response to either cytoplasm extract (Figure 6B, blue column) or the control (Figure S6B, black column), while high O.D. is detected in response to nucleus (Figure S6B, red column) and whole cell extracts (Figure S6B, green column), respectively. The value of relative O.D. in response to nucleus and whole cell extracts is calculated to be 64.2- and 66.9-fold higher than that in response to cytoplasm extract (inset in Figure S6B), respectively, implying that hAAG indeed locates in the nucleus of human cells, consistent with the results obtained by western blotting (Figure S5), and hAAG enzyme can be separated accurately from cytoplasm, nucleus, and whole cell by using the nuclear extract kit (40010) (Active Motif, Carlsbad, CA, USA).

The above western blotting (Figure S5) and ELISA (Figure S6) experiments clearly demonstrate the presence of a small amount of hAAG proteins in cytoplasm, but the cytoplasmic proteins do not exist in the nucleus extract which is used in this research. Thus, the proteins existing in cytoplasm do not influence the applications of the proposed method in real sample analysis.

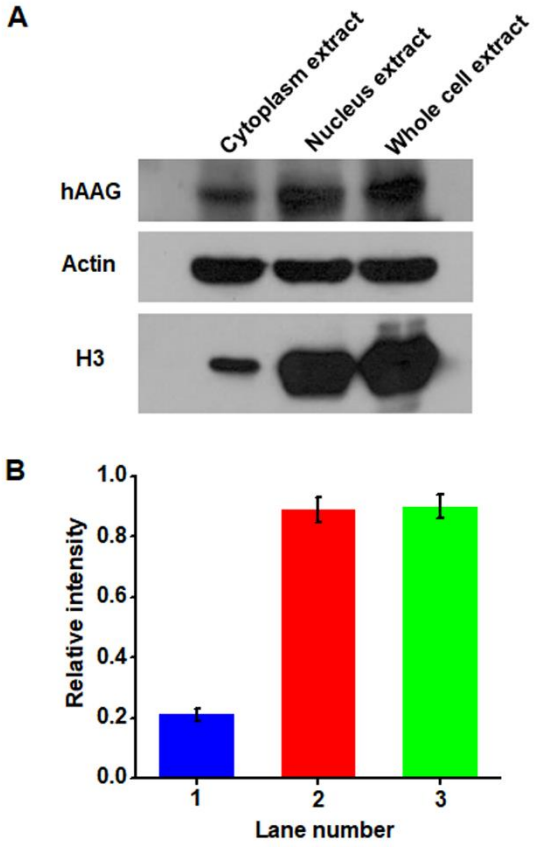

Figure S5. (A) Analysis of hAAG expression in cytoplasm (lane 1), nucleus (lane 2), and whole cell (lane 3), respectively. (B) Relative intensities of bands in response to cytoplasm (blue column), nucleus (red column), and whole cell extracts (green column), respectively. The relative intensity is the ratio value of I_t / I_i (I_t is the band intensity in response to target sample (i.e., cytoplasm, nucleus, and whole cell extract), and I_i is the band intensity in response to the internal reference protein (i.e., actin)). Error bars represent the standard deviations of three experiments.

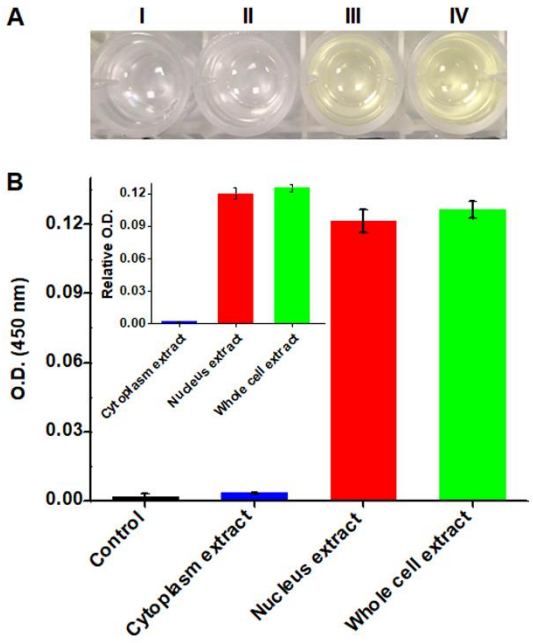

Figure S6. (A) Color changes in response to the control (I), cytoplasm (II), nucleus (III), and whole cell extracts (IV), respectively. (B) Measurement of O.D. in response to the control (black column), cytoplasm (blue column), nucleus (red column), and whole cell extracts (green column), respectively. Inset shows the dependence of relative O.D. on cytoplasm, nucleus, and whole cell extracts, respectively. The relative O.D. is the ratio value of $O.D._t$ — $O.D._c$ ($O.D._t$ is the optical density in the presence of target (i.e., cytoplasm, nucleus, and whole cell extract), and $O.D._c$ is the optical density in the presence of the control (i.e., only lysis buffer)). Error bars represent the standard deviations of three experiments.

Detection of hAAG activity in the spiked human serum

To evaluate the feasibility of the proposed method for complex biological sample analysis, we measured the recovery of hAAG by spiking different-concentration hAAG (1.0×10^{-5}-0.1 U/μL) to the normal human serum. As shown in Table S1, the recovery ratio is calculated to 99.6%-101.0% with a relative standard deviation (RSD) of 0.98%-2.34%, consistent with the values (recovery ratio of 96.6-106.0% and RSD of 1.53-5.38%) obtained by base-excision repair-mediated triple

amplification-based fluorescence assay [7]. These results demonstrate that the proposed method can be applied for accurate quantification of hAAG activity in complex biological samples.

Table S1. Recovery studies in spiked human serum samples

sample	added (U/μL)	measured (U/μL)	recovery (%)	RSD (%)
1	1.0×10^{-5}	1.01×10^{-5}	101.0	2.34
2	2.5×10^{-4}	2.49×10^{-4}	99.6	1.23
3	5.0×10^{-3}	5.04×10^{-3}	100.8	1.39
4	2.5×10^{-2}	2.51×10^{-2}	100.4	0.98
5	0.1	9.96×10^{-2}	99.6	1.08

References

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