Gold Island-Enhanced Multiplex Quantum Dots Fluorescent System for Biomedical Analysis of Circulating Tumor Nucleic Acids

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Research

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Abstract

Background: Circulating tumor nucleic acids (CTNAs) have been employed as the potential marker for tumor diagnosis and management, which are highly related to the tumorigenesis, progression and metastasis processes. Therefore, it is of significance to develop a highly-sensitive and reliable methods for detection of CTNAs, especially the multiplex point mutation detection of blood-derived CTNAs.

Results: Herein, a gold island-enhanced multiplex quantum dots (QDs) fluorescent platform was constructed for highly-sensitive detection of CTNAs in serum. The gold island-enhanced multiplex fluorescent strategy was designed as the highly-efficient signal giving-out mode which could amplify the fluorescence of QDs, realized a homogeneous nano-platform for the enrichment, multiplex detection and point mutations monitoring of CTNAs with the principle of base-stacking. A high sensitivity of 10 amol and desirable specificity were achieved, and the performance index for analysis of clinical CTNAs samples indicated that the gold island-enhanced multiplex QDs fluorescent strategy could realize multiplex point mutations detection of CTNAs in complex blood samples.

Conclusions: Hence, this platform achieved high detection rate in clinical samples that suitably met the clinical-requirements for multiplex detection and point mutations monitoring of CTNAs, and thus has the potential to serve as the tumor liquid biopsy strategy based on CTNAs.

Introduction

Circulating tumor nucleic acids (CTNAs),[1-3]the cell-free nucleic acids released from tumor lesion,[4-6] have been approved to be the potential marker for tumor diagnosis and management.[7-10] The diagnosis strategy that relies on CTNAs in blood samples could serve as a liquid biopsy approach that potentially becoming a supplement or replacing of the tumor tissue biopsies.[11-13] The existing researches indicated that tumor patients generally have relatively higher concentration and mutation probability of CTNAs than healthy groups.[14, 15] However, the concentration and mutation probability of CTNAs are drastically varied in plasma or serum samples, and it is very difficult to differentiate tumor patients from healthy person. Furthermore, the low expression level of CTNAs leads to the requirements for large volume of serum in clinical detection process, typically larger than 10 mL to obtain sufficient target CTNAs substrates.[5, 11] Thus, it is of great significance to develop a highly-sensitive, reliable strategy for the detection and mutation analysis of CTNAs.

The existing researches have proved that detection strategies based on the expression level and mutated sequences of CTNAs are the efficient assay for liquid biopsy of tumors.[5, 16-22]These strategies require highly sensitive and specific monitoring approach to detect the low abundance of CTNAs and the mutant fragments in high abundance of wild-type sequences from tumor patients.[23, 24] Among them, the detection methodology based on polymerase chain reaction (PCR) and DNA sequencing provided the possibility for CTNAs.[25, 26] PCR-based CTNAs detection provided sensitive and specific assays for clinical samples, but is not effective for the detection of point mutations in CTNAs. [5, 25, 26] Otherwise,

DNA sequencing provided an excellent assay for detections of the mutant fragments in CTNAs, but the whole implementation was expensive that could not meet the low-cost demands of routine clinical analysis, and time-consuming (2-3 weeks) greatly limited its applications.[16, 27, 28] Therefore, the development of a novel method that is more accurate, and able to detect CTNAs and its mutations directly in serum, especially multiplex point mutation detection in blood, is thus required urgently.

Herein, a homogeneous nanoplatform based on gold island [29-31] -enhanced multiplex quantum dots (QDs)[32-36] fluorescent strategy (**Figure 1**) was constructed to multiplex detection and point mutations monitoring of CTNAs in serum. This strategy constructed a homogeneous nanoplatform for enrichment and detection of CTNAs in the blood, and the principle of base stacking [37-40]was employed as the force to realize point mutations detection. 8 nt DNA recognition domains were connected to three kind of quantum dots (QDs) with the emission at 525nm (QDs 1), 585nm (QDs 2) and 650nm (QDs 3), to construct multi-functional recognition probes for the detection of multiplex point mutations. The multi-functional recognition probes can be easily excited by a single ultraviolet (UV) light source, [41]and three point mutations can be simultaneously detected with the emission at 525nm, 585nm and 650nm, respectively. With this strategy, a high sensitivity of 10 amol and desirable specificity was achieved. The performance index of gold island-enhanced multiplex QDs fluorescent strategy for analysis of clinical CTNAs samples indicated that it could simultaneously realize multiplex detection and point mutations monitoring of CTNAs in blood samples. Hence, this platform suitably met the strict clinical-requirements for multiplex detection and point mutations monitoring of CTNAs and thus has the potential to serve as an accurate paradigm for the liquid biopsy of tumor.

Materials And Methods

Reagents.

All the chemical reagents, such as $HAuCl_4$, ammonium hydroxide, $NaBH_4$ were obtained from Alfa Aesar Co., Ltd. Diethylpyrocarbonate (DEPC)-treated water and RNase inhibitor were obtained from Takara Biotechnology (Dalian) Co., Ltd. Streptavidin-modified magnetic beads were synthesized by New England BioLabs. Reagents related to electrophoresis and DNA probes were purchased from Shanghai Sangon Biotechnology Co. Ltd. SYBR I and SYBR II were purchased from Invitrogen. Invitrogen synthesized all oligonucleotides.

Synthesis of Gold-Island.

The synthesis of gold-island was based on the previously reported methods. [30] The glass slides were firstly submerged in $HAuCl_4$ (3 mM) followed by the addition of ammonium hydroxide with shaking for 1 min, the Au ions were absorbed by positive charge on glass slides. The treated glass slide was then washed three times with deionized water to remove the unbound Au ions. And 1 mM $NaBH_4$ was added to reduce the gold clusters to gold seeds. At the end, the slides were incubated in a solution of HAuCl4 and

hydroxylamine at a 1:1 ratio and shaken for 5 min before washing twice with water, followed by incubation for 10 min.

Construction of QDs Probes.

The biotin-labeled ssDNA probes and streptavidin-labeled QDs (QDs1 to QDs3, 1 μM) were mixed at a molar ratio of 30:1, and then subjected to thermostatic reactions at 37 °C for 30 min. The free ssDNA probes were filtered with a Nanosep 100K OMEGA tubular ultrafiltration membrane (Pall Corporation, Port Washington, NY). After twice being spun and washed with PBS buffer (1×), the final products were re-dissolved in PBS buffer (1×) and stored at 4 °C.

Base-Stacking Hybridization Model.

The key part of base-stacking hybridization model is the 8 nt DNA recognition domain of signal probe. 50 nM of signal probe was employed as the signal giving-out complex. 50 nM of capture probe was mixed with 10 μL Magnetic beads (1mg/mL) for 30 min. Then, the target was captured by capture probe, and signal probe was added in to construct base-stacking hybridization model at the temperature of 37 °C.

Cell Culture.

Tumor cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin, 100 ug/ml streptomycin (GIBCO, Invitro- gen), at 37 ℃ with an atmosphere of 5% CO_2 and 95% air.

Results And Discussion

Design of Gold Island-Enhanced Multiplex Quantum Dots Fluorescent System.

A homogeneous nanoplatform based on gold island-enhanced multiplex QDs fluorescent system (**Figure 1**) was constructed to detect the content and multiplex point mutations of CTNAs. As shown in **Figure 1A**, the gold ions were reduced to the gold island on the surface of poly-L-lysine-coated glass sheet, and sulfydryl-labeled capture probes were connected to the gold island by Au-S bond for capture of target CTNAs, to form chip-like operation site. Three kind of QDs, with the emission at 525nm, 585nm and 650nm, were labeled with the biotin on three recognition DNAs to form signal probes. The principle of base stacking (**Figure S1 to S3**) was employed as the force to realize multiplex CTNAs detection and point mutations monitoring. The 3’ terminal of the four recognition DNA were designed as C (wild-type probe), T (135A probe), G (135C probe) and A (135T probe) to recognize the wild-type of 135G and the point mutation of 135A, 135C and 135T in the Kirsten rat sarcoma-2 virus (KRAS) genes of CTNAs. Mutated KRAS gene are proved to be associated with lung cancer, colorectal cancer and ovarian cancer. While the target exists, the capture probe on the gold island can immobilize the target, and then the mixture of signal probes was added in for identifying the target CTNAs with wild-type and point mutation sequence. After the washing steps, the signal probes were excited by UV light (300 nm), and the specific

fluorescent signals were enhanced by the gold island with the principle of surface plasmon resonance. The enhanced signals for target CTNAs were detected by the fluorescence spectrophotometer. The structure of gold island-enhanced multiplex QDs fluorescent system was shown in **Figure 1B**, the QDs probes were excited by the UV light, and the fluorescent signals were collected by the microscopic system that could recognize the single photon level difference. And the X-Y axis scan was executed to cover all the area of gold island chip.

Principle Validation and Optimization of Gold Island-Enhanced Multiplex Quantum Dots Fluorescent System.

In this section, we verified the feasibility of this system. The TEM results in **Figure 2A and B** presents the morphology of the synthesized gold island and the QDs-loaded gold island chip. The sulfydryl-labeled DNA probe (capture probe) was firstly labeled on the gold island, and the QDs probe was added after the target was immobilized by the capture probe. The results indicated that the gold island was punctate distributed (**Figure 2A**), and larger size particles appeared after the QDs loaded with the target (**Figure 2B**). These results proved the feasibility of gold island-enhanced multiplex QDs fluorescent system. Meanwhile, the QDs probe used in this work (QDs 1 to 3) were characterized by the dynamic light scattering (**Figure 2C and Figure S4**) and Zeta potentials (**Figure S5**). While the loaded QDs excited by the laser source, red dots were emerged. Herein, we observed the single dot in **Figure 2D** with the extension of time. And the fluorescent signal was detected and treated by the superposition of single photon level charge coupled device (CCD). The results indicated that the fluorescence increased with the data-acquisition time and reached a plateau at the time of 18s. Definitively, we verified the multiplex detection performance with three QDs probes. The results in **Figure 2E** indicated that the multiplex signals with three kinds of colors were acquired while the target presented, comparing with control group and single target. Hence, the principle of gold island-enhanced multiplex QDs fluorescent system was validated. Then, we evaluated the key factors of experiment, shown in **Figure S6, S7 and S8**. The results indicated that the optimal experiment conditions of hybridization time, washing time and hybridization temperature were set as 30 min, 4 times and 38℃.

Sensitivity and Specificity of Gold Island-Enhanced Quantum Dots Fluorescent Strategy.

With the optimized condition, the sensitivity and specificity were evaluated with the fluorescence spectrograph and gold island-enhanced multiplex QDs fluorescent system. Firstly, we verified the feasibility of multiplex detection for three circulating tumor microRNAs with gold island-enhanced multiplex QDs fluorescent system. The results in **Figure 3A** approved that the QDs probes could stably response to multiplex circulating tumor microRNAs, and the TEM results in **Figure 3B** revealed that the three QDs probes were immobilized on the surface with the targets. Then, the ‘golden standard’ fluorescence spectrograph was used for further verification of gold island-enhanced multiplex QDs fluorescent system (**Figure 3C**). The results in **Figure 3D**indicated that the QDs1, QDs2 and QDs3 probes could stably response to multiplex circulating tumor microRNAs, and the fluorescence intensity increased with the concentration of circulating tumor microRNAs. Differentiable signals of three emission peaks

corresponding to specific target were obtained. Meanwhile, a good linear response was achieved from 10 to 10^5 pmol (**Figure 3E, F and G**), and a high sensitivity of 1 pmol was achieved. Furthermore, the specificity was also evaluated by comparing with non-target CTNAs M1, M2 and random sequences (RS1 to RS3). The sequences were listed in **Table S1**. The results in **Figure 3H and I** indicated that the specific signals were only detected with targets, and the gold island-enhanced multiplex QDs fluorescent system achieved excellent specificity. Hereto, the feasibility of multiplex detection for three circulating tumor microRNAs with gold island-enhanced multiplex QDs fluorescent system was confirmed by fluorescence spectrograph.

Subsequently, the sensitivity and specificity of gold island-enhanced multiplex QDs fluorescent strategy were evaluated. The results in **Figure 4** indicated that the QDs1 probe achieved a high sensitivity of 0.1 pmol (**Figure 4A**), and the QDs2, QDs3 probes were 0.01 pmol (**Figure 4B and C**). The linear regression analysis was executed, R^2 values of 0.9984, 0.9957 and 0.9811 were achieved for QDs1 to QDs3. These results revealed a good linear relation. Meanwhile, excellent specificity was achieved by the gold island-enhanced multiplex QDs fluorescent system with the specific targets (**Figure 4A to C**). The results in **Figure 4D** indicated that the gold island-enhanced multiplex QDs fluorescent strategy could specifically response to targets (T1 to T3). The above results showed that the gold island-enhanced multiplex QDs fluorescent strategy realized high sensitivity and excellent specificity, which could respectively response to single target and multiplex targets of CTNAs.

Detection of CTNAs from Cultured Tumor Cell Lines and Blood of Tumor Patients.

To test the performance of detecting complex samples, three tumor cell lines (HepG2, A549, MCF-7) with high microRNA21 expression levels were detected by the gold island-enhanced multiplex QDs fluorescent system. The cell samples were processed by a total RNA extraction kit after cell counting (10^5 cells). The results in **Figure 5A, B and C** revealed that this system could stably response to the target from the tumor cell lines. Furthermore, the blood samples from tumor patients were detected in **Figure 5D, E and F**, the stable signals were also obtained. The outstanding performance for detecting complex samples with gold island-enhanced multiplex QDs fluorescent system was proved.

Furthermore, the multiplex detection of single-base mutations was constructed for lung cancer with gold island-enhanced multiplex QDs fluorescent strategy. The single-base mutations of 135A, 135C, 135T of the KRAS gene were detected with the designed strategy. The detailed strategy was listed in the Supporting Information (**Figure S2 to S3**). The results in **Figure 6A** (135A)**, B** (135C) **and C** (135T) indicated that the specific signal was obtained while the target of single-base mutation presented. Meanwhile, the multiplex signals were obtained with the three mutated targets (**Figure 6D**). Definitively, the three mutations were detected with the blood samples of lung cancer patients (**Figure 6E to H**). The results showed that the gold island-enhanced multiplex QDs fluorescent strategy could stably response to mutations of blood samples. Furthermore, we calculated the positive rates of the detections based on single mutation, two mutations and three mutations, the results in **Figure 6I** revealed that the positive rates were increased with the amount of detected mutations, the positive rates of detecting three

mutations was much higher than the two mutations, and the lowest is the single mutation detection. Therefore, the multiplex detection of single-base mutations based on gold island-enhanced multiplex QDs fluorescent strategy could response to multiplex targets with single test process. Hence, this platform achieved high detection rate in clinical samples that suitably met the strict clinical-requirements for multiplex point mutations detection of CTNAs, and thus has the potential to serve as an accurate paradigm for the tumor liquid biopsy based on CTNAs.

Conclusions

A gold island-enhanced multiplex QDs fluorescent platform was constructed for highly-sensitive detection of CTNAs that could also recognize the multiplex point mutations detection of CTNAs in serum. This strategy realized a homogeneous nano-platform for the enrichment, multiplex detection and point mutations monitoring of CTNAs with the principle of base-stacking. Furthermore, a gold island-enhanced multiplex QDs fluorescent strategy was employed as the highly-efficient signal giving-out mode, a high sensitivity of 10 amol and desirable specificity was achieved. The performance index of gold island-enhanced multiplex QDs fluorescent strategy for analysis of clinical CTNAs samples indicated that it could simultaneously detect the multiplex CTNAs and point mutations detection of CTNAs in blood samples. Hence, this platform suitably met the strict clinical-requirements for multiplex point mutations detection of CTNAs and thus has the potential to serve as an accurate paradigm for the liquid biopsy of tumor.

Declarations

Supplementary Information

Supplementary Information The online version contains supplementary material available at

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Authors' contributions

The manuscript was written through contributions of all authors. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated and analyzed during this research are included in this published article.

Ethics approval and consent to participate

All animal experiments were approved by the Ethics Committee of Southern Medical University.

Consent for publication

All authors agree for publication.

Competing interests

The authors declare no conflict of interest.

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Figures

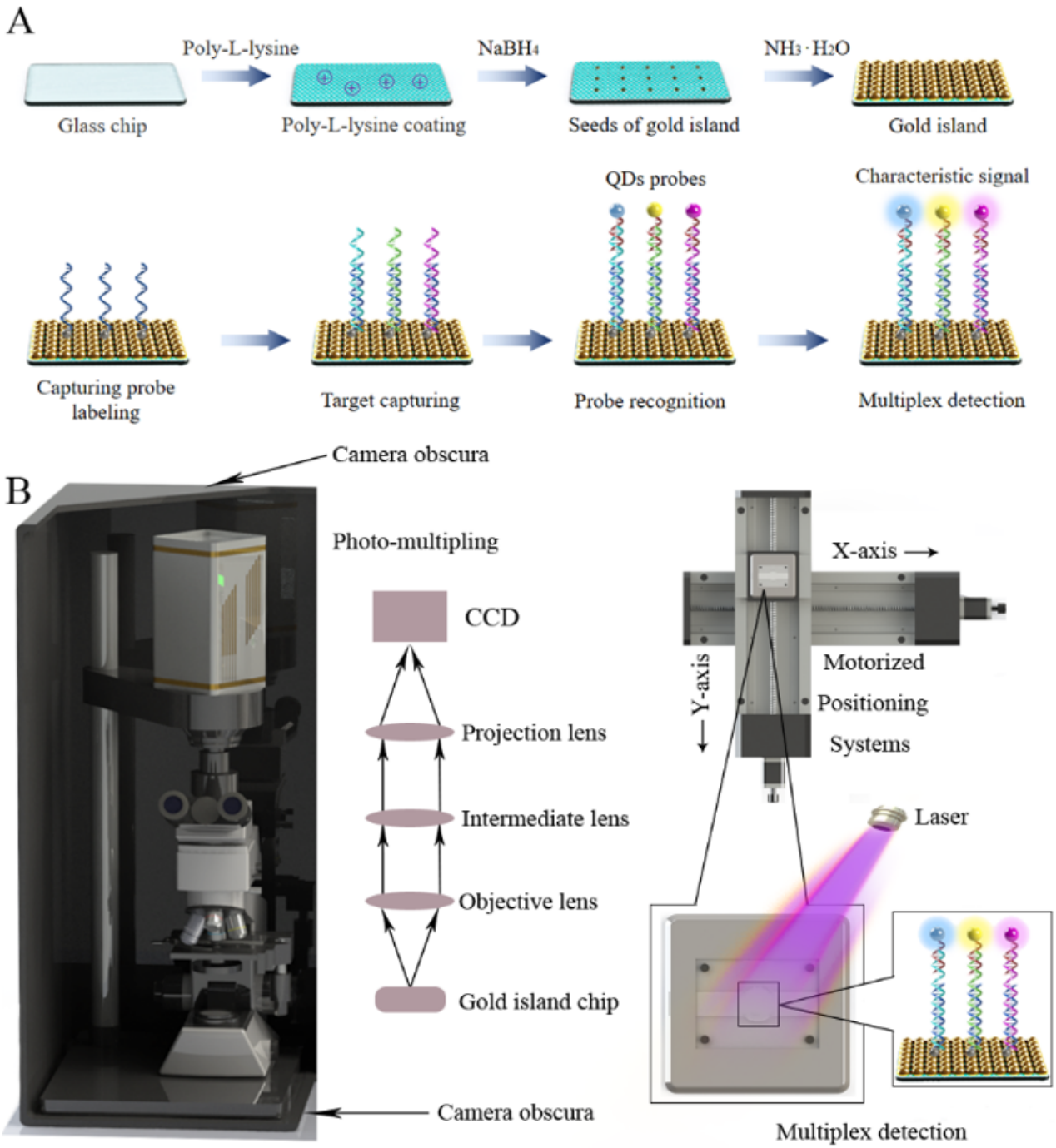

Figure 1

Gold Island-Enhanced Multiplex Quantum Dots Fluorescent System for Combined Diagnosis of Multiplex Circulating Tumor Nucleic Acids. A. Formation of gold island chip and detection process for CTNAs. B. Schematic diagram of gold island-enhanced quantum dots fluorescent system.

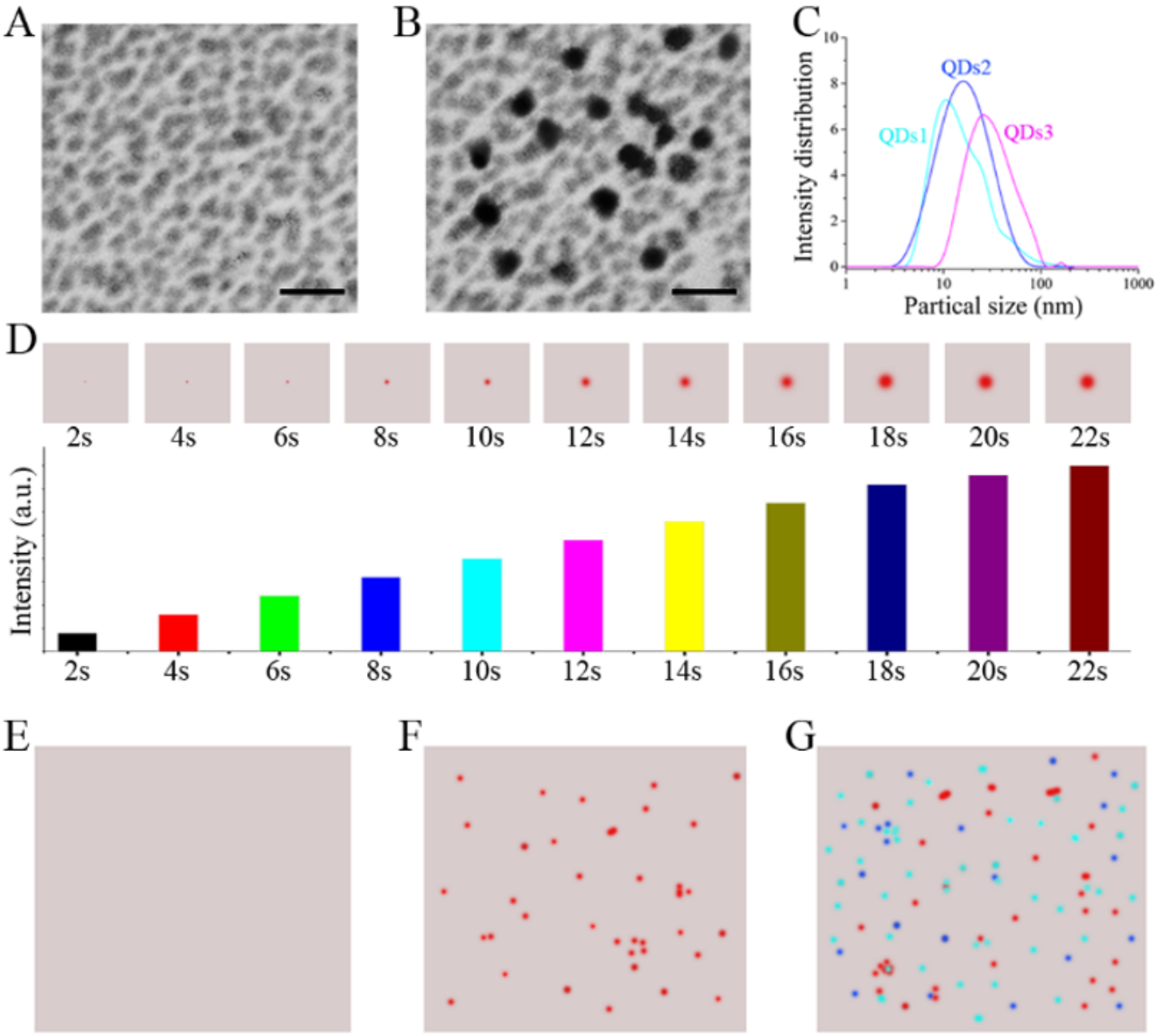

Figure 2

Principle Validation of Gold Island-Enhanced Multiplex Quantum Dots Fluorescent System. A. TEM morphology of the synthesized gold island. B. TEM morphology of the QDs-loaded gold island chip. C. Particle size of QDs probes. D. Optimization of the time of data acquisition. E. Multiplex detection performance of gold island-enhanced multiplex quantum dots fluorescent system.

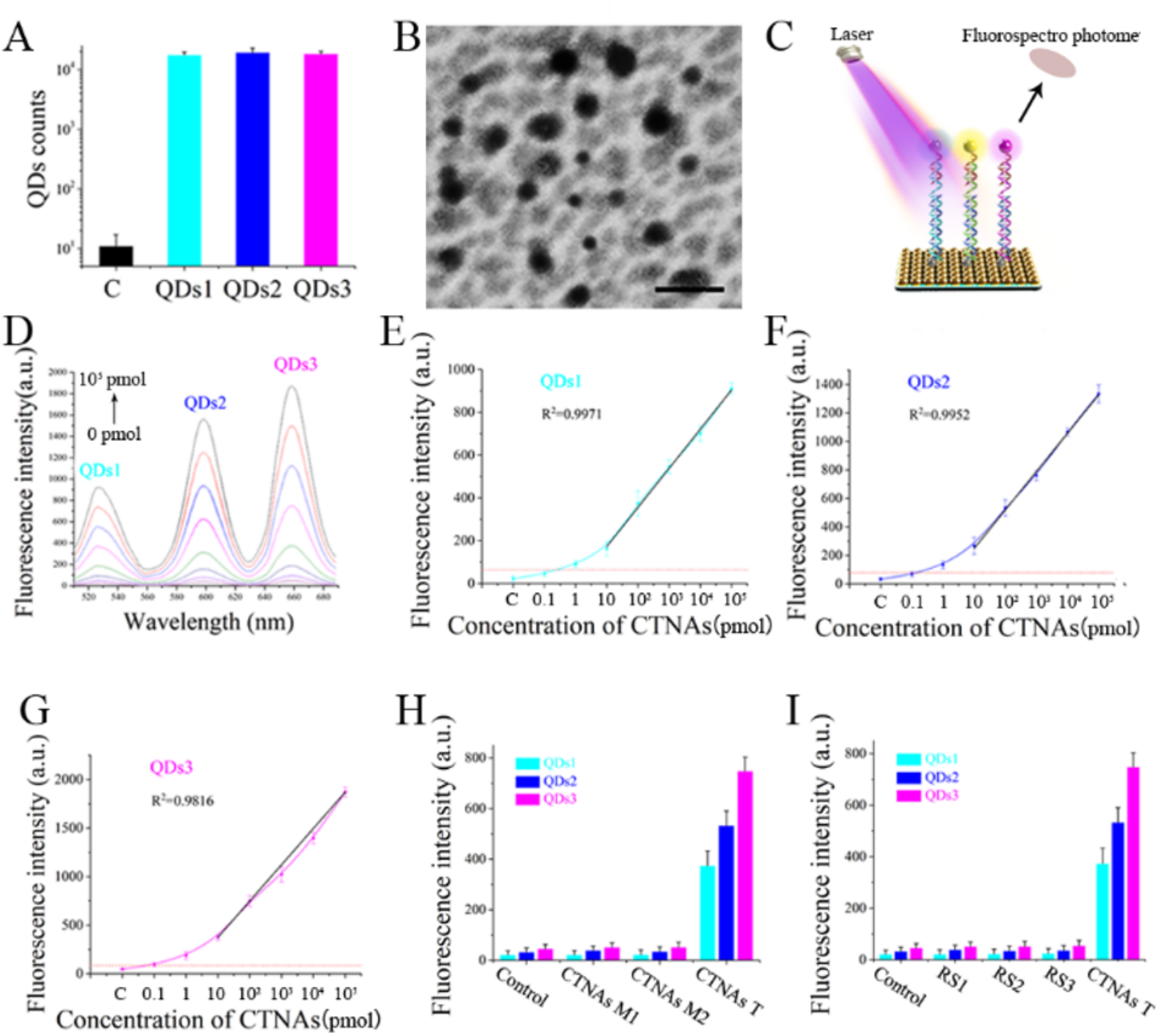

Figure 3

Sensitivity and Specificity of Gold Island-Enhanced Multiplex Quantum Dots Fluorescent System with Fluorescence Spectrograph. A. Detection of multiplex circulating tumor microRNAs. B. TEM morphology of the gold island-enhanced multiplex quantum dots chip with three circulating tumor microRNAs. C. Principle of multiplex circulating tumor microRNAs detection with fluorescence spectrograph. D. Sensitivity of multiplex circulating tumor microRNAs detection with fluorescence spectrograph. E. Linear analysis of QDs1 in Figure 3D. F. Linear analysis of QDs2 in Figure 3D. G. Linear analysis of QDs3 in Figure 3D. H. Specificity of gold island-enhanced multiplex quantum dots fluorescent system with CTNAs

M1 and M2. I. Specificity of gold island-enhanced multiplex quantum dots fluorescent system with random sequences (RS1 to RS3).

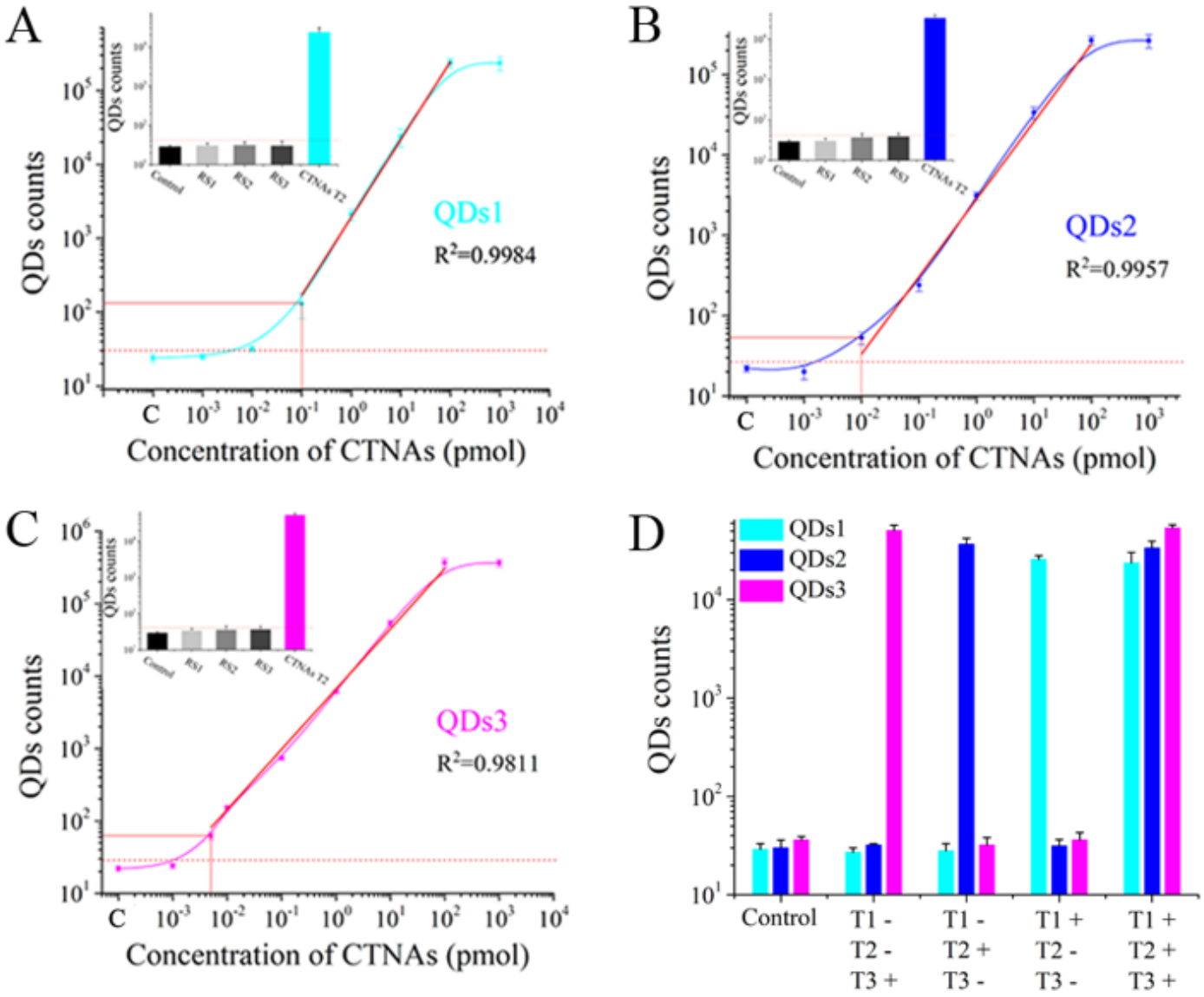

Figure 4

Sensitivity and Specificity of Gold Island-Enhanced Multiplex Quantum Dots Fluorescent Strategy. A. Detection of T1 target with different concentration and specificity of QDs1 probe. B. Detection of T2 target with different concentration and specificity of QDs2 probe. C. Detection of T3 target with different concentration and specificity of QDs3 probe. The concentration of T1 to T3 was varied from 10-3 pmol to 103 pmol. D. Single and multiplex target detection of T1, T2 and T3 targets.

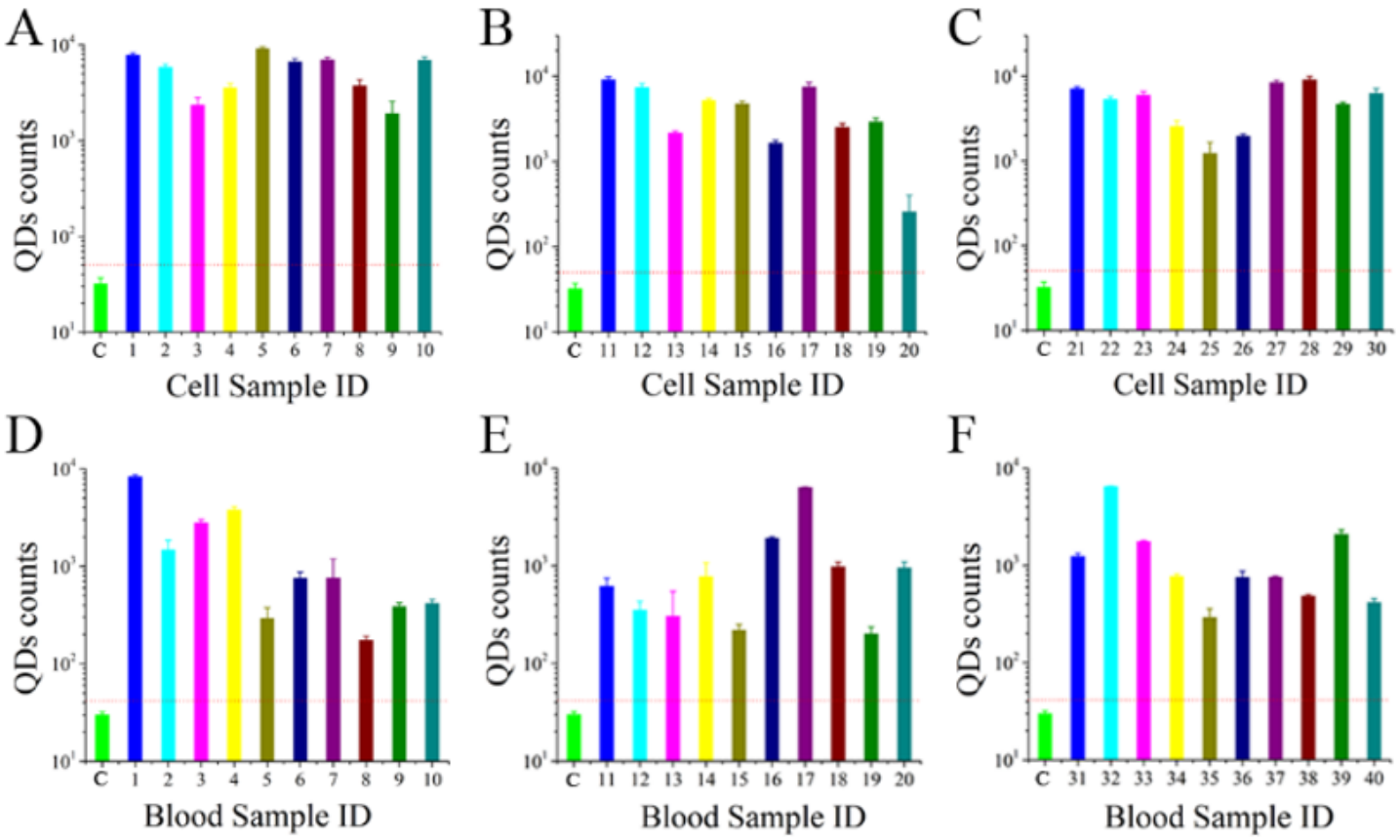

Figure 5

Detection of MicroRNA from Cell Lines (HepG2, A549, MCF-7) and CTNAs in Blood of Tumor Patients. A. Detection of microRNA from HepG2 cell lines. B. Detection of microRNA from A549 cell lines. C. Detection of microRNA from MCF-7 cell lines. D. Detection of CTNAs in blood of liver cancer patients. E. Detection of CTNAs in blood of lung cancer patients. F. Detection of CTNAs in blood of breast cancer patients.

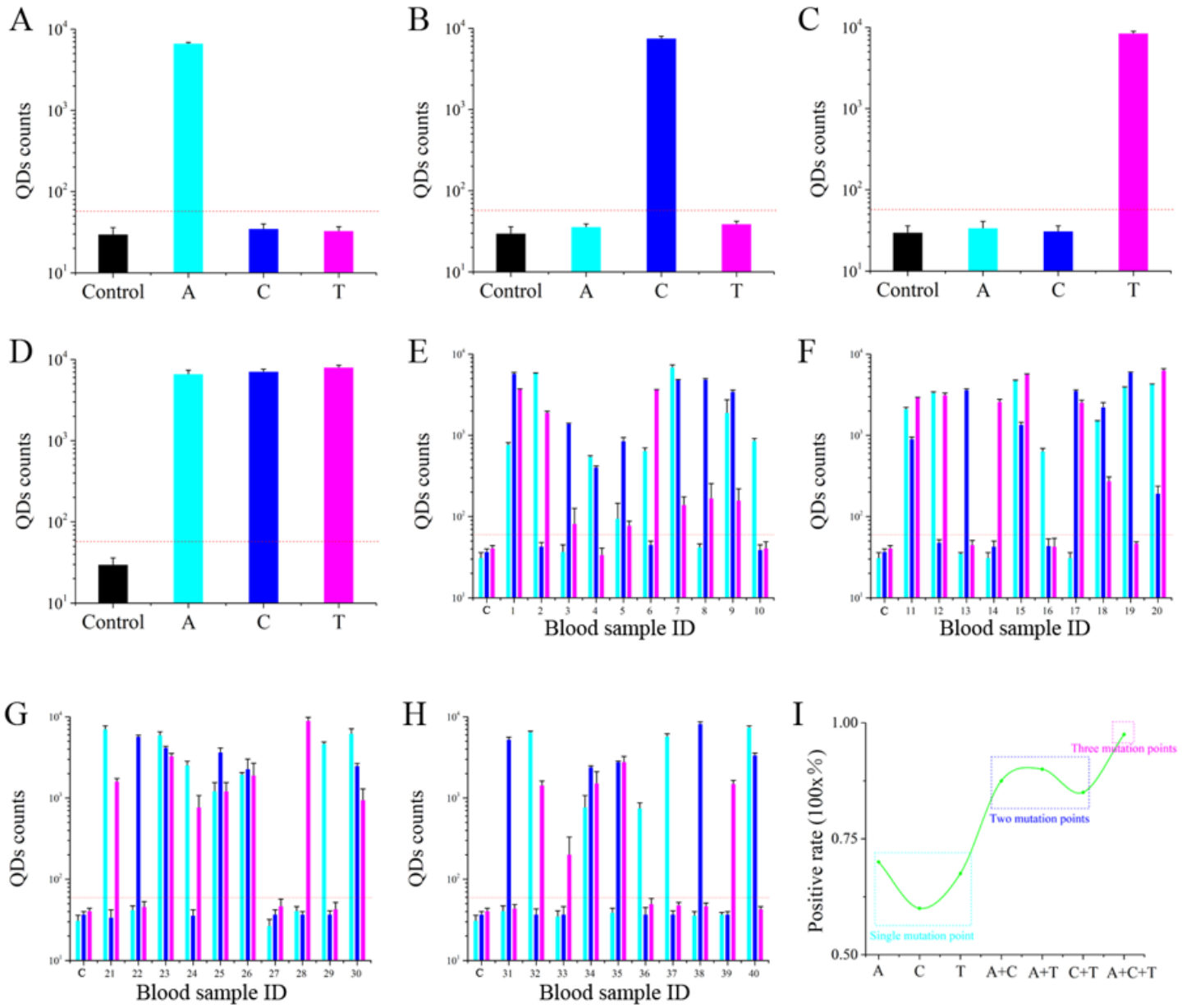

Figure 6

Multiplex Detection of Single-Base Mutations. A. Detection of 135A mutation with gold island-enhanced multiplex quantum dots fluorescent strategy. B. Detection of 135C mutation with gold island-enhanced multiplex quantum dots fluorescent strategy. C. Detection of 135T mutation with gold island-enhanced multiplex quantum dots fluorescent strategy. D. Multiplex detection of 135A, C and T with gold island-enhanced multiplex quantum dots fluorescent strategy. E. Detection of multiplex single-base mutations in sample ID 1 to 10. F. Detection of multiplex single-base mutations in sample ID 11 to 20. G. Detection of multiplex single-base mutations in sample ID 21 to 30. H. Detection of multiplex single-base mutations in

sample ID 31 to 40. I. Positive rate of the single, two and three mutation detection results in Figure 6E to H.

Supplementary Files

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- SupplementaryMaterial.docx