Electronic Supplementary Information

A Novel, Rapid, and Sensitive Homogeneous Sandwich Detection Method of Glypican-3 as a Serum Marker for Hepatocellular Carcinoma

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Materials and apparatus. The fetal bovine serum (FBS) and DMEM medium were purchased from HyClone, GE Healthcare Life Sciences (Logan, Utah). The carboxyl graphene was purchased from XF Nano Inc. (Nanjing, China). Ethylene diamine tetraacetic acid (EDTA) and bull serum albumin (BSA) were purchased from AMRESCO (Solon, USA). Superpositively charged fluorescent protein (ScGFP) was expressed and purified as described previously.[1,2] An expression plasmid of GPC3 (pEFGPC3) was kindly provided by Professor Jorge Filmus. GPC3 and anti-GPC3 monoclonal antibodies (mAbs) αGCN and αGCC were individually prepared as described below. Human serum samples were obtained from the Qilu Hospital (Shandong, China) with informed consent from 31 individuals with a normal liver (NL), 42 chronic hepatitis (CH) and 42 patients with hepatocellular carcinoma (HCC). FBS, BSA, skim milk powder, GPC3, mAb and ScGFP stock solutions were prepared with phosphate buffered saline (PBS) (10 mM Na_2HPO_4, 2 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl, and pH = 7.4).

The HCC cell line (HepG2) was obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China); grown in DMEM medium with 10% FBS, 2 mM L-glutamine, and 2 mM non-essential amino acids; and maintained at 37 °C in a humidified atmosphere with 5% CO_2.

The female Balb/c mice (6-8 weeks old) were obtained from Center for New Drug Evaluation of Shandong University. All of the experiments were performed under the experimental protocol approved by the local animal care committee of Shandong University.

The fluorescence intensity (at 509 nm) was measured using an EnSpire™ 2300 Multilabel Reader with excitation at 470 nm. A 384-well white plate (PerkinElmer plate) was used as a sample reservoir.

Purification of GPC3. The GPC3 was purified by affinity chromatography with the anti-GPC3 mAb coupled to agarose, which was prepared by coupling anti-GPC3 mAb to NHS-activated Sepharose™ 4 Fast Flow agarose (GE Healthcare) according to the manufacturer's protocols. Briefly, the culture supernatant of HepG2 was collected and loaded on a column containing anti-GPC3 agarose. The column was washed with 50 mM Tris-HCl buffer containing 0.5 M NaCl (first wash at pH = 8.0 and second wash at pH = 9.0) to remove the nonspecific bound impurities, and then, GPC3 was eluted with 100 mM trimethylamine, followed by immediate neutralization by 1 M NaH_2PO_4. Finally, the eluate of GPC3 was desalted by Microcon YM-10 (Millipore), followed by quantification using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and a BCA protein assay kit (Kangwei, China).

Preparation of anti-GPC3 mAbs. To prepare the mAbs αGCC and αGCN, the gene fragment encoding the 70 amino acids of the C-terminal and the remaining part of the GPC3 gene were cloned, expressed and purified to use as antigens for preparing anti-GPC3 mAbs. Female Balb/c mice (6-8 weeks old) were immunized with each prepared antigen three times, and then the one with a titer higher than 2000 was dissected. Its spleen cells were fused with the myeloma cell line P3x63Ag 8.653 to prepare hybridoma cells. By screening, two clones stably secreting high affinity mAbs against the C- and N-terminals of GPC3 were obtained and named αGCC and αGCN, respectively. To prepare enough mAbs, these two clones were individually inoculated in mice celiac, and the result led to a high concentration of mAbs used for purifying αGCN or αGCC with protein G-sepharose (GE Healthcare).

Preparation of the mAb-coated carboxyl graphene. To prepare αGCN- or αGCC-coated carboxyl graphene, 10 μg of αGCN or αGCC was dispersed in 100 μL of carboxyl graphene (1

mg/mL), and then, 5 mM EDTA was added to prevent the precipitation of the carboxyl graphene. After mixing, BSA was added to reach a final concentration of 1% (m/v) to block the unbound site, completing the preparation of αGCN- or αGCC-modified carboxyl graphene. This solution was stored at 4 °C and diluted 100-fold with Tris-HCl buffer (10 mM Tris-HCl, 100 mM NaCl and pH = 10.0) before use.

Determination of the optimal reagent adding sequence. Adding sequence of ScGFP, αGCN-carboxyl graphene, αGCC-carboxyl graphene, and GPC3 is an important factor affecting fluorescence quenching efficiency in this method. To determine the optimal reagent adding sequence, seven different orders were investigated, and the final content of αGCN-carboxyl graphene, ScGFP, GPC3 and αGCC-carboxyl graphene were 0.1 μg, 0.015 μg, 0.04 ng and 0.1 μg in 40 μL PBS buffer, respectively. During the analysis, after adding each reagent the mixture was incubated for 5 min at room temperature. The results were shown in Figure S1, in which the data was shown as the percentage of the quenching degree of that obtained in the control without GPC3. The results showed that the A sequence resulted in the highest fluorescence quenching rate, and thus this adding sequence was chosen for all following experiment.

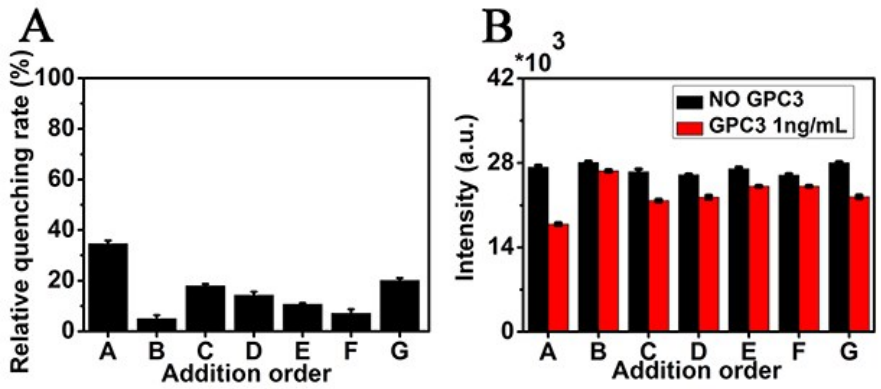

Fig. S1. Effect of reagent adding sequence on fluorescence quenching rate. A, αGCN-carboxyl graphene and GPC3 were mixed first followed by adding ScGFP and αGCC-carboxyl graphene sequentially; B, αGCN-carboxyl graphene and GPC3 were mixed first, and then αGCC-carboxyl graphene and ScGFP was added orderly; C, αGCN-carboxyl graphene and ScGFP were mixed first followed by adding GPC3 and αGCC-carboxyl graphene sequentially; D, αGCN-carboxyl graphene and ScGFP were mixed first followed by adding αGCC-carboxyl graphene and GPC3 sequentially; E, αGCN-carboxyl graphene and αGCC-carboxyl graphene were mixed first followed by adding GPC3 and ScGFP sequentially; F, αGCN-carboxyl graphene and αGCC-carboxyl graphene were mixed first by adding ScGFP and GPC3 sequentially; G, GPC3 and ScGFP were mixed first followed by adding αGCN-carboxyl graphene and αGCC-carboxyl graphene sequentially. The panel B displays the corresponding raw data of fluorescence intensity used for the calculation of relative quenching rate in panel A.

Determination of the optimal reaction time in each step. To determine the reaction time, 10 μL αGCN-carboxyl graphene (0.1 μg), 5 μL GPC3 (0.04 ng) were added to 10 μL PBS buffer in 384-well white plate, and incubated at the room temperature for different times. Then, 5 μL ScGFP (0.015 μg) was added to the mixture and incubated for 5 min in the dark. Finally 0.1 μg αGCC-

carboxyl graphene was added to the mixture and incubated for another 15 min. From the results, we found that the fluorescence intensity gradually decreased with incubation time increase until it became stable after 5 min. Therefore, the reaction time of αGCN-carboxyl graphene and GPC3, was determined as 5 min (Fig. S2A).

Similarly, the reaction time of the αGCN-carboxyl graphene-GPC3 complex and ScGFP was determined as 15 min (Fig. S2B), and the reaction time of αGCN-carboxyl graphene-GPC3-ScGFP complex with αGCC-carboxyl graphene was 10 min (Fig. S2C).

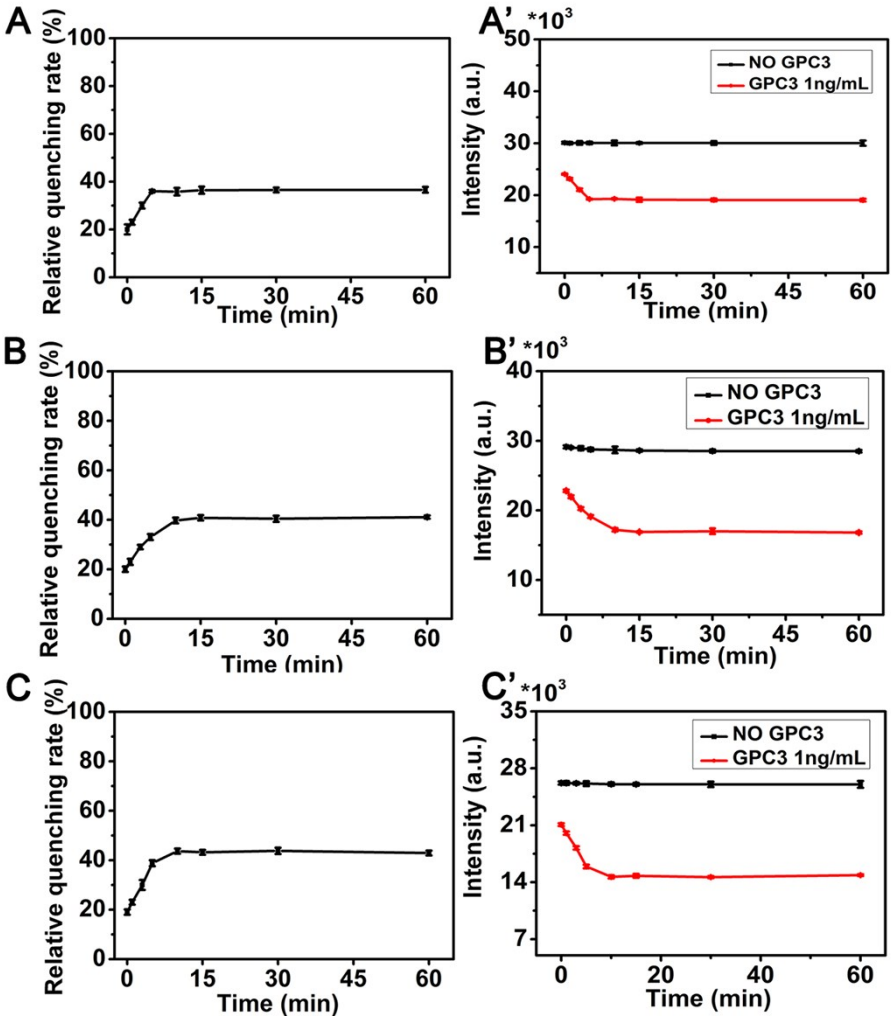

Fig. S2. (A-C) Effect of reaction time in each step on fluorescence quenching rate. A, the reaction time of αGCC-carboxyl graphene and GPC3; B, the reaction time of αGCC carboxyl graphene-GPC3 complex and ScGFP; C, the reaction time of αGCN-carboxyl graphene-GPC3-ScGFP complex and αGCC-carboxyl graphene. The final concentrations of αGCC-carboxyl graphene, GPC3, ScGFP and αGCN-carboxyl graphene were 2.5 μg/mL, 1ng/mL, 0.375 μg/mL and 2.5 μg/mL, respectively. Panels A', B' and C' display the corresponding raw data of fluorescence intensity used for the calculation of relative quenching rate in panels A, B and C, respectively.

Determination of the optimal reaction buffer. Effects of buffers with different pH were investigated, including 10 mM NaAc-HAc buffer (pH 5.0-6.0), 10 mM NaH_2PO_4-Na_2HPO_4 buffer (pH 6.0-8.0), and 10 mM Tris-HCl buffer (pH 7.0-10.0). Results were shown in Figure S3A, the

highest fluorescence quenching rate was found in Tris-HCl buffer (pH 10.0), which were similar to the research of ScGFP for the detection of Glycosaminoglycan.[2]

To get the better relative fluorescence quenching degree, different concentrations of NaCl were examined in 10 mM Tris-HCl, pH 10.0. Data were shown in Figure S3B as the percentage of the quenching activity. With the increasing of concentration of NaCl, the fluorescence quenching degree firstly increased and then decreased, which indicated that the nonspecific binding of ScGFP to other molecular could be inhibited by increasing salt concentration but the specific binding to GPC3 could be interfered by too high salt concentration. Therefore, 10 mM Tris-HCl, 100 mM NaCl, pH 10.0 was selected as the optimal reaction buffer for the following studies.

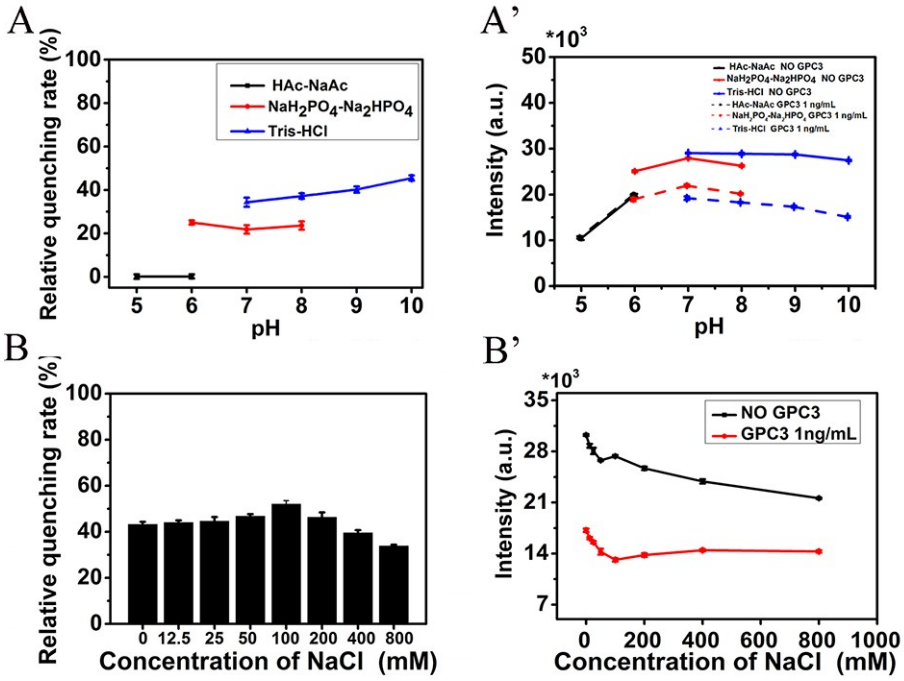

Fig. S3. Determination of the optimum reaction buffer. A, Relative fluorescence quenching rate in different buffers with varying pH values from 5 to 10. B, Effects of NaCl concentration on fluorescence quenching rate. Panels A' and B' display the corresponding raw data of fluorescence intensity used for the calculation of relative quenching rate in panels A and B, respectively.

Determination of the optimal concentration of mAb coated to carboxyl graphene. To determine the optimal concentration of the mAb coated to carboxyl graphene, different concentration of mAb was incubated with carboxyl graphene to prepare the mAb-carboxyl graphene and used to perform the fluorescence quenching experiment at the optimal reaction conditions as described above. The results show that the fluorescence quenching rate increase with the increase of mAb concentration until 0.1 mg/mL, corresponding to 0.01 μg mAb being coated to 10 μL carboxyl graphene (10 μg/mL), and then decrease gradually (Fig. S4), which suggest that the amount of mAb is too low to capture most of the ScGFP, and on the contrary when the amount of mAb is too high the free mAb will completely bind to the ScGFP to prevent fluorescence quenching by carboxyl graphene. Therefore, the 0.1 mg/mL mAb was chosen to coat carboxyl graphene in this study.

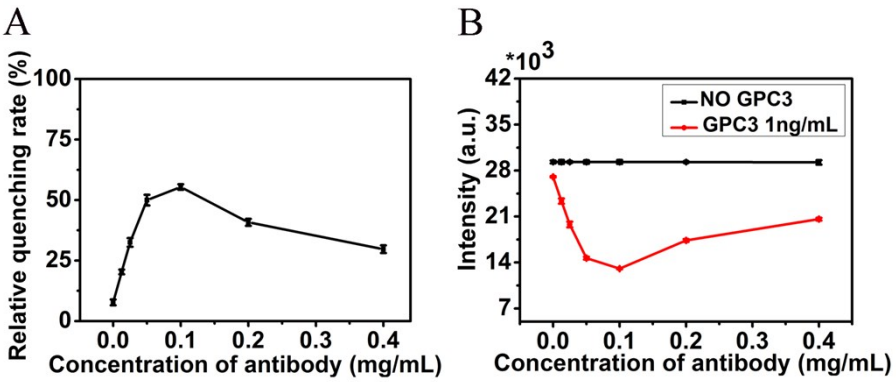

Fig. S4. A, Effect of concentration of mAb coated to carboxyl graphene on fluorescence quenching rate. B, The corresponding raw data of fluorescence intensity used for the calculation of relative quenching rate in panel A.

Determination of the optimum blocking condition. After mAb coated to carboxyl graphene, the unbound sites in carboxyl graphene were blocked with blocking reagent. To find the best blocking conditions, different concentrations of BSA and skim milk power in Tris-HCl buffer were investigated. As shown in Figure S5, the final concentration of 1% BSA and skim milk power individually showed the best performance. In this study, 1% BSA was selected as blocking reagent for subsequent experiments.

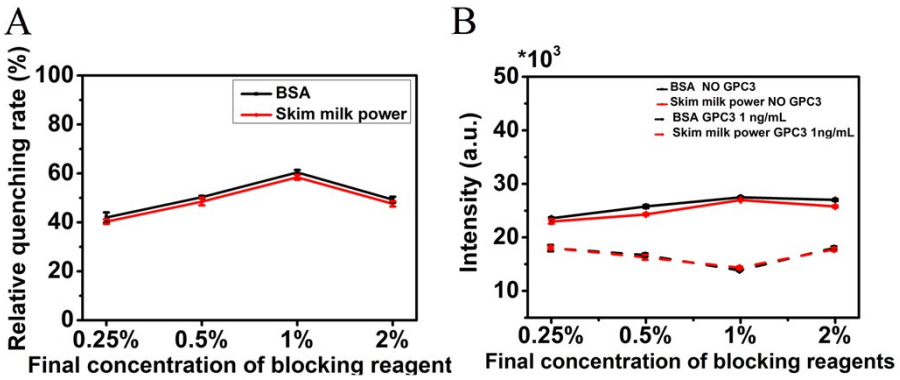

Fig. S5. A, Determination of the optimum blocking conditions. The final concentrations of αGCC-carboxyl graphene, GPC3, ScGFP and αGCN-carboxyl graphene were 2.5 μg/mL, 1ng/mL, 0.375 μg/mL and 2.5 μg/ mL, respectively. B, The raw data of fluorescence intensity for the preparation of panel A.

Determination of the optimal concentration of ScGFP. The amount of ScGFP introduced into the system is critical. A series of concentrations of ScGFP were tested to get the optimal quenching rate. The indicated concentration of GPC3 (5 μL) diluted with Tris-HCl buffer and 10 μL of αGCN-carboxyl graphene (0.1 μg) were added to 10 μL of Tris-HCl buffer in a 384-well white plate, followed by incubation at room temperature for 5 min. Afterwards, 5 μL different amount of ScGFP diluted with Tris-HCl buffer was added to the solution,

respectively, which was further incubated for 15 min in the dark, followed by the addition of 0.1 μg of αGCC-carboxyl graphene and incubation for another 10 min. Finally, the fluorescence intensity (at 509 nm) was measured by the microplate reader with excitation at 470 nm. From the results, we can see that if the concentration of the ScGFP is too high or too low the quenching rate is not well. Therefore, the 0.375 μg/mL was selected as the optimal concentration.

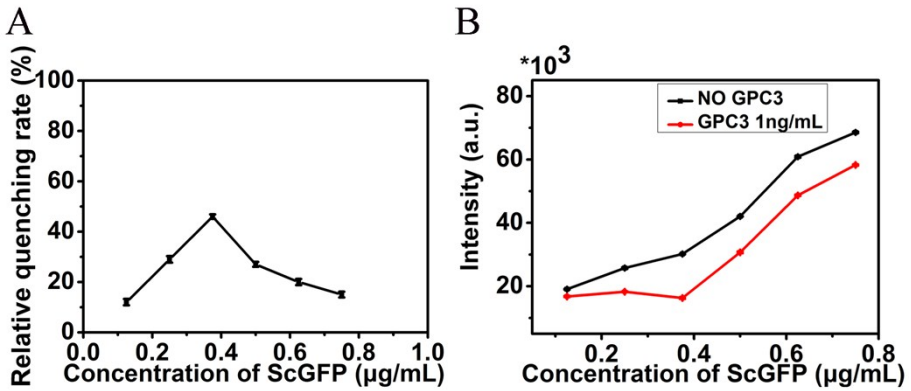

Fig. S6. A, Determination of the optimal concentration of ScGFP. The final concentrations of αGCC-carboxyl graphene, GPC3 and αGCN-carboxyl graphene were 2.5 μg/mL, 1ng/mL and 2.5 μg/ mL, respectively. B, The raw data of fluorescence intensity for the preparation of panel A.

Determination of the optimal fluorescence quencher. To get the better quenching rate, we tested three commonly used nanomaterial fluorescence quenchers: carboxyl graphene, graphene oxide and collaurum. From the results, we can see that the graphene in particular carboxyl graphene has the better quenching rate. This may be because the graphene has a sheet structure, which makes it easy to form the sandwich construction to result to the maximum quenching extent. Thus, carboxyl graphene was selected as the quencher in this study.

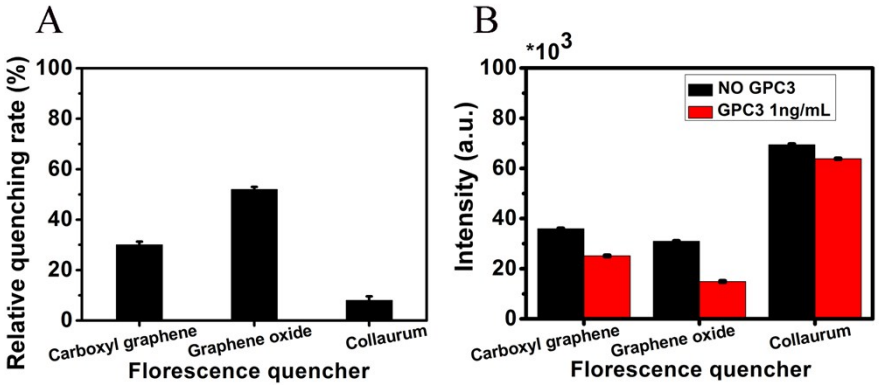

Fig. S7. A, Determination of the optimum fluorescence quencher. The final concentrations of αGCC-indicated quencher, GPC3, ScGFP and αGCN-indicated quencher were 2.5 μg/mL,

1ng/mL, 0.375 μg/mL and 2.5 μg/ mL, respectively. B, The raw data of fluorescence intensity for the preparation of panel A.

GPC3 measurement in buffer or serum. Different amounts of GPC3 (5 μL) diluted with Tris-HCl buffer and 10 μL of αGCN-carboxyl graphene (0.1 μg) were added to 10 μL of Tris-HCl buffer in a 384-well white plate, followed by incubation at room temperature for 5 min. Afterwards, 5 μL of ScGFP (0.015 μg) diluted with Tris-HCl buffer was added to the solution, which was further incubated for 15 min in the dark, followed by the addition of 0.1 μg of αGCC-carboxyl graphene and incubation for another 10 min. Finally, the fluorescence intensity (at 509 nm) was measured by the microplate reader with excitation at 470 nm. To show the key role of HS chains for the binding of ScGFP to GPC3 and the specificity of this method for GPC3, GPC3ΔGAG, or heparinase I- and heparinase III-treated GPC3 were also analyzed by this method.

To investigate the possibility of this method for detecting GPC3 in complex biological samples, the GPC3 dissolved in Tris-HCl buffer containing 20% human serum was measured using the same procedure as described above.

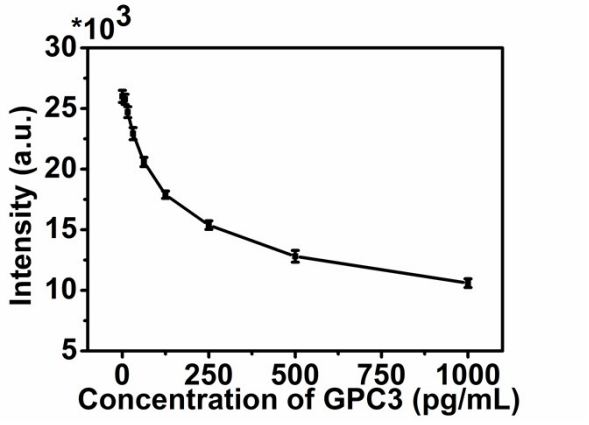

Fig. S8. The raw data of fluorescence intensity used for the calculation of relative quenching rate in Fig. 1A.

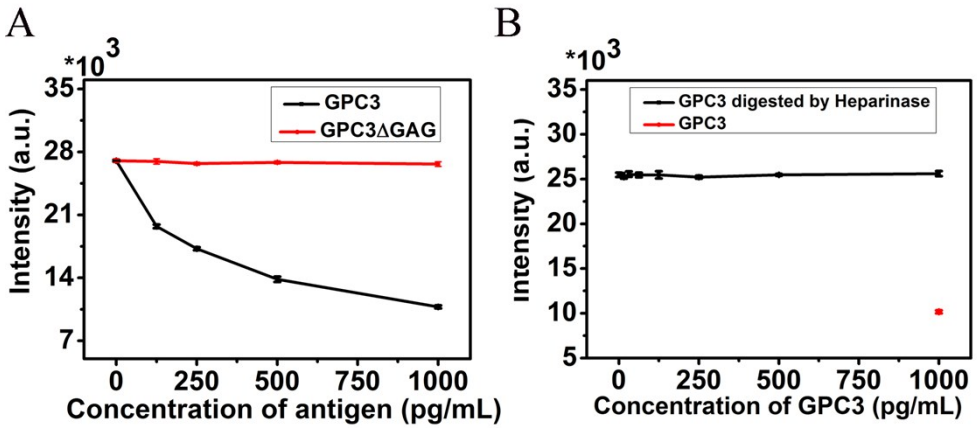

Fig. S9. The corresponding raw data of fluorescence intensity used for the calculation of relative quenching rate in Fig. 2A and 2B.

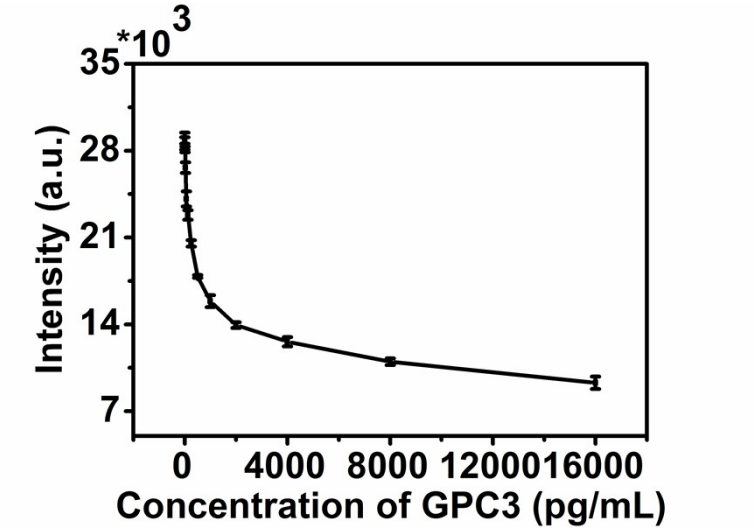

Fig. S10. The raw data of fluorescence intensity used for the calculation of relative quenching rate in Fig. 3.

Interfering test of this method. To investigate the interferences of substances derived from blood, two commonly serum interferents, hemolysate and triglyceride, were tested for their interferences to GPC3 detection. The indicated amount of human haemoglobin and triglycerides were added into to the GPC3 (1 ng/mL) in serum, and the concentrations of GPC3 in these samples were measured using this method. From the data shown in Table S1, we can see that these substances do not show significant interference to the detection of GPC3 in serum.

Table S1. Interference from addition of Hemolysate and Triglyceride to GPC3 sample.

Interferent	Concentration (ng/mL)	Measured value (ng/mL)	Recovery Rate (%)
Hemoglobin	0	0.98	98
	5	1.02	102
	10	1.18	118
Triglycerides	0	1.03	103
	5	0.98	98
	10	1.06	106

Detection of GPC3 in clinical samples. The serum of 31 individuals with NL, 42 patients with CH, and 42 patients with HCC were diluted 5-fold with Tris-HCl buffer and were evaluated using the homogenous detection technology.

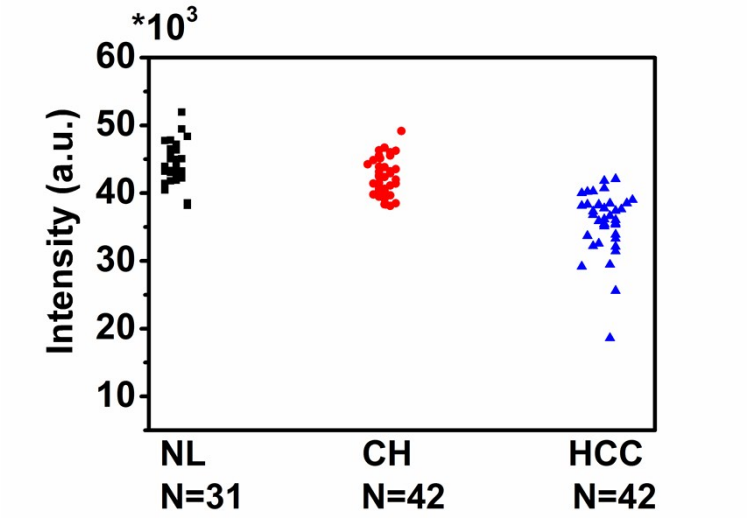

Fig. S11. The raw data of fluorescence intensity used for the calculation of relative quenching rate in Fig. 4.

Detection of GPC3 in clinical samples with conventional sandwich ELISA method. We have done a conventional sandwich ELISA to validate the data shown in the Figure 4.[3] The ninety-six-well ELISA plates were covered with 0.5 μg α GCN and incubated overnight at 4 °C. After washing, the 1% BSA in PBS were added to block the well for two hours. Then the 50 μL of diluted serum samples (1:4 in PBS) was added and incubated for four hours. After washing, the anti-GPC3 camel polyclonal antibody were added. After washing, horseradish peroxidase–conjugated sheep anti-camel IgG were incubated and hydrogen peroxide were as substrates. In the meantime, the purified GPC3 were added to the 5 times diluted normal sera to prepare the calibration curve. From the concentration (Fig S12B), we can find that the concentration of GPC3 in normal liver is 6.25±3.17 ng/mL and in chronic hepatitis is 8.79±5.75 ng/mL. However, GPC3 in HCC has elevated levels (28.70±29.90 ng/mL) ranging from 5.24-201.72 ng/mL. Using the mean±2SD (~20.29 ng/mL) of the GPC3 value in the chronic hepatitis as cut-off point, 57.14% of HCC (24 of 42) has the significantly elevated value. Comparing with our sandwich method (Fig. 4), the results are not totally same but similar. The difference may be caused by the different sensitivity and detection limition of this two methods.

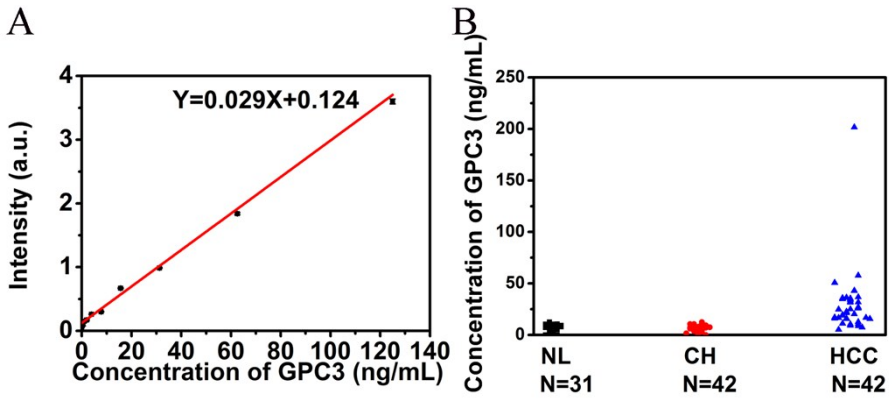

Figure S12. Detection of GPC3 in clinical samples with sandwich ELISA method. A, The standard curve. B, The concentration of GPC3 in clinical samples.

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