Supporting Information

Multitag-Regulated Cascade Reaction: A Generalizable Ultrasensitive MicroRNA Biosensing Approach for Cancer Prognosis

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Experimental Section

1 Materials and instruments

Reagents. Quartz optical-fiber (6 cm in length, 600 μm in diameter) was purchased from Chunhui Science & Technology Industrial Co., China. The optical-fibers were etched to tapered-structures by hydrofluoric acid (HF) before further chemical modification. Streptavidin (SA), desthiobiotin (DTB), EZ-link NHS-desthiobiotin (NHS-DTB), PBS buffer packs and streptavidin-coupled magnetic beads (MBs, 1 μm diameter, 10 mg/mL) were purchased from Thermo Fisher Scientific Company. Bovine serum albumin (BSA), (3-mercaptopropyl)trimethoxysilane (MTS), (3-Aminopropyl)triethoxysilane (APTS), 4-maleimidobutyric acid N-hydroxysuccinimide ester (GMBS), sodium citrate ($Na_3C_6H_5O_7 \cdot 3H_2O$), citric acid ($C_6H_8O_7 \cdot H_2O$), sodium chloride (NaCl), tris-HCl and formamide were purchased from Sigma-Aldrich company. RNA Lysis buffer was purchased from Promega (Z3051). Amicon Ultra 3K and 10K centrifugal filters were from Merck Millipore, Life Science. Cy5.5-ester was obtained from GE Healthcare, Life Sciences. Cy5.5 labeled SA (SA-F) was synthesized according to previously reported method, and the labeling ratio was calculated to be 8 based on UV measurements.[1] All acids and other organic solvents were purchased from Beijing Chemical Works. All aqueous solutions were prepared using molecular biology grade USP sterile purified water (Corning Cellgro, NY, USA).

Cancer patient samples. Urine samples of patients with non-small cell lung cancer (NSCLC) and unspecified lung cancer (USLC) were collected from Beijing Aizhen hospital (N = 9) and Beijing Cancer Hospital (N = 9), respectively.

Oligonucleotides. All nucleic acid oligonucleotides (HPLC-purified) were purchased from Sangon Biotechnology Co. Ltd. (Shanghai, China), as listed in **Table S1**. The design of hairpin sequences are refered to the work of Ma et.al.[2]

Buffers

PBS buffer: 100 mM PBS, 150 mM NaCl, pH 7.4;
Equilibration buffer: 10 mM PBS buffer, 15 mM NaCl, pH 7.4;
SPSC buffer: 0.75 M NaCl, 50 mM Na_2HPO_4, pH 7.4;
Washing buffer: 0.05% SDS, pH 1.9;
Isoelectric BSA blocking solution: 2 mg/mL of BSA in 0.1 M citrate buffer, pH 4.6;

Instruments. Fluorescence (FL) spectra were obtained by Hitachi F-7000 spectrometer. Unless specified otherwise, the excitation wavelength for FAM and Cy5.5 are set at 495 nm and 675 nm, respectively. UV absorbances were measured by Hitachi U-3900 spectrometer. The optical-fiber

diameter was monitored with a microelectrode polisher (model 2002-C, Inbio Life Science Instrument Co., Ltd., Hubei, China). Atomic force microscopy (AFM) measurements were carried out using Bruke (Dimension edge) at Tianhe Scientific Center.

2 Optical-fiber based fluorescent biosensor

The newly developed optical-fiber biosensor consumes less reagents and is more automated than previously developed ones.[3] There are two major updates for this biosensor: (1) the manual injection system has been replaced with an automatic injection system; (2) A small-sized cylindrical flow cell is used. The minimum volume needed for each sample injection is 250 μL (decreased by ~44%) compared to previously developed prototypes. The pump speed in sample injection and elution processes is set at 12 μL/s during the whole study.

3 Verification of TSD1 and TSD2 processes

First of all, 5 μM of hairpin strands (H1 beacon, H1 and H2-F) were individually denatured at 90 ^{o}C for 5 min, and then cooled at room temperature (25 ^{o}C) for over an hour to obtain annealed hairpins.

For TSD1 verification, 5 nM of In was added into 50 nM of annealed H1 beacon in 1 mL SPSC buffer, the obtained solution was incubated at 25 ^{o}C for different times (30-150 min) before FL measurement.

For TSD2 verification, 5 nM of Bio-In was added into 1 mL SPSC buffer containing annealed H1 (50 nM) and H2-F (50 nM), the solution was incubated at 25 ^{o}C for 1 h. Next, 50 μL of 10 mg/mL streptavidin-coupled magnetic beads (MBs) was added into the solution to capture biotinylated MCR assemblies (AB), and the MB-involved mixture was gently rotated for 20 min at 25 ^{o}C. Then the AB-conjugated MBs were separated from unreacted hairpins using a magnetic stack, and the supernatant was collected for further FL measurement.

4 Characterization of MCR assemblies by AFM measurements and gel electrophoresis

AFM Measurement. MCR assemblies for AFM characterization was prepared by incubating 0.5 μM Bio-In, 5 μM Bio-H1 and 5 μM H2-F at room temperature for 48 h (Bio-H1 and H2-F were independently annealed). For AFM control group, only 5 μM Bio-H1 and 5 μM H2-F are mixed at room temperature for 48 h. Then the obtained mixture containing MCR assemblies (MCR mixture) was stored at 4 ^{o}C before AFM measurements. For AFM measurements, a freshly cleaved mica surface was prepared for sample mounting. Next, 40 μL of 0.5 % APTS aqueous solution was added to the center of the mica surface for 2 min. The mica surface was cleaned with water and dried in air. Then 20 μLMCR mixture was added to the center of the mica surface and dried in air for AFM measurements. The sample was scanned under tapping mode.

Native Gel Electrophoresis. A 15% freshly prepared polyacrylamide gel was prepared with 5× TBE buffer. Electrophoresis was run at 60 V for 1 h in 1× TBE buffer. Gels were stained with GelRed Acid Gel Stain (Biotium, US) and imaged under UV illumination using FLS-5100 film (Fuji Photo Film Co., Ltd., Tokyo, Japan).

5 Preparation of DTB functionalized optical-fiber sensing surface

Firstly, DTB-labeled BSA (BSA-DTB) was synthesized using BSA and N-Hydroxysuccinimide (NHS)-activated desthiobiotin (NHS-DTB) as starting materials (**Figure S2**). Initially, 10 mM NHS-DTB in DMF and 1 mg/mL BSA (c_0) in 100 mM PBS buffer were prepared as stock solutions. (Please note that this labelling reaction must be conducted using amine-free buffer such as PBS at pH 7.2-8.0.) To investigate the optimal labeling ratio, 0X, 5X, 10X, 15X 50X and 75X molar excess of NHS-DTB to BSA were incubated with 1 mg/mL BSA (c_{before}) in PBS buffer for 1 h at 25 °C (0X as blank control). During the incubation, NHS moiety reacted efficiently with primary amine groups of BSA to form stable amide bonds. After that, unreacted NHS-DTB molecules were removed out of the system by Amicon-10K centrifugal filter (4 °C, 12000 rpm, 8 times). After purification, the synthesized BSA-DTB were collected and diluted to 1 mL. The protein recoveries could be calculated based on the changes of UV absorbance at the characteristic peak (278 nm) of BSA before ($Abs._{before}$) and after ($Abs._{after}$) Amicon purification. Recovery = $Abs._{after}/Abs._{before}$ based on Beer-Lambert law (**Table 2**), and the concentration of BSA after purification (c_f) can be calculated by c_f = recovery × c_0.

Taking advantages of remaining amine groups, BSA-DTB could be covalently modified onto the optical-fiber surface based on previously reported protocols with slight changes.[4] Before surface modification, the tapered optical-fiber was cleaned in hot piranha solution (v/v H_2SO_4: 30% H_2O_2 = 3:1). As shown in **Figure S3**, the cleaned fiber was first immersed in 2 % MTS toluene solution for 2 h at 25 °C. Next, sulfhydryl activated fiber was immersed in 1 mg/mL GMBS ethanol solution for 1 h at 25 °C. Finally, the GMBS-fiber was incubated with 0.05 mg/mL BSA-DTB (0-75X) at 4 °C overnight. Before usage, the DTB-fiber was further blocked in isoelectric BSA blocking solution for more than 6 hours to reduce nonspecific adsorption.[5]

6 Detection of miR in buffer/human urine using the optical-fiber based biosensor

For miR detection in SPSC buffer using MCR approach, different concentrations of *let-7a* were incubated with independently annealed Bio-H1 and H2-F (both 1 μM) in 1 mL SPSC buffer at room temperature (25 °C) for 4 h. For its monotag-regulated control group, Bio-I and H1 were used instead. The optical-fiber based detection proceeded as follows: (1) 20 nM of unlabeled SA was first pumped into the flow cell to react with DTB surface for 2 min; (2) then unbound SA were washed off by SPSC

buffer elution; (3) MCR assemblies were pumped into the flow cell and designed to reacted with surface-bound SA for 10 min; (4) SDS washing buffer containing SDS was pumped into the flow cell to wash off surface bound SA together with MCR assemblies; (5) Equilibration buffer was pumped into the flow cell to regenerate the sensing surface for next round of detection. All original sensorgrams are presented without deducting the baseline.

Human urine samples pretreatment: After fresh urine collection in hospital, 0.1 % v/v of RNA lysis buffer was immediately added into the urine samples to resist possible enzymatic degradation. After gently but sufficiently mixing, the samples were frozen at − 80 ^{o}C before further treatment.

Detection miR in human urine samples: Firstly, urine samples were unfrozen to room temperature. For each test, 0.5 mL of urine sample was concentrated to 30 μL using Amicon-3K centrifugation (12000 rpm, 4 ^{o}C). 1 μL of the concentrated urine was added into 9 μL of SPSC buffer, and the obtained solution was incubated with independently annealed H1 and H2 (both 1 μM) in 1 mL SPSC buffer at room temperature (25 ^{o}C) for 4 h. The following detection procedure was the same as the above. The statistical significance of the net signals obtained from different groups of urinary samples were analyzed by Origin 9.0 using One-Sample t-Test.

7 Logistic fitting of the calibration curve

Normalized net signals (NNS) were used in preparing the calibration curve, which was fitted with a four-parameter Logistic model using Origin 9.0:

$$S = \frac{A_1 - A_2}{1 + \left([c]/[c_0]\right)^p} + A_2$$

Where [c] represents analyte concentration; S represents normalized net signal (NNS), [c_0] represents the analyte concentration at the midpoint or infection point (IC50); and p is the slope of the tangent at IC50. A_1 and A_2 represent the minimum and maximum signal of the calibration curve, respectively. Linear working range was defined as analyte concentrations corresponding to 20%-80% of the maximal signal (A_2); Limit of detection (LOD) was defined as the analyte concentration corresponding to 10% of the maximal signal (i.e. NNS = 10 %).

The fitted parameters corresponding to **Figure 4D** are:

$A_1 = – 15.7 \pm 3.7$; $A_2 = 114.9 \pm 3.9$; $c_0 = 0.28 \pm 0.06$; $p = 0.25 \pm 0.02$ ($R^2 = 0.99971$)

The calculated LOD and linear range are:

LOD = 0.8 fM; Linear range: 1 fM - 71 pM

Supplementary Tables

Table S1 Sequences used in this developed MCR approach

Name	Sequence (5'-3')
Bio-In	**Biotin**-TGAGGTAGTAGGTTGTATAGTT
H1	AGTAGGTTGTATAGTTCAAAGTAACTATACAACCT ACTACCTCA
Bio-H1	AGTAGGTTGTATAGTTCAAAGTAACTATACAACCT ACTACCTCA-**biotin**
H1 beacon	**FAM**-AGTAGGTTGTATAGTTCAAAGTAACTATACA ACCTACT**(BHQ1)**ACCTCA
H2-F	**Cy5.5**-ACTTTGAACTATACAACCTACTTGAGGTAGT AGGTTGTATAGTT
Hsa-let-7a	UGAGGUAGUAGGUUGUAUAGUU
Hsa-let-7b	UGAGGUAGUAGGUUGU**G**U**G**GUU
Hsa-let-7e	UGAGGUAG**G**AGGUUGUAUAGU
Hsa-let-7f	UGAGGUAGUAG**A**UUGUAUAGUU
Hsa-let-7g	UGAGGUAGUAG**U**UUGUA**C**AGU
Hsa-let-7i	UGAGGUAGUAG**U**UUGU**GCU**GUU
Hsa-miR-122a	UGGAGUGUGACAAUGGUGUUUG
Hsa-miR-151a	CUAGACUGAAGCUCCUUGAGG
Hsa-miR-181a	AACAUUCAACGCUGUCGGUGAGU

Table S2 Protein recovery rates of different BSA-DTB conjugates

DTB/BSA molar ratio	$Abs._{278\ nm}$	Recovery (%)	c_f (mg/mL)
0X	0.592	88	0.88
5X	0.556	83	0.83
10X	0.550	82	0.82
15X	0.584	87	0.87
50X	0.553	82	0.82
75X	0.546	81	0.81

Table S3 Sensing performances of some reported miR assays/biosensors

Entry	Platform	Method	LOD	Cancer patient sample application	Ref.
1	optical-fiber FL biosensor	multitag-regulated reaction (MCR), enzyme-free	0.8 fM	YES	this work
2	homogeneous FL assay	T4 RNA ligase involved	10 fM	NO	Ref. 6
3	homogeneous FL assay	polymerase-assisted exponential amplification reaction (EXPAR)	0.01 fM	NO	Ref. 7
4	on-particle	exonuclease III-powered stochastic DNA walker	10 fM	NO	Ref. 8
5	homogeneous FL assay	photoinduced electron transfer, rolling circle amplification	6 aM	YES	Ref. 9
6	electrochemical biosensor	enzyme-based isothermal exponential amplification	98.9 fM	NO	Ref. 10
7	localized surface plasmon resonance (LSPR) sensor	gold nanoprisms modified LSPR surface	23-35 fM	YES	Ref. 11
8	nanophotonic biosensor	direct detection, amplification-free	23 aM	YES	Ref. 12
9	nanopore-based	alpha-hemolysin protein based, amplification-free	0.1 pM	YES	Ref. 13
10	electrochemical biosensor	magnetobiosensors based on viral protein p19	0.04 nM	YES	Ref. 14
11	DNA logic gate platform	near-infrared Ag_2S quantum dots-based DNA logic gate	12.0 fM	NO	Ref. 15
12	electrochemical biosensor	gold-loaded nanoporous superparamagnetic iron oxide nanocubes (Au-NPFe_2O_3NC)	100 aM	YES	Ref. 16
13	electrochemical biosensor	gold-loaded nanoporous superparamagnetic nanocubes (Au@NPFe_2O_3NC) for signal amplification	100 fM	YES	Ref. 17
14	electrochemical biosensor	poly(A) extension strategy, amplification-free	10 fM	YES	Ref. 18

Supplementary Figures

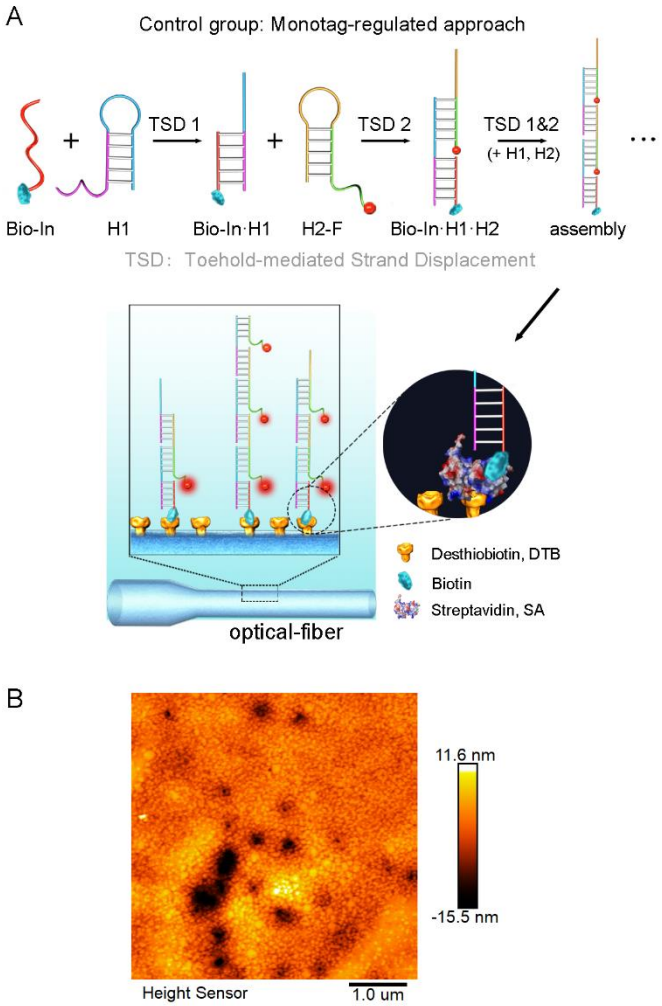

Figure S1 (A) Schematic illustration of the monotag-regulated approach (control group of MCR). (B) Control AFM image (mixture of H1 and H2 in the absence of In). No linear assemblies can be observed without adding In.

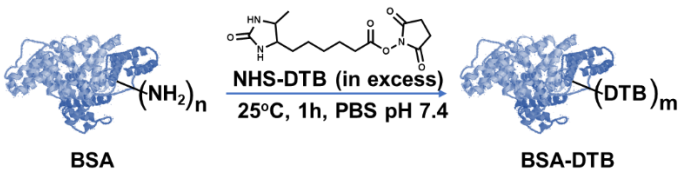

Figure S2 Schematic illustration of the synthesis reaction of BSA-DTB conjugate

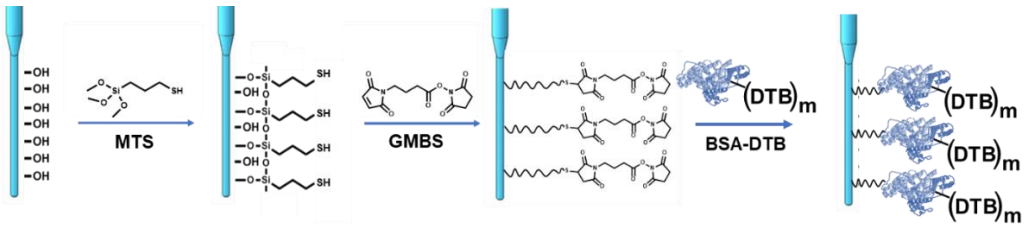

Figure S3 Schematic illustration of the preparation of DTB functionalized optical-fiber

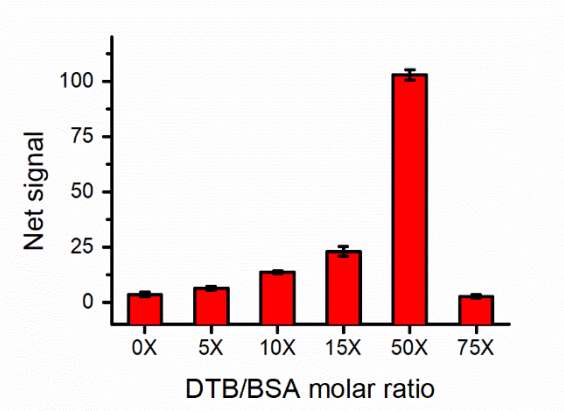

Figure S4 Net signals induced by 15 nM of SA-F (in 1 mL SPSC buffer) on optical fibers modified with different types of BSA-DTB conjugates (0X-75X)

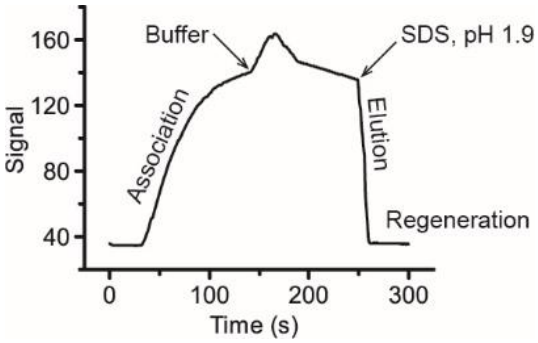

Figure S5 Sensorgram during the association, buffer washing and elution of 15 nM SA-F

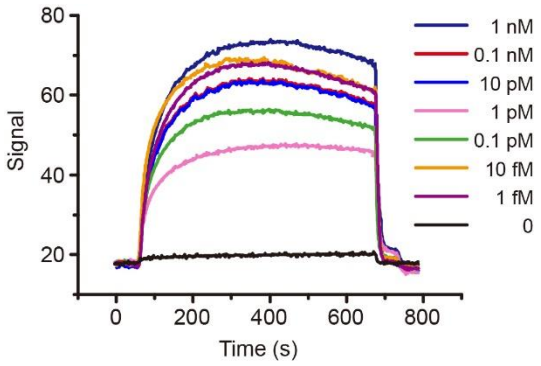

Figure S6 Origianl sensorgrams corresponding to **Figure 4C**

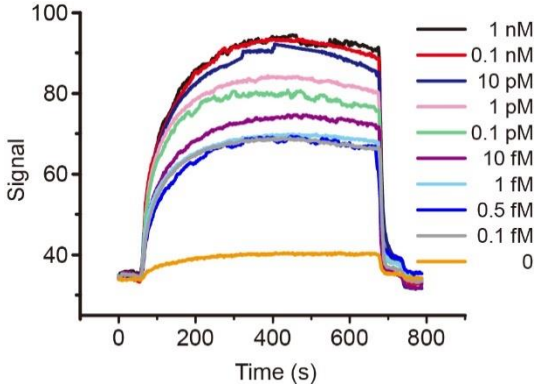

Figure S7 Origianl sensorgrams corresponding to **Figure 4D**

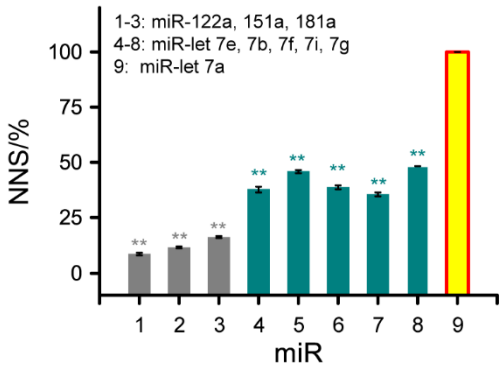

Figure S8 Selectivity of the developed MCR approach. c(miR) =1 nM for all miRs. NNS = normalized net signal (signals generated by 0 and 1 nM of *let-7a* were set as 0 and 100%, respectively). For group 1-3 (miR-122a, 151a and 181a), $p = 0.05$; For group 4-8 (miR-let 7e, 7b, 7f, 7i and 7g), $p = 0.01$.

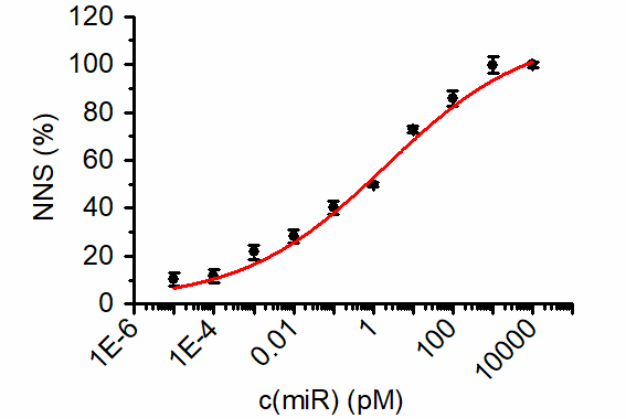

Figure S9 Normalized net signals (NNS) against different concentrations of miR in urine samples

The fitted parameters corresponding to **Figure S9** are: A_1 = 1.0 ± 2.4; A_2 = 113.8 ± 7.2; c_0 = 1.99 ± 1.28; p = 0.24 ± 0.04 (R^2 = 0.99058).

The calculated LOD and linear range are:

LOD = 0.1 fM; Linear range: 6 fM - 614 pM

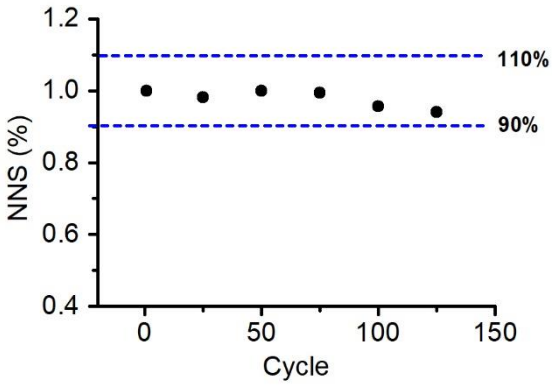

Figure S10 Normalized net signals (NNS) against 4 nM of SA-F in different regeneration cycles (1st, 25th, 50th, 75th, 100th and 125th) with a standard deviation 2.5%.

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