nature materials

SUPPLEMENTARY INFORMATION

DOI: 10.1038/NMAT4377

Phage-mediated counting by the naked eye of miRNA molecules at attomolar concentrations in a Petri dish

Supplementary Figure 1-24

Supplementary Table 1

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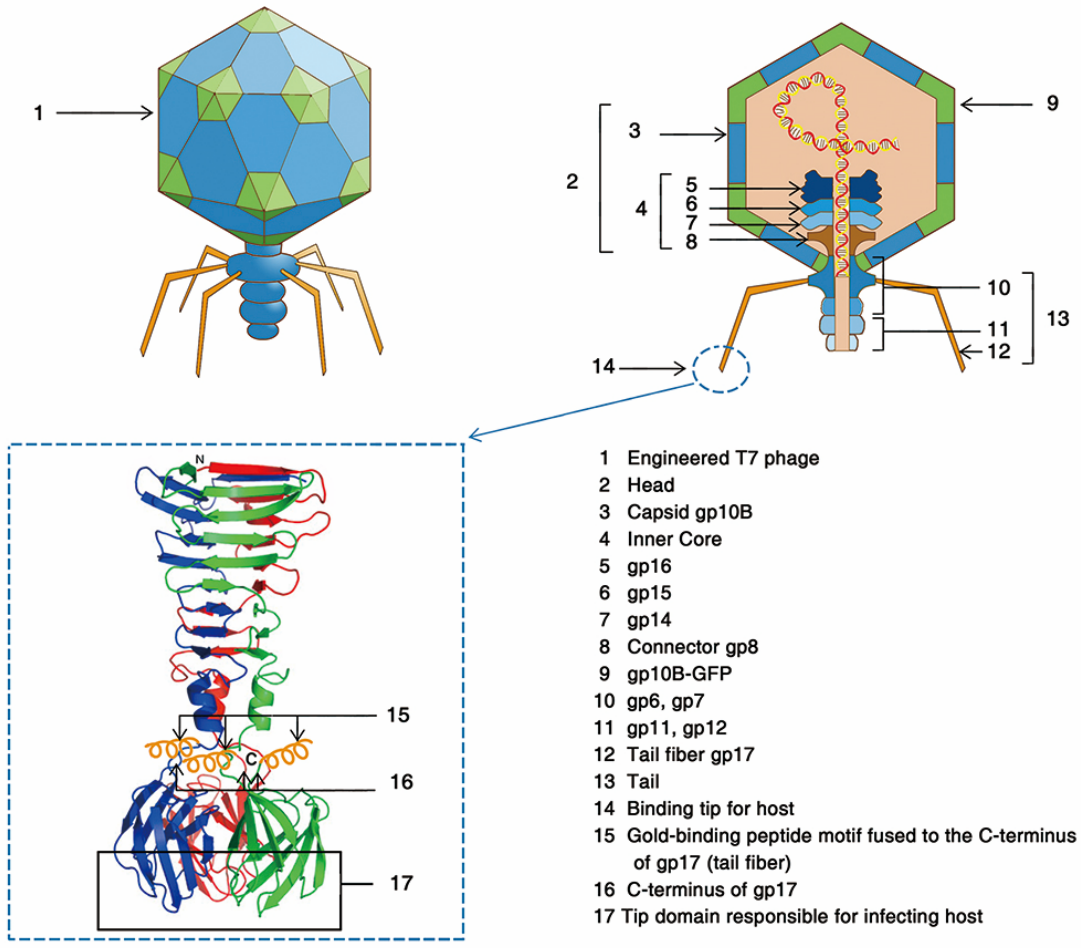
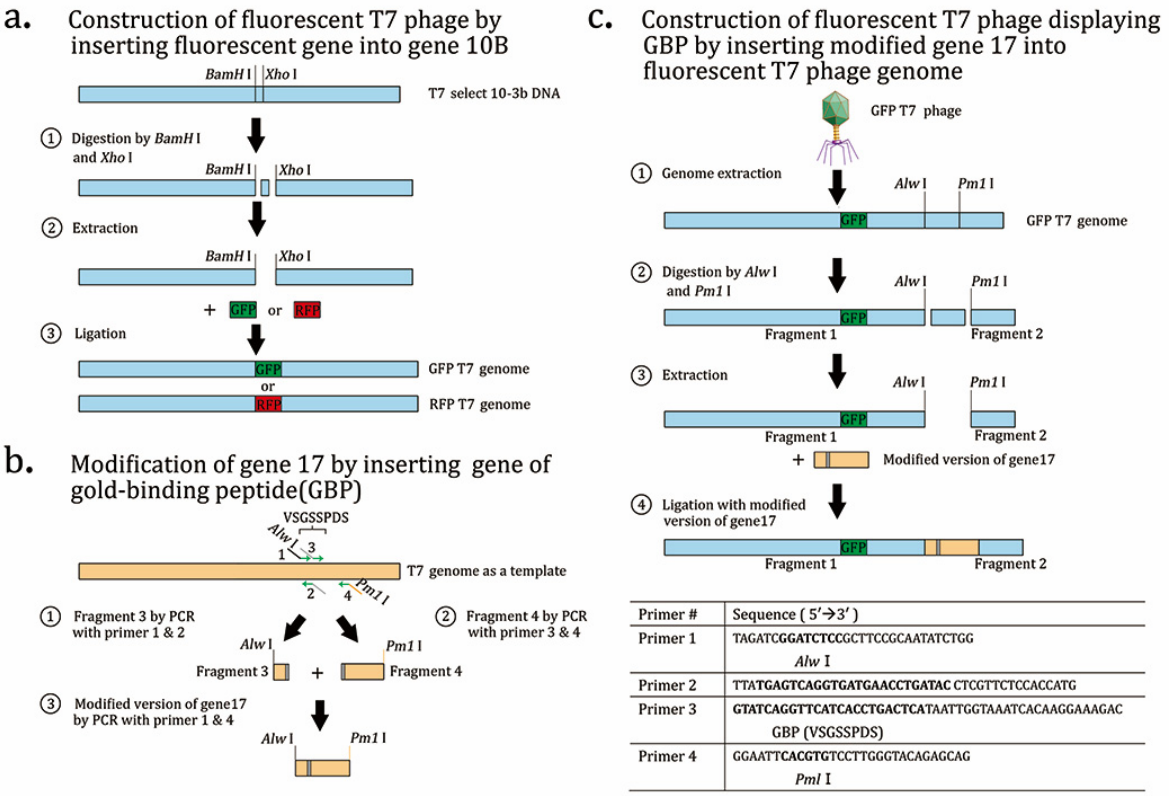
Supplementary Figure 1



Supplementary Figure 1. **The schematic diagram of the structure of T7 phage double displaying EGFP and gold-binding peptide.** Capsid gp10 of T7 phage was engineered to display EGFP to generate the fluorescent T7 phage and the gp17 gene was further modified to display gold-binding peptides for constucting the GNP probe where one GNP is attached to one T7 phage.

Supplementary Figure 2



Primer #	Sequence (5′→3′)
Primer 1	TAGATC**GGATCTCC**GCTTCCGCAATATCTGG *Alw* I
Primer 2	TTAT**GAGTCAGGTGATGAACCTGATAC** CTCGTTCTCCACCATG
Primer 3	**GTATCAGGTTCATCACCTGACTCA**TAATTGGTAAATCACAAGGAAAGAC GBP (VSGSSPDS)
Primer 4	GGAATT**CACGTG**TCCTTGGGTACAGAGCAG *Pml* I

Supplementary Figure 2. The schematic diagram of the construction of fluorescent T7 phage with or without GBP displayed. a, The procedure for construction of fluorescent T7 phage. b, The steps to obtain the modified version of gene 17 with GBP incoporated. c, Construction of fluorescent T7 phage displaying GBP by inserting modified gene 17 into fluorescent T7 phage genome.

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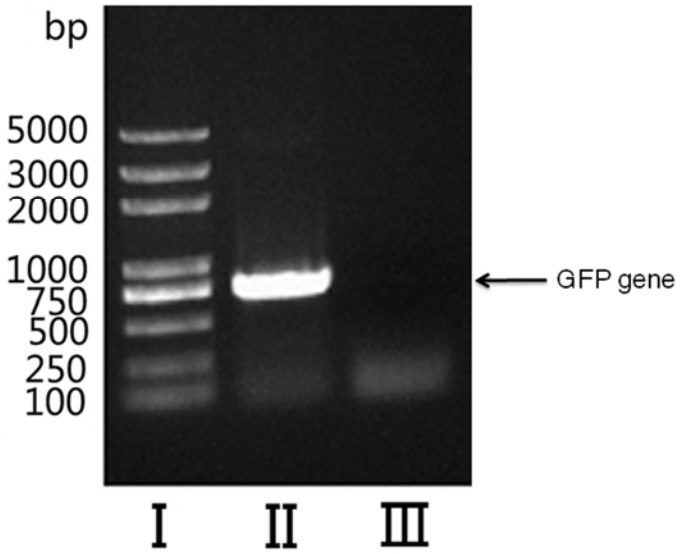
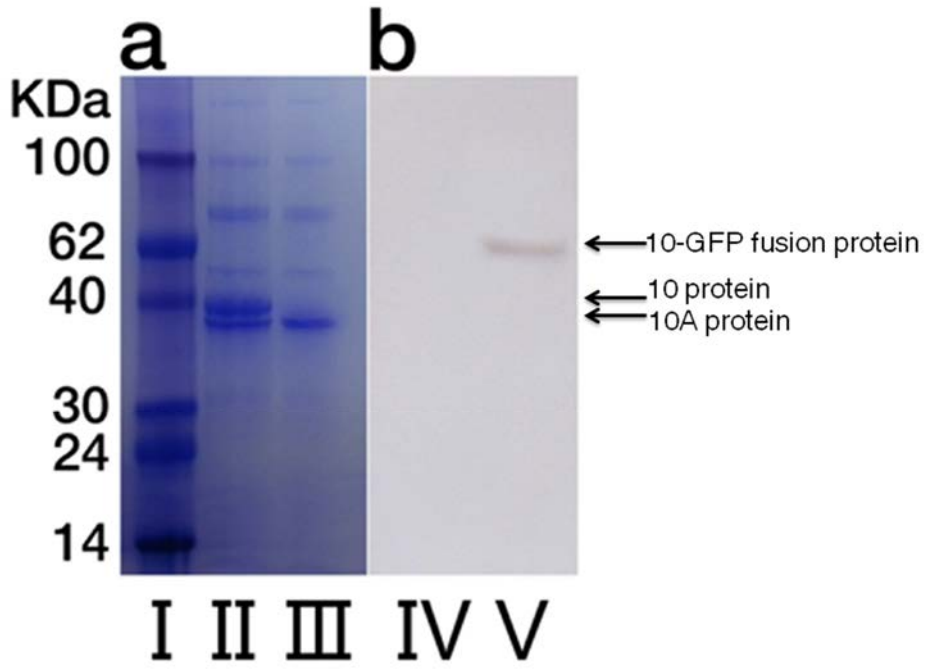
Supplementary Figure 3



Supplementary Figure 3. Verification of recombinant green fluorescent T7 phage by PCR. Agarose (1.0%) gel electrophoresis diagram of PCR reaction containing 1 μl of T7 phages, 20 pmol primers (forward: 5' ATATGGATCCATGGTGAGCAAGGGCGAGGA 3', reverse: 5' ATCGCTCGAGTTACTTGTACAGCTCGTCC 3', the underlined nucleotides are *Bam*H I and *Xho* I recognition sites, respectively) and other reaction components in 50 μl PCR reaction volume. Lane I: DNA marker. Lane II: the recombinant fluorescent T7 phage as the DNA template. Lane III: the non-modified T7 phage as the DNA template. This data shows the successful fusion of GFP to gene 10 (encoding capsid protein) of T7Select10-3b vector. It should be noted that the 10-GFP fusion protein is the only capsid produced from the modified T7 genome due to the deletion of the translational frameshift sequence in T7Select 10-3b vector and the 10-GFP fusion-protein is a minority of the recombinant T7 phage capsid protein. The majority is the 10A protein, which is produced from the plasmid in host bacteria BLT5403.

Supplementary Figure 4



Supplementary Figure 4. The successful construction of the green fluorescent T7 phage verified by gel electrophoresis and Western blotting analysis. 20 μl of purified T7 phage solution was subjected to SDS PAGE (4%~12% gradient gel, Genscript), and analyzed by Coomassie blue staining (**a**) and Western blotting with anti-GFP antibodies (**b**). Lane I: Protein marker. Lane II: non-modified T7 phage containing capsid 10A (348 aa) from helper plasmid 5403 in host BLT5403 and the gene 10 product (369 aa) from non-modified T7 Select 10-3b genome. Lane Ⅲ: the capsid of the recombinant GFP fluorescent T7 phages. Lane Ⅳ: Western blotting of Lane II by anti-GFP antibodies. Lane Ⅴ: Western blotting of Lane Ⅲ by anti-GFP antibodies. The bands on the gel of capsid protein 10A (~38 kDa), protein 10 (~40 kDa) and fusion protein 10-GFP (~65 kDa) are indicated by arrowheads. This data further confirms the successful fusion of GFP to capsid protein 10 of T7 phage.

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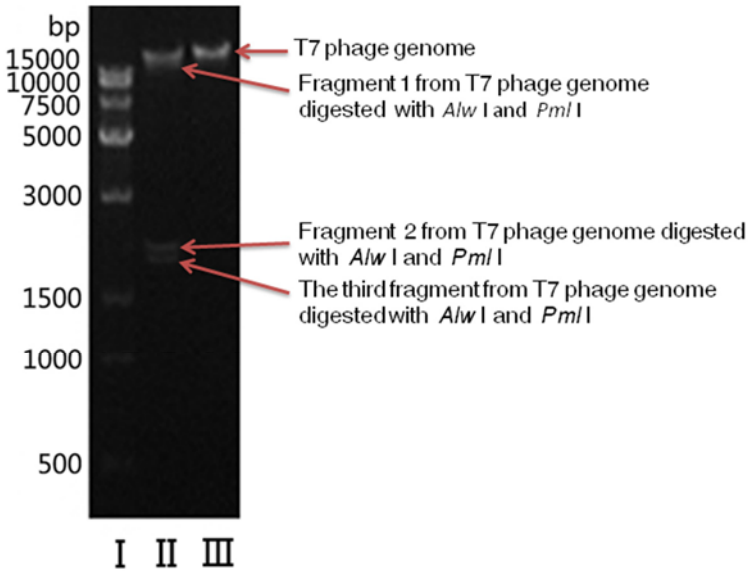
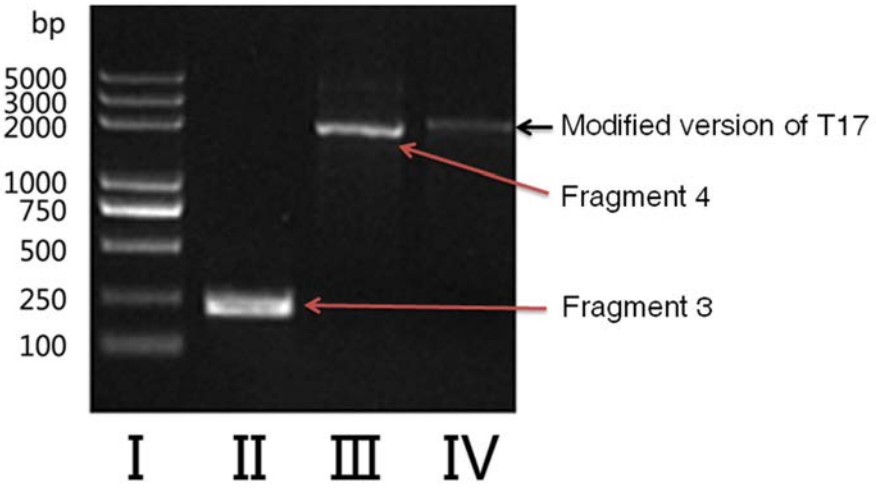
Supplementary Figure 5



Supplementary Figure 5. Agarose (1.0%) gel electrophoresis diagram of the product of T7 phage genome digested by *Alw* I and *Pml* I enzyme. Lane I: DNA marker; Lane II: the product of 200 ng T7 phage genome digested by *Alw* I and *Pml* I enzyme; Lane III: 200 ng T7 genome. The T7 genome was extracted from T7 phage with the T7 genome extract kit (Genmed, US) and quantified by UV absorbance at 260 nm (an optical density of 1.0 at 260 nm reflects a concentration of dsDNA solution of 50 μg/mL). This data shows that T7 phage genome was successfully cleaved into three fragments by *Alw* I and *Pml* I enzyme.

Supplementary Figure 6



Supplementary Figure 6. Construction of the recombinant tail fiber gene 17 fused with gold-binding peptide sequence. Lane I**,** DNA marker. Lane II, Fragment 3 (~200 bp) generated by PCR with primers (forward: 5' TAGATCGGATCTCCGCTTCCGCAATATCTGG 3'; reverse: 5' TTATGAGTCAGGTGATGAACCTGATACCTCGTTCTCCACCATG 3'; The underlined nucleotide is *Alw* I recognition site) and using T7 genome DNA as a template. Lane Ⅲ, Fragment 4 (~1800 bp) generated by PCR with primers (forward: 5' **GTATCAGGTTCATCACCTGACTCA**TAATTGGTAAATCACAAGGAAAGAC 3'; reverse: 5' GGAATTCACGTGTCCTTGGGTACAGAGCAG 3'; The bold nucleotide is the oligonucleotide sequence which is responsible for coding the gold-binding polypeptide "VSGSSPDS". The underlined nucleotide is *Pml* I recognition site. Lane Ⅳ, The modified version of gene17 (~2000 bp) was obtained by overlapping PCR technology with primers (forward:5' TAGATCGGATCTCCGCTTCCGCAATATCTGG 3'; reverse:5' GGAATTCACGTGTCCTTGGGTACAGAGCAG 3') and using fragment 3 (Lane II) and fragment 4 (Lane III) as DNA templates. This data shows the successful fusion of the gold-binding peptide to the tail fiber gene 17 product.

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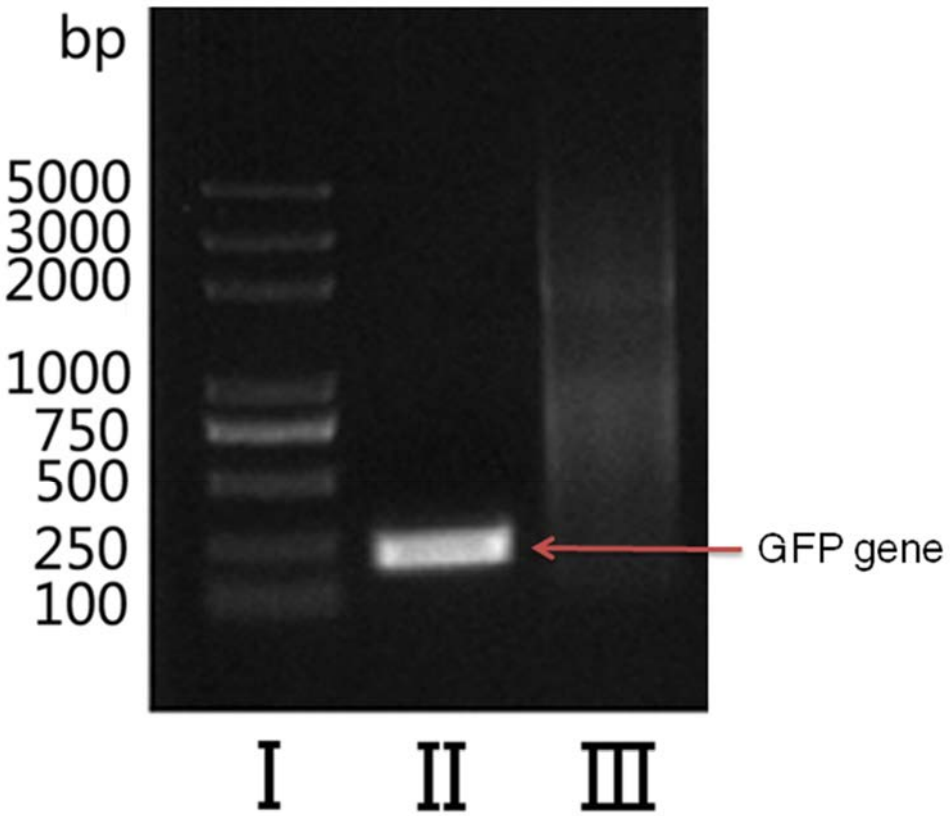
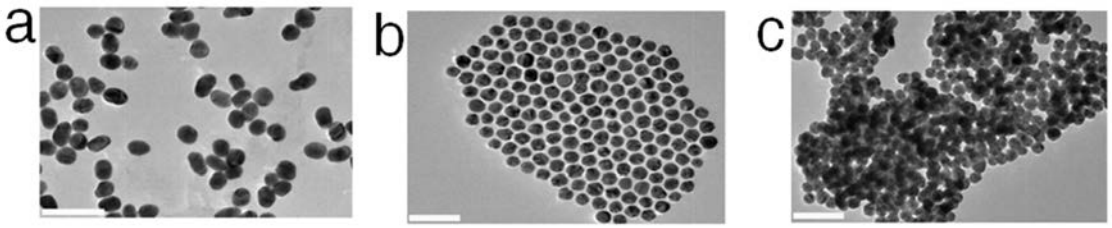
Supplementary Figure 7



Supplementary Figure 7. Agarose (1.0%) gel electrophoresis diagram of PCR product of one of positive T7 phage plaques. The sequence size (~200 bp in recombinant T7 phage genome) was amplified by PCR with primers (forward: 5′ TAGATCGGATCTCCGCTTCCGCAATATCTGG 3′; reverse: 5′ TTATGAGTCAGGTGATGAACCTGATAC 3′). Lane I, DNA marker. Lane II, the recombinant fluorescent T7 phages with gold-binding peptide as the DNA template for PCR. Lane III, the fluorescent T7 phages without gold-binding peptide as the PCR template. This data shows that the positive T7 phage clone is the recombinant fluorescent T7 phages with gold-binding peptide.

Supplementary Figure 8



Supplementary Figure 8. **a**, TEM image of GNPs. **b**, Representative TEM image of oligonucleotide **1** or **2** modified GNPs. **c**, TEM image of network-like structures formed due to the hybridization of **1**-modified GNPs (40 µl, 2.5 nM) with **2**-modified GNPs (40 µl, 2.5 nM) in the presence of let-7a (20 µl, 1 µM). This data shows that oligonucleotide functionalized GNPs have a good dispersibility and can hybridize with target miRNA molecules. Scale bar: 100 nm in **a, b**, and **c**.

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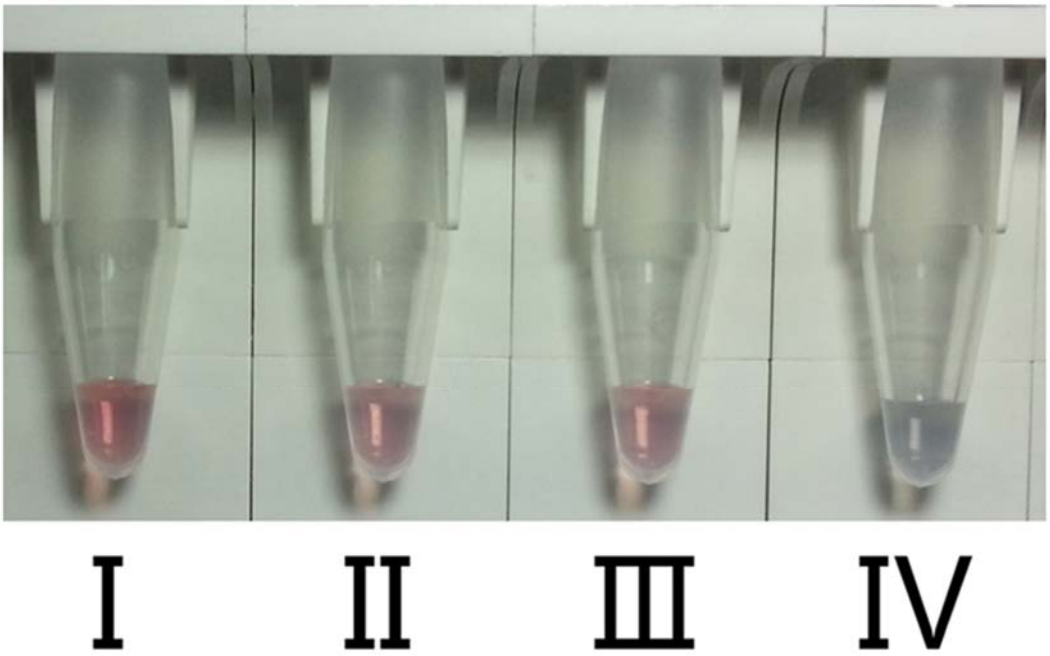
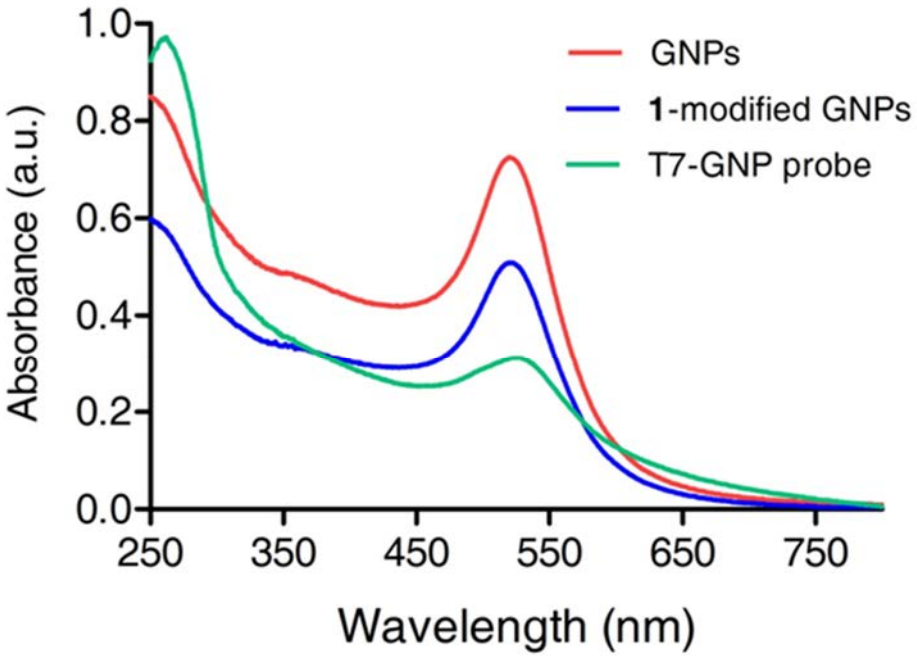
Supplementary Figure 9



Supplementary Figure 9. The fabrication of GNP pre-probe. I, Macroscopic image of 20 nm GNPs at 5 nM. II, Macroscopic image of **1** or **2**-modified GNPs at 2.5 nM. III, Macroscopic image of hybridization reaction system composed of 40 μl of **1**-modified GNPs at 2.5 nM, 40 μl of **2**-modified GNPs at 2.5 nM and 20 μl of H_2O. Ⅳ, Macroscopic image of hybridization reaction system composed of 40 μl of **1**-modified GNPs at 2.5 nM and 40 μl of **2**-modified GNPs at 2.5 nM in the presence of 20 μl of let-7a at 1 μM. This data shows that oligonucleotide functionlized GNPs have a good dispersibility and can hybridize with target miRNA molecules.

Supplementary Figure 10



Supplementary Figure 10. Ultraviolet-Visible absorption spectra of modified or non-modified GNPs. Red curve: 20 nm GNPs. Blue curve: **1**-modified GNPs. Green curve: GNP probe (i.e., made of **1**-modified GNPs and green fluorescent GNP-binding T7 phages in one-to-one ratio). By measuring the peak absorbance value and assuming an extinction coefficient of 8.78×10^{8} $M^{-1}cm^{-1}$ for 20 nm GNPs (*Reference: Liu X., Atwater M., Wang, J. H. & Huo Q. Extinction coefficient of gold nanoparticles with different sizes and different capping ligands. Colloids Surf. B: Biointerfaces 58, 3-7 (2007)*), the concentration of GNPs, oligonucleotide-modified GNPs and GNP probes were calculated to be ~0.83 nM, ~0.58 nM and ~0.35 nM, respectively. This data indicates that T7 phage was successfully connected with GNPs.

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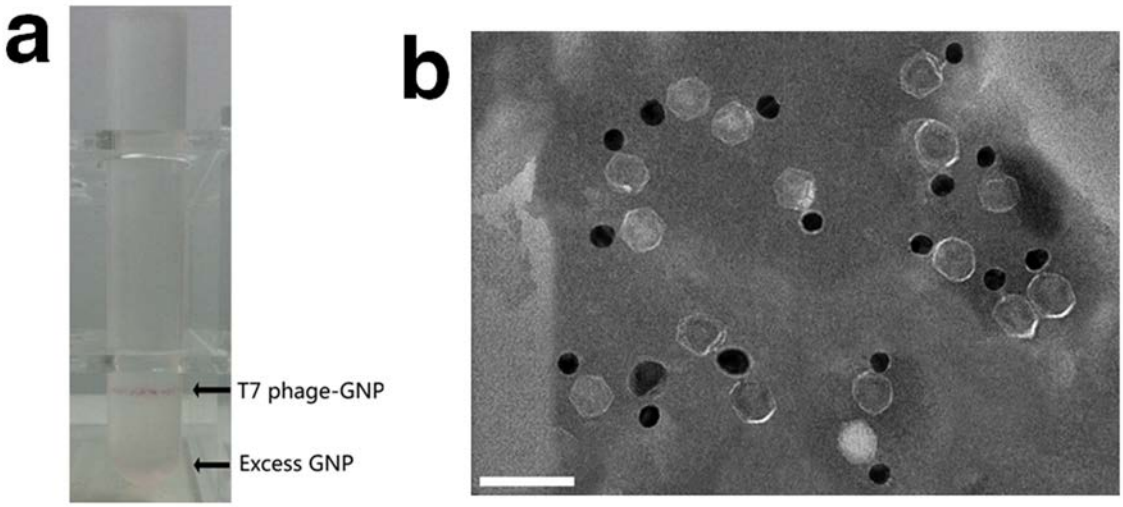
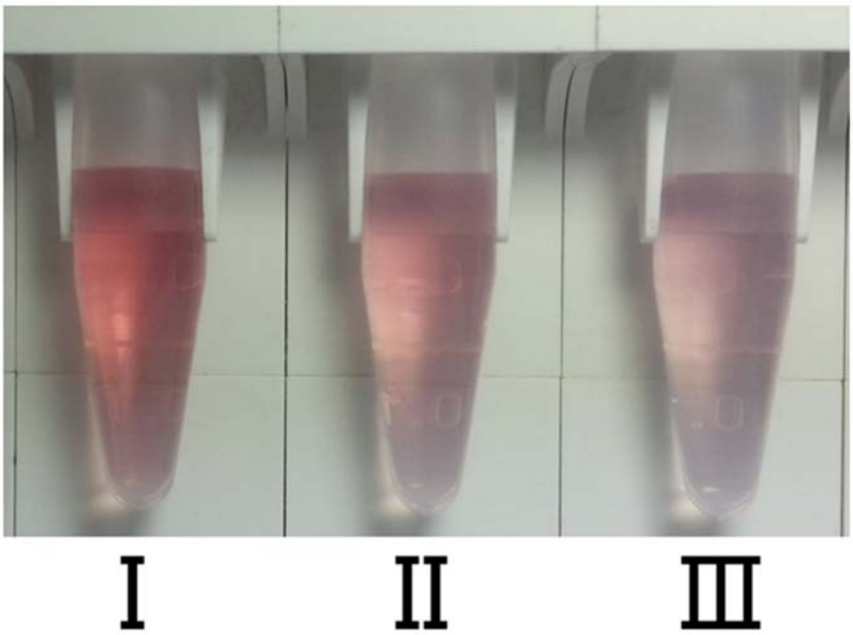
Supplementary Figure 11



Supplementary Figure 11. CsCl gradient centrifugation for obtaining T7-GNP probes. a. The CsCl solution after gradient ultracentrifugation. Two arrows point to the positions of T7-GNP probes and excess GNPs after ultracentrifugation, respectively. **b, TEM image of prepared T7-GNP probes with rare case where two T7 phages are attached to one GNP.** TEM image of prepared T7-GNP probes formed after excess GNPs and T7 phage displaying GBP motif were mixed and purified by CsCl gradient centrifugation. Scale bar, 100 nm.

Supplementary Figure 12



Supplementary Figure 12. The fabrication of GNP probes. I, Macroscopic image of 20 nm GNPs. II, Macroscopic image of GNP pre-probes (with **1** incorporated). III, Macroscopic image of GNP probes (with both **1** and the green fluorescent T7 phage displaying gold-binding peptide incorporated). The fabrication procedure of GNP probes is described as follows: recombinant T7 phages were mixed with excess oligonucleotide-functionalized GNPs (about 5 times that of the T7 phage) for a few hours at room temperature. Then the resultant mixture was purified by CsCl gradient centrifugation (134434 g, 30 min, Beckman SW41 rotor). CsCl gradient consists of four concentrations: CsCl solution/TE buffer (v/v)=1:0, 2:1,1:1 and 1:2, respectively. CsCl solution: 50g CsCl soluted in 30 mL H_2O. TE buffer: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA). After GNP probes were selected from the gradient centrifugtion tubes, they were subjected to dialysis against an assay buffer (0.2 M NaCl, 10 mM phosphate buffer, 0.1% Tween 20, pH 7.4). Finally the purified GNP probes were observed by TEM imaging. This data shows the successful fabrication of GNP probes.

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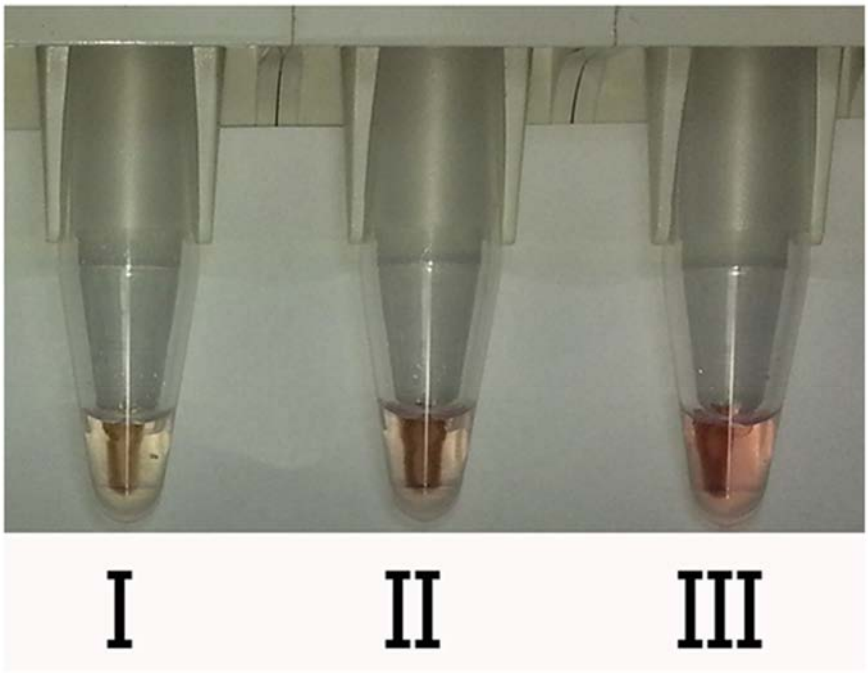
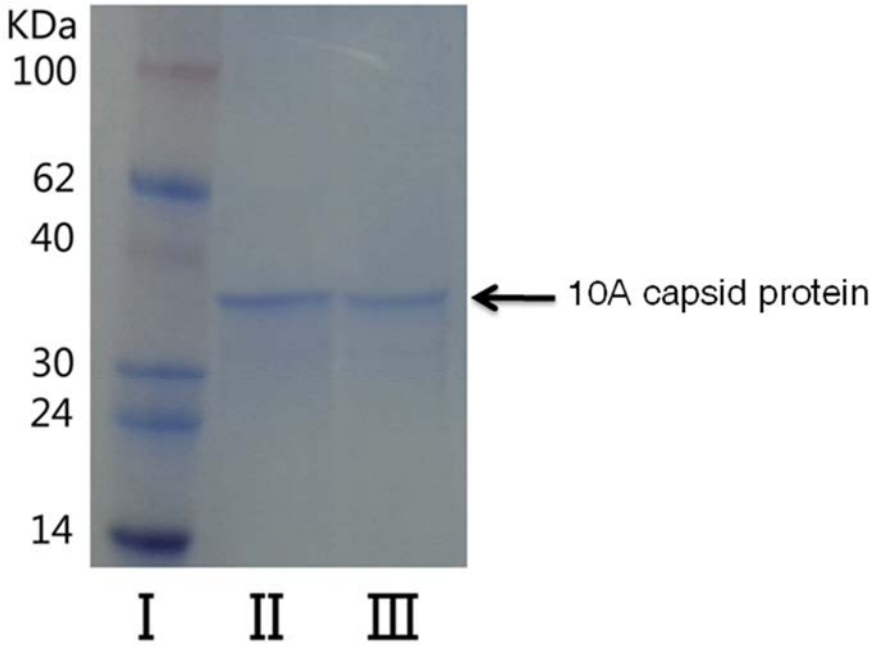
Supplementary Figure 13



Supplementary Figure 13 Macroscopic image verifying the formation of sandwich complex between GNP and MMP probes in the presence of target miRNA. I, The solution containing 40 μl of **2**-functionalized MMP probes at 2.5 mg/mL and 60 μl of assay buffer (0.2 M NaCl, 10 mM phosphate buffer, 0.1% Tween 20, pH 7.4). II, The solution containing 40 μl of **2**-functionalized MMP probes at 2.5 mg/mL, 40 μl of **1**-functionalized GNP probes (with recombinant EGFP-displayed gold-binding T7 phages) at 2.5 nM, 10 μl of let-7a at 1 μM and 10 μl of the assay buffer. III, The solution containing 40 μl of **2**-functionalized MMP probes at 2.5 mg/mL, 40 μl of **1**-functionalized GNP probes (with recombinant EGFP-displayed gold-binding T7 phages) at 2.5 nM and 20 μl of assay buffer. All of the above reaction tubes were gently shaken for 30 min at room temperature in an incubator before placed on the magnetic stand. This data shows that the sandwich was successfully formed between the GNP and MMP probes in the presence of target miRNA.

Supplementary Figure 14



Supplementary Figure 14. Gel electrophoresis for semi-quantifying the T7 phages from hybridization complex. Lane I, Protein marker. Lane II, 10 µl of the recombinant fluorescent T7 phage stock (~10^{12} pfu/ml by titering assay). Lane III, The T7 phages were released from the sandwich complexes in a hybridization reaction system composed of 40 µl of **2**-functionalized MMP probes at 2.5 mg/mL, 40 µl of **1**-functionalized GNP probes at 2.5 nM, 10 µl of let-7a at 1 µM and 10 µl of the assay buffer. The resulting sandwich complexes were drawn out again by a magnetic force and washed with the assay buffer six times. T7 phages were released from the sandwich complexes by adding 0.1 mL of 2 µM synthesized gold-binding peptide (VSGSSPDS) followed by incubation for 15 min. 10 µl of the resulting supernatant was subjected to SDS-PAGE (4%~12% gradient gel, Genscript) analysis, followed by Coomassie blue staining. This data shows that T7 phages were released effectively from the sandwich complex.

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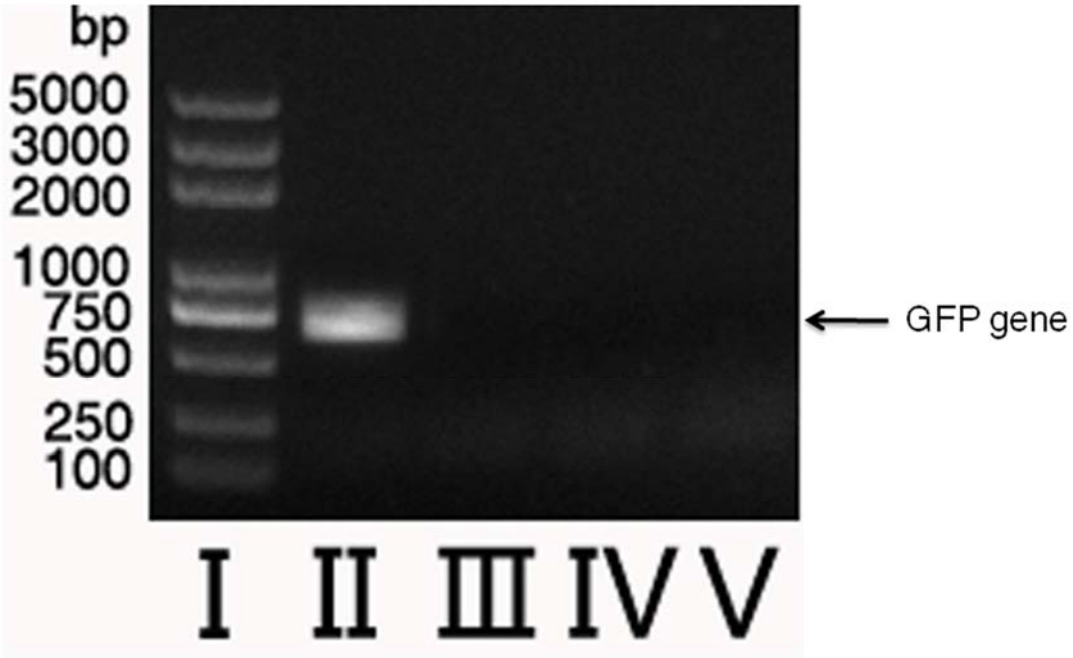
Supplementary Figure 15



Supplementary Figure 15. Gel analysis showing that there is no non-specific adsorption between GNP probes or T7 phages and MMP Probes. Lane I, DNA marker. Lane II, 5 μl of 2.5 nM GNP Probes (with recombinant EGFP-displayed T7 phages) as DNA template for PCR amplification. Lane III, 5 μl of MMP probes at 2.5 mg/mL as the DNA template for PCR amplification. Lane IV, The reaction solution containing 40 μl of **2**-functionalized MMP probes at 2.5 mg/mL, 40 μl of GNP probes (with recombinant EGFP-displayed T7 phages) at 2.5 nM, 10 μl of let-7a at 1 μM, and 10 μl of the assay buffer. After hybridization, the sandwich complexes were separated by a magnetic force and washed six times with the assay buffer, followed by adding 0.1 mL of 2 μM synthesized gold-binding polypeptides to release T7 phage into supernatant under shaking at room temperature for 15 min. Then the mixture was subjected to centrifugation at 12000 rpm for 10 min. Finally the supernatant was decanted and the pellets at the bottom of tube were resuspended with 20 μl of assay buffer. 5 μl of resuspension solution was used as DNA

template for PCR amplification. Lane Ⅴ, The operation and solution are the same as Lane Ⅳ except the absence of target molecule 1et-7a in the reaction. All PCR reaction tubes contained 5 μl of aforementioned template, 20 pmol EGFP gene primers (forward: 5' ATATGGATCCATGGTGAGCAAGGGCGAGGA 3', reverse: 5' ATCGCTCGAGTTACTTGTACAGCTCGTCC 3') and other reaction components in 50 μl PCR reaction system. This data shows that (1) in the presence of target mRNA, T7 phages in the sandwich complex coud be released by using GBP at a high concentration to elute them from the surface of GNPs the complex, and thus T7 phages with EGFP displayed could not be detected in the pellet (precipitated complex) (i.e., EGFP band could not be detected); (2) in the absence of target mRNA, GNP probe (i.e., T7-GNP complex) did not form a sanwidch complex with MMP probe and thus the pellet did not contain T7 phage displaying EGFP (i.e., EGFP band could not be detected).

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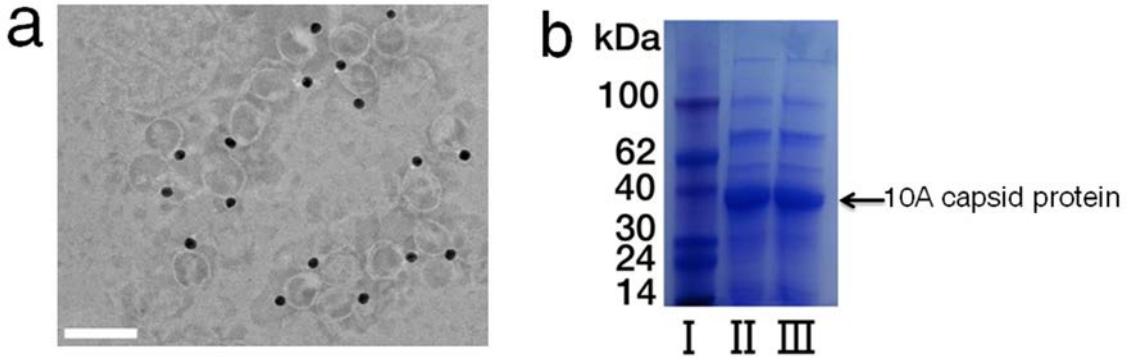
Supplementary Figure 16



Supplementary Figure 16. Assay of T7-GNP probes released from hybridization complex and then collected by using magnetic force to remove the MMP probes. A hybridization reaction mixture, composed of 40 μl of **2**-functionalized MMP probes at 2.5 mg/mL, 40 μl of **1**-functionalized GNP probes at 2.5 nM, 10 μl of let-7a at 20 nM and 10 μl of the assay buffer, was gently shaked for 30 min at 25 ℃. Then the magnetic force was used to pull the hybridization complex towards the tube wall from the reaction solution. The liquid portion was discarded and then 100 μl of 2 μM competition oligonucleotide (5′ ACTATACAACCTACTACCTCA 3′, which is perfectly complementrary to let-7a and can thus bind competitively to let-7a, enabling the T7-GNP probes to be released from the hybridization complex) was added. The resultant mixture was incubated for 15 min at 35 ℃ and then cooled to 20 ℃ for another 15 min. Then the reaction mixture was subjected to magnetic separation to remove the MMP probes. Finally, the liquid portion, which contained T7-GNP probes, was collected and subjected to either TEM or SDS-PAGE assay. The above liquid portion was concentrated by centrifugation at 13,000 g for 5 min. The resultant precipitate was added in 20 μl of loading buffer and boiled for 5 min, followed by SDS analysis. **a**, TEM of the T7-GNP probes released from the hybridization complex. Scale bar, 100 nm. **b**, SDS analysis of T7-phage probes released from hybridization complex. Lane I: 10 μl of DNA marker. Lane II: 10 μl of T7 phage used to

form T7-GNP probes of (~10^{13} pfu/ml by titering assay). Lane Ⅲ: T7-GNP probes released from the hybridization complex. The TEM image shows that T7 and GNP are still coupled in one-to-one manner after released from the hybridization complex. The SDS-PAGE assay confirms that the phage used to form the T7-GNP probes is still present in the probes after the probes are released.

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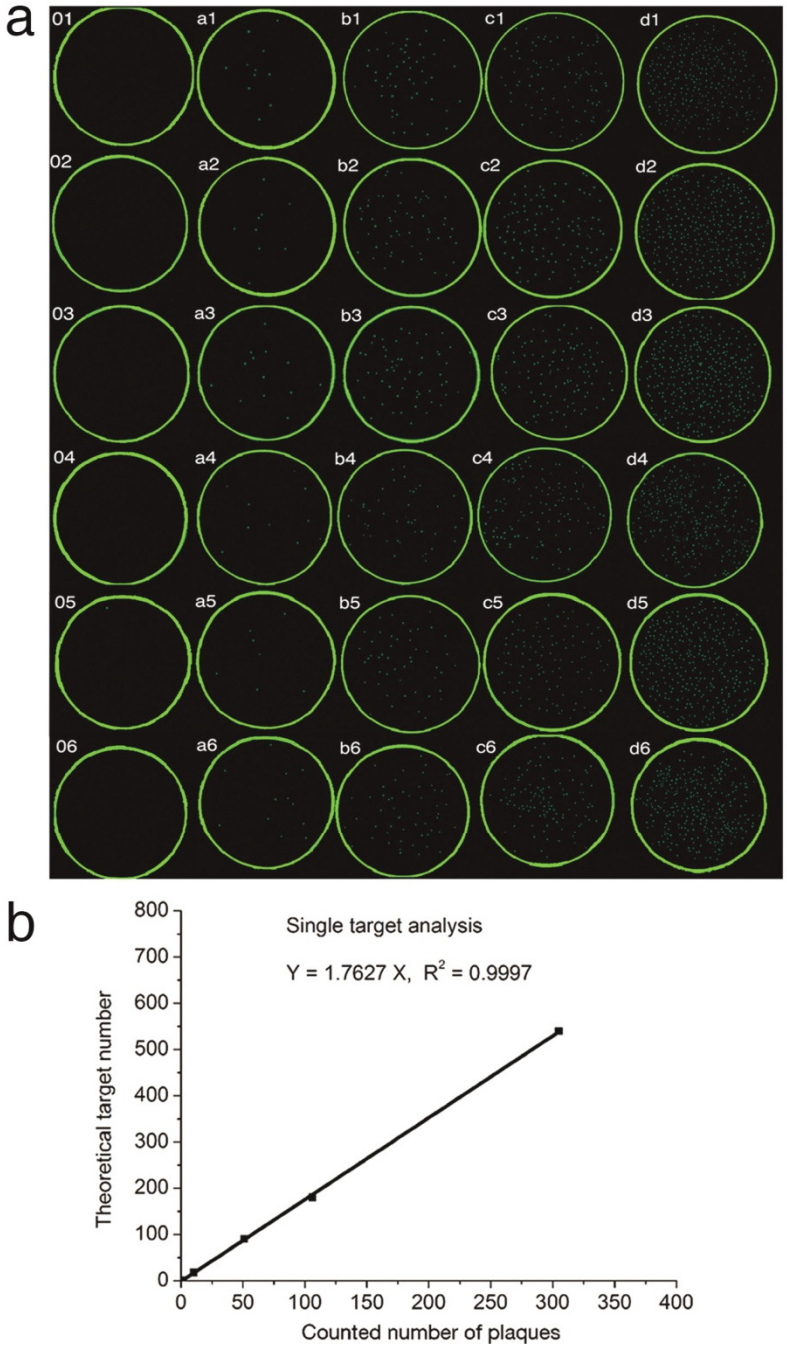
Supplementary Figure 17



Supplementary Figure 17. a, Counting single target microRNA at various concentrations. a, Plaque plates under the fluorescent scanner in the presence of single target let-7a at various concentrations. 01~6, In the absence of target molecules;

a1~6, In the presence of 3 aM of let-7a; b1~6, In the presence of 15 aM of let-7a; c1~6, In the presence of 30 aM of let-7a; d1~6, In the presence of 90 aM of let-7a; N=6 ($P<0.05$). Number 1~6 indicate the label for the six samples in parallel experiments for a particular concentration of the miRNA, respectively. **b**, The linear fitting of the counted number of plaques and the corresponding theoretical number of target miRNA for single target miRNA analysis. This data shows that the counting strategy has good reproducibility.

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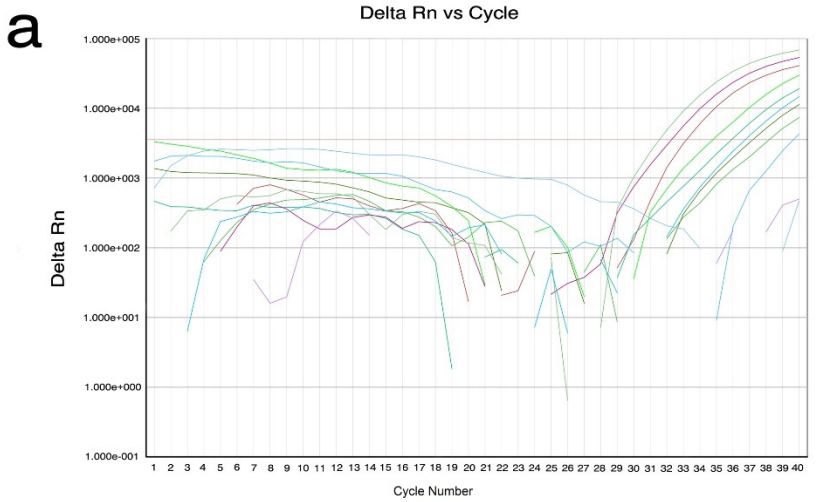
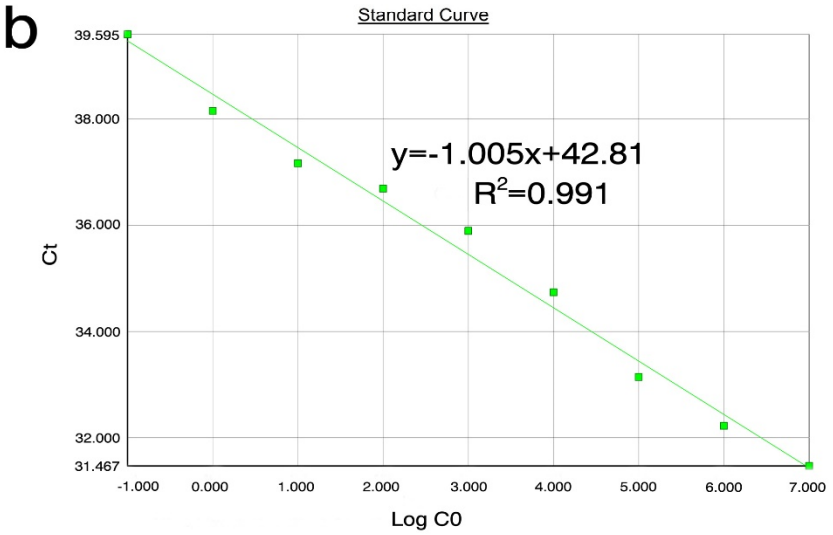
Supplementary Figure 18





c

Concentration of sample	Ct value by PCR	Calculated amount of target in 10 µL volume
3.0 aM	38.40	21
30.0 aM	35.70	166

Supplementary Figure 18. qPCR method for quantifying the let-7a samples used in counting strategy. a, The profile (let-7a) was presented by qPCR machine for testing the serial standard sample (copy number: 2560, 1280, 640, 320, 80, 40, 20 and 10). **b**, The standard equation (Y= -1.005X+42.81) was drawn by Microsoft Excel with the values of copies and Ct value. X is $\log_2$(copy number), Y is Ct value. **c**, The Ct values of two samples

(3 aM or 30 aM) used in counting strategy and determined by qPCR. The detection procedure by qPCR can be described as follows: First the let-7a molecule was reverse transcribed with PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara) and the Reverse primer (5′ GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAACTAT 3′). And then q-PCR (ABI-9700) was employed to amplify and detect the resultant product with SYBR® Premix Ex Taq™ II amplification primers (Takara) and primers (forward: 5′ TGAGGTAGTAGGTTGTATAG 3′, reverse: 5′ GTGCAGGGTCCGAGGT 3′). Finally the standard equation was drawn by the standard samples and corresponding Ct values. The samples (3 aM and 30 aM) used in our counting strategy were subjected to the above procedures to get the Ct values (38.4 and 35.7) of the two samples. The number of the miRNA in the two samples calculated according to the above standard equation of qPCR are 21 and 166, respectively. They are very similar to the theoretical values of the number of mRNA in 3 aM sample (~20) and 30 aM (~180). This data shows that the concentrations of miRNA molecules in the samples used in our counting strategy were accurate.

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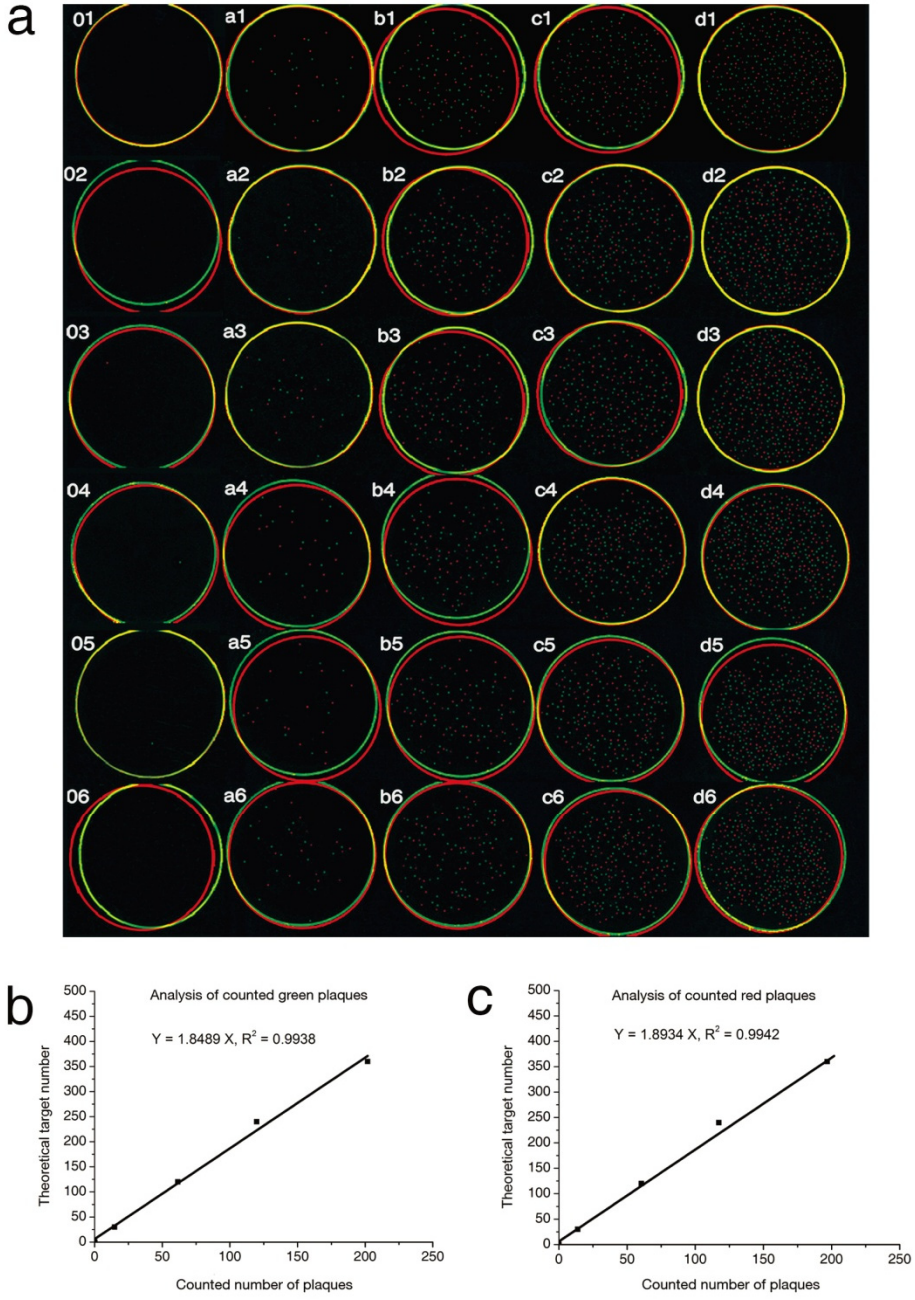
Supplementary Figure 19



Supplementary Figure 19. Plaques in the plates under the fluorescent scanner in the presence of both let-7a and miR-195 at various concentrations. a, 01~6, In the absence of target molecules; a1~6, In the presence of 5 aM each of let-7a and miR-195;

b1~6, In the presence of 20 aM each of let-7a and miR-195; **c**, In the presence of 40 aM each of let-7a and miR-195; d1~6, In the presence of 60 aM each of let-7a and miR-195. N=6 ($P<0.05$). Number 1~6 indicate the label for the six samples in parallel experiments for a particular concentration of the miRNA, respectively. **b** and **c**, The linear fitting of the counted number of plaques and the corresponding theoretical number of target miRNA for two-target analysis at various concentrations. This data shows that the counting strategy for multiple mRNAs also has good reproducibility.

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Supplementary Figure 20

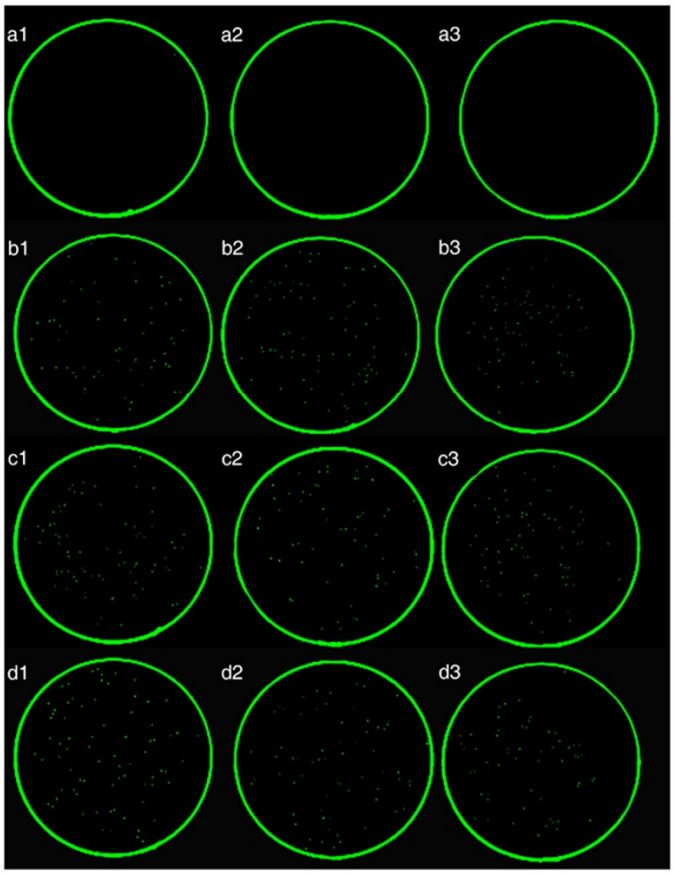
a

Has-let-7a	UGAGGUAGUAGGUUGUAUAGUU
Mut-1	UGAGAUAGUAGGUUGUAUAGUU
Mut-2	UGAGGUAGUAGGUUUUAUAGUU

b

Sample	Components of sample solution (in 10 μL volume)	Counted number of let-7a in the presence of mutants (mean±s.d.)
a	Without let-7a or Mut-1 or Mut-2	0.3±0.5
b	20 aM let-7a	66±8.5
c	20 aM let-7a and 200 aM Mut-1	69±9.0
d	20 aM let-7a and 200 aM Mut-2	67±8.8

c



Supplementary Figure 20. Distinguishing single nucleotide polymorphisms of miRNAs by counting strategy. a, The sequences of target miRNA let-7a and its two mutants (Mut-1 and Mut-2). The underlined base in mutants is the single mismatch base.

b, The result for counting let-7a at 20 aM in the presence (c and d) or absence (b) of a mutant at 200 aM in 10 μl sample solution along with a blank control (a). All of these reaction mixtures were incubated at 29 ℃. **c**, The corresponding plaque plates under the fluorescence scanner (at 488 nm excitation wavelength). Number 1~3 indicate the label for the three samples in parallel experiments for a particular concentration of the miRNA. N=3 ($P<0.05$). Data in **b** are derived from those shown in **c**. These data confirm that our counting strategy can distinguish target miRNAs and their mutants with single base mismatch.

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Supplementary Figure 21

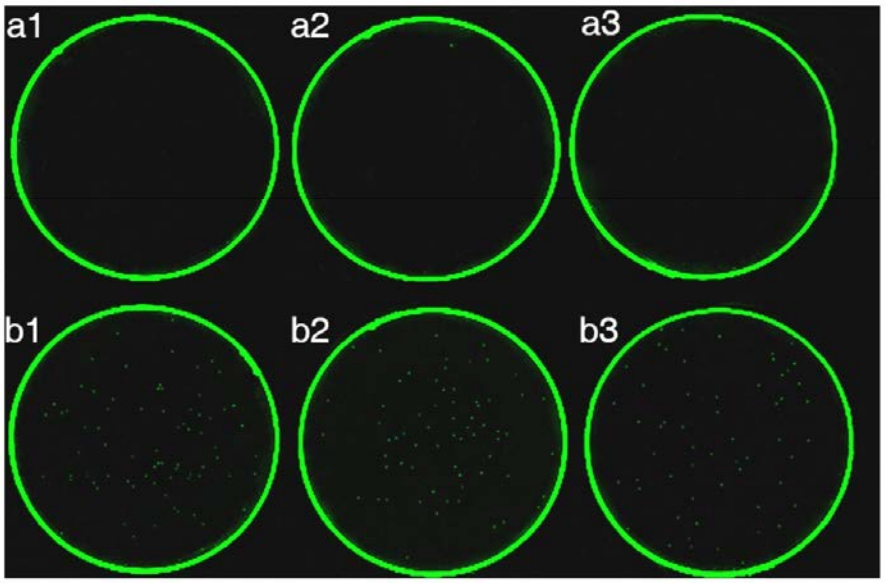
a

miR-21	UAGCUUAUCAGACUGAUGUUGA
miR-95	UUCAACGGGUAUUUAUUGAGCA

b

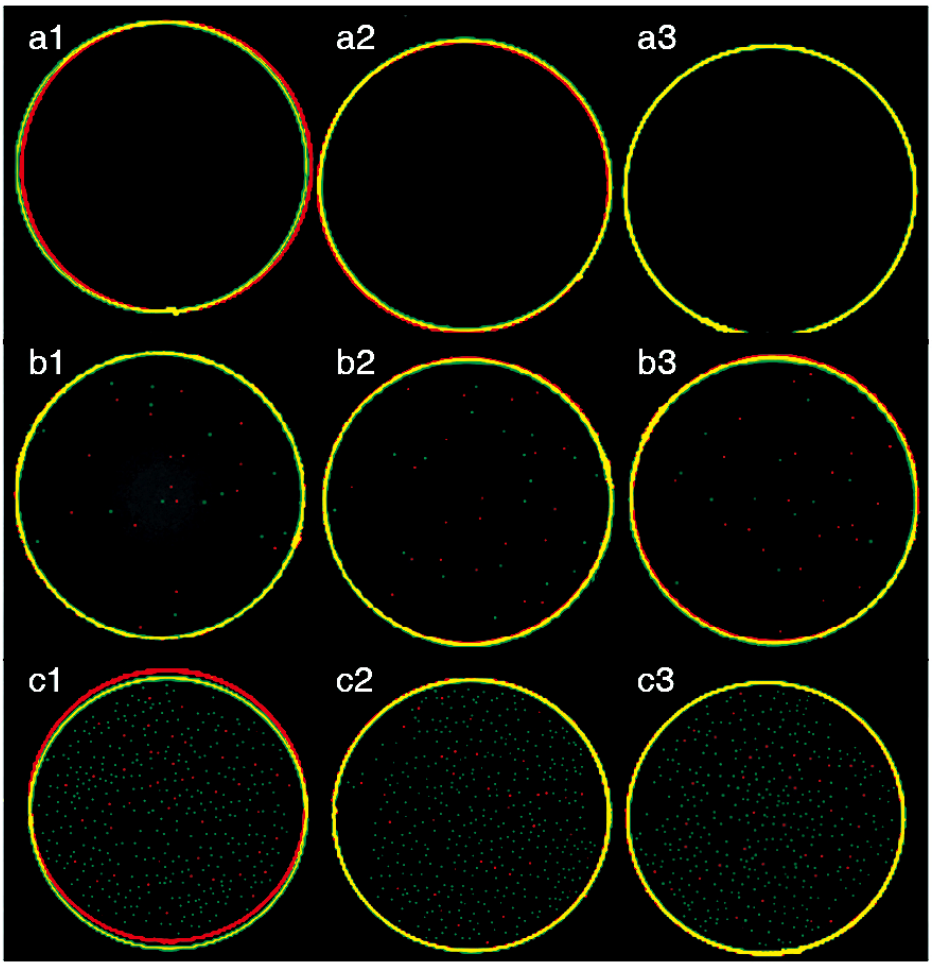
Sample composition	Theoretical value of miR-21 (in 10 μL volume)	Counted number of miR-21 by counting strategy (mean±s.d.)
miR-21（20 aM） miR-95（200 aM）	120	66±8.5
miR-21（0 aM） miR-95（200 aM）	0	0.3±0.5

c



Supplementary Figure 21 Distinguishing miRNA and its scrambled sequence by counting strategy. a, the sequence of target miRNA (miR-21-5p) and one of its scrambled sequences (which happens to be the sequence of another miRNA, miR-95-3p). **b**, Results from counting miR-21-5p (20 aM) in the presence of 200 aM miR-95-3p. **c**, The corresponding plaque plates under the fluorescence scanner. Number 1~3 indicate the label for the three samples in parallel experiments for a particular concentration of the miRNA, respectively. N=3 ($P<0.05$). Data in **b** are derived from those shown in **c**.

Supplementary Figure 22



Supplementary Figure 22. Counting two target miRNAs (has-miR-210-5p and has-miR-1246) to determine the level of their altered expression in HepG2 cancer cells by TNF-α treatment. Plaque plates under the fluorescent scanner for the control and samples extracted from HepG2 Cell before and after TNF-α treatment (300 ng per culture plate). **a,** Blank control with PBS substituted for cancer cells. **b,** Before TNF-α treatment. **c,** After TNF-α treatment. Green and red fluorescence plaques represent has-miR-1246 and has-miR-210, respectively. Samples were obtained by using a commercial miRNA extraction kit (Mivana mirna isolation kit, Ambion) to extract miRNAs from cultured HepG2 cell (~10^7 cell on a 10 cm plate) and then diluted 10^8 times for carrying out the counting method.

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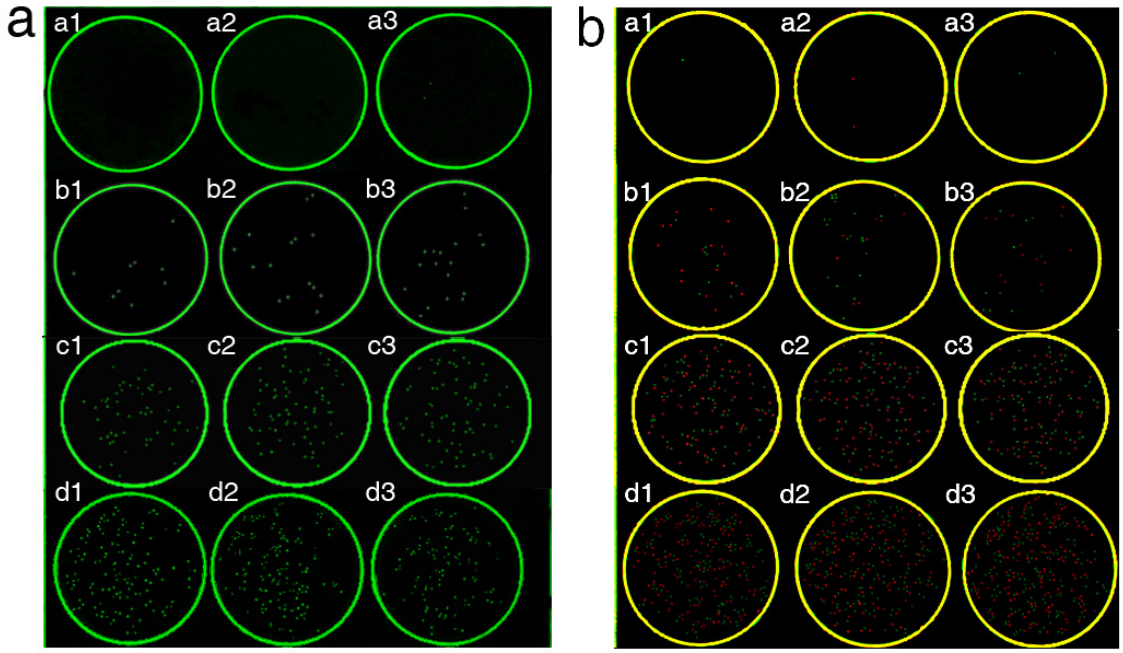
N=3 ($P<0.05$). Number 1~3 indicate the label for the three samples in parallel experiments for a particular concentration of the miRNA. These data confirm that our counting method can reliably detect the level of miRNAs in cancer cells and thus evaluate the altered miRNA express arising from the drug treatment of cancer cells.

Supplementary Figure 23



Supplementary Figure 23. Counting single or multiple target miRNAs in the serum sample by counting strategy. The serum from a healthy person was made devoid of the traget miRNAs (let-7a or both let-7a and miR-195) by using the magnetic microparticle probe functionalized with the sequence complementary to the target miRNAs to capture and magnetically remove the target miRNAs. Then we added the target miRNAs of known content to form serum with specific concentrations of the target miRNAs for our counting method. **a**, Plaque plates under the fluorescent scanner for counting miRNAs in the sera with different theoretical concentrations of let-7a. a, Blank control**.** b, In the presence of 4

aM let-7a. c, In the presence of 20 aM let-7a. d, In the presence of 40 aM let-7a. **b**, Plaque plates under the fluorescent scanner for counting miRNAs in the sera with different theoretical concentrations of let-7a and miR-195. a, Blank control**.** b, In the presence of 4 aM each of let-7a and miR-195. c, In the presence of 20 aM each of let-7a and miR-195. d, In the presence of 40 aM each of let-7a and miR-195. N=3 ($P<0.05$). Number 1~3 indicate the label for the 3 samples in parallel experiments for a particular concentration of the miRNA.

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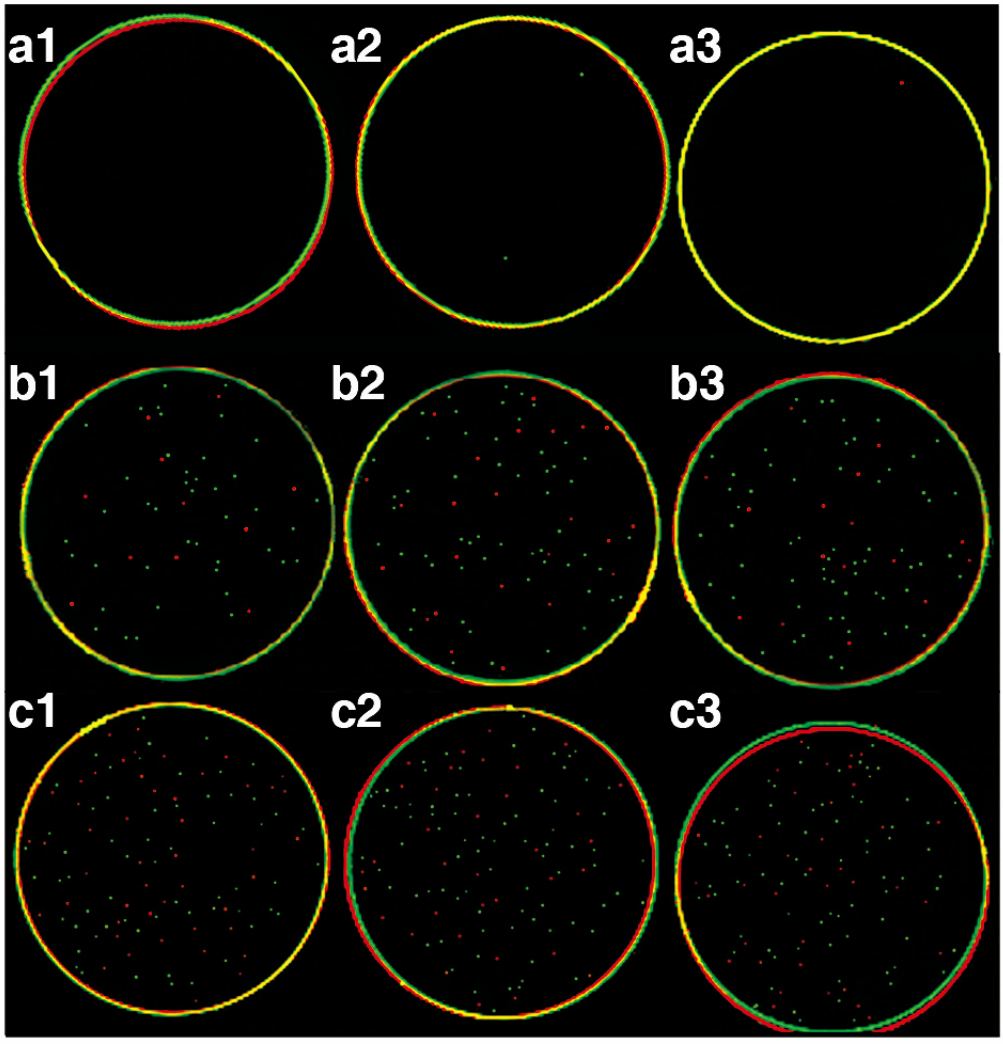
Supplementary Figure 24



Supplementary Figure 24. Quantifying miRNAs (hsa-let-7b-5p and has-miR-21-5p) in tumor tissue and its nearby healthy tissue (i.e., para-carcinoma tissue) from a non-small cell lung cancer patient by counting strategy. Plaque plates under the fluorescent scanner for the control and samples extracted from tissues using a commercial miRNA extraction kit (Mivana mirna isolation kit, Ambion). a, Blank control with PBS substituted for the sample from tissue. b, Tumor tissue. c, Para-carcinoma tissue (Green and red fluorescence plaques represent hsa-let-7b-5p and has-miR-21-5p, respectively). Samples were obtained by using Mivana mirna isolation kit (Ambion) from tissues (~100 mg weight

per block tissue) and were diluted 10^9 times (carcinoma tissue) or 10^7 times (para-carcinoma tissue) for counting strategy. N=3 ($P<0.05$). Number 1~3 indicate the label for the three samples in parallel experiments for a particular concentration of the miRNA.These data confirm that our counting method can reliably quantify the level of miRNAs in human tissues.

Supplementary Table 1

The sequences used in spiked serum assay by counting strategy

hsa-let-7a-5p	5'UGAGGUAGUAGGUUGUAUAGUU 3'
hsa-miR-195-5p	5'UAGCAGCACAGAAAUAUUGGC 3'
The sequence immobilized on the magnetic particle probe for clearing let-7a in serum	5'AACTATACAACCTACTACC TCAAAAAAAAAAA 3'
The sequence immobilized on the magnetic particle probe for clearing mir-195 in serum	5' GCCAATATTTCTGTGCTGC TAAAAAAAAAAA 3'