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Supplementary information

One-step scalable fluorescent microgel bioassay for endogenous viral miR-US4-5p ultrasensitive detection.

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S1 Folding simulation

Folding simulation were predicted by UNAfold software, setting oligonucleotide concentration 50nM and NaCl 200mM at 25°C. In figure 2A and B are showed the potential folding simulations of the quencher strand, while in 2C is reported the tail strand folding. As clearly perceivable, such structures are very instable compared to the stable one formed in presence of their partially or totally complementary sequence (QF ΔG=-23.75 kcal.mole^{-1}; QT ΔG =-45.29 kcal.mole^{-1})

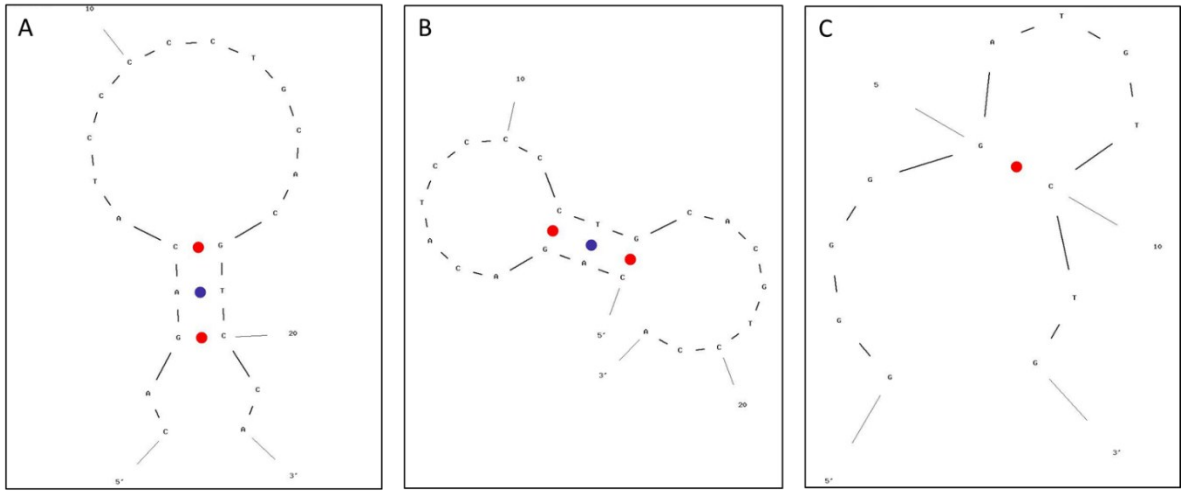


Fig. 1 A-B) Quencher strand (Q) and C) Tail strand (T) folding simulations and their relative free energy

S2 Target alignment

Target sequences blast provide information about the sequences similarity. In particular, in table 1 are reported their alignment score (as raw score and E-value) and oligonucleotide sequence.

Table 1 Blast analysis of the target miRNA

MATURE MIRNA	ALIGNMENT	SEQUENCE
hcmv-miR-US4-5p	Score:110 Evalue: 9e-04	hcmv-miR-US4-5p 1 uggacgugcagggggaugucug 22 |||||||||||||||||||| hcmv-miR-US4-5p 1 uggacgugcagggggaugucug 22
mmu-miR-5620-3p	Score:66 Evalue: 4	hcmv-miR-US4-5p 8 gcagggggaugucug 22 ||||||||||-||| mmu-miR-5620-3p 16 gcagggggaugacug 2
cre-miR1148.1	Score:65 Evalue: 4,8	hcmv-miR-US4-5p 4 acgugcaggggga 16 ||||||||||||| cre-miR1148.1 4 acgugcaggggga 16

Table 2 Needleman-Wunsch alignment between the target and non-specific oligonucleotide sequences used in our study

MIRNA		SEQUENCE
miR-143-3p	IDENTITY 10/21 (45%)	UGGACGUGCAGGGGGAUGUCUG 22 || | || || | || UG-AGAUGAAGCACTGUAGCUC 21

S3 Quenching efficiency and assay performance in homogeneous assay

Fluorescence spectra are measured exciting the sample at 647nm and collecting the emission intensity from 667 to 750nm. Efficiency of quenching in hybridization buffer has been calculated by dividing the fluorescence intensity of ds-probe complex (QF) by the fluorescence intensity of the fluorescent tail (F), multiplying the result by 100 and then subtracting the results from 100. In homogeneous condition, quenching efficiency for the selected probe is 89.1% (figure 2A).
The Forster distance between the donor fluorophore ATTO647N and the acceptor BHQ2 has been predicted as follow:

$$R_o = 0.21 \cdot (\kappa^2 \cdot n^{-4} \cdot \Phi_D \cdot J(\lambda))^{\frac{1}{6}}$$

where κ^2 is a factor describing the relative orientation in space of the transition dipole moments of the donor and acceptor and it is usually assumed to be equal to 2/3, n is the refractive index, typically assumed to be 1.3 for biomolecules in aqueous solution, Φ_D is donor fluorescence quantum yield, and J (λ) is the spectral overlap between area normalized donor emission and acceptor molar absorptivity calculated using a Python Script (http://www.pymolwiki.org/index.php/forster_distance_calculator). Substituting the J value obtained (9.60172e^{15} $M^{-1}cm^{-1}nm^4$) in the equation reported above, R_0 of 69.91 Angstrom is predicted.
Melting temperature is calculated monitoring the fluorescence in function of the temperature. In particular, 500µL of hybridization buffer containing 50nM of QF probe is initially denatured (3 min

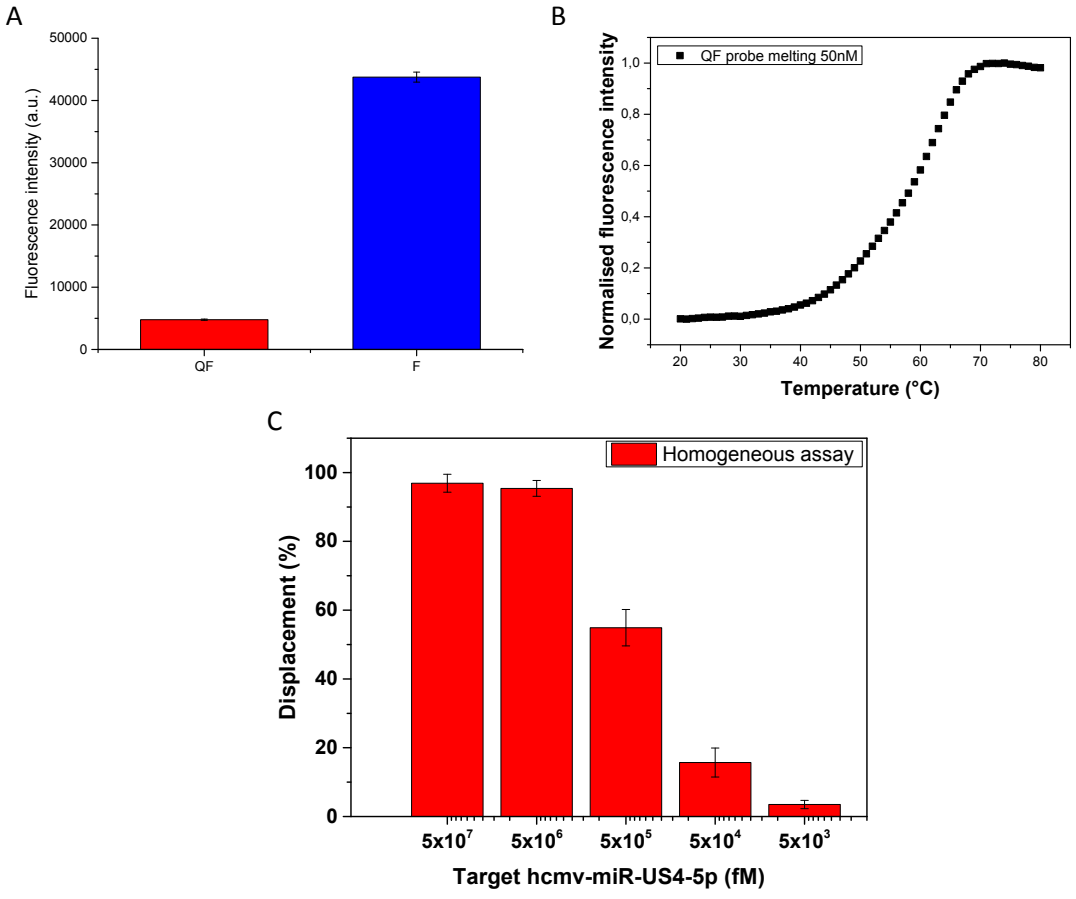


Fig. 2 A) Fluorescence emission intensity of the tail labelled strand(F, 5nM) and duplex tail-quencher strand (QF, 5nM 1:1 ratio); B) Melting curve of QF double strand probe (50nM); C)

95°C), then the temperature is decreased from 95°C to 20°C, with scan rate of 1°C/2min. Melting temperature of our probe is 59 °C (figure 2B).

Displacement efficiency was measured in homogeneous assay mixing 5nM of ds-probe with the viral target (from 10^{6} to 10^{-15}M) at room temperature. From the experiments results that the minimum target concentration distinguishable from the background in homogenous assay is in pM order (figure 2C).

S4 Microgel characterization

-Size and Surface charge

Measurements were conducted using Dynamic light scattering (Malvern Zetasizer Nano ZS instrument, 633 nm laser, 173° scattering angle) total of 3 runs (each comprised 3 cycles) were conducted.

Table 3 Hydrodynamic diameter (Dh) and ζ potential measurement of microgels.

	ZAVERAGE (D_H, nm)	PDI	ZETAPOTENTIAL (MV)
CORE	416.2±0.900	0.006	-3.67±0.287
FIRST SHELL	889.4±18.05	0.078	-6.52±0.0557
SECOND SHELL	1002±16.3	0.28	-15.1±0.379

Table 4 Synthesis parameters. All numbers are reported as mM concentrations of the final solution.

	PEGDMA	Rhodamine	Fluoresceine	AAc	KPS	PVA
CORE	18.2	0.1	--	--	2.2	48
FIRST SHELL	9.1	--	--	--	1.1	48
SECOND SHELL	9.1	--	0.1	36	1.1	48

-Carboxyl acid group titration

Sample was equipped by suspending 50mg of microgels in 50 mL of 10^{-3}M KCl solution. Titrations were run in a thoroughly cleaned 100 mL beaker equipped with a pH electrode while NaOH 0.1M was used as titrant. Carboxyl groups content of the microgel is in a range of 1.71 ± 0.1 μmol/mg particles.

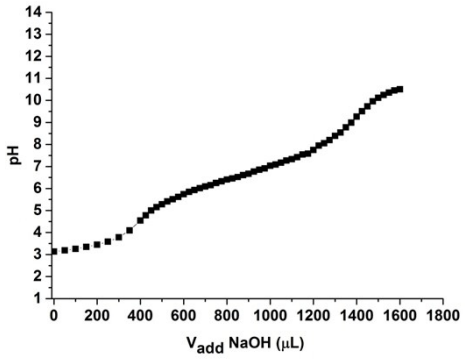


Fig. 3 Titration curve of COOH immobilised on the second shell of microgels

-Calibration curve

The concentration of tails bound to the microgel is calculated analyzing the fluorescence intensity of the supernatant after the coupling reaction. The fluorescence intensity is then quantified using a calibration curve. For this purpose, tails are firstly mixed with coupling solution (MES 0.1mM pH 4.8 and EDC 500mM), then are diluted in MES 0.1mM pH 4.8 as reported in figure 4.

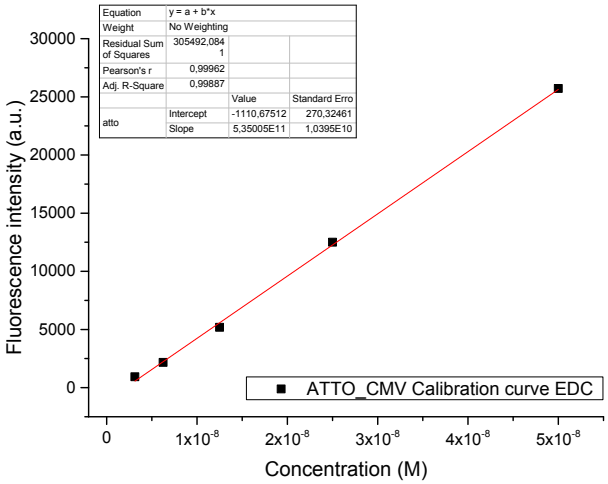
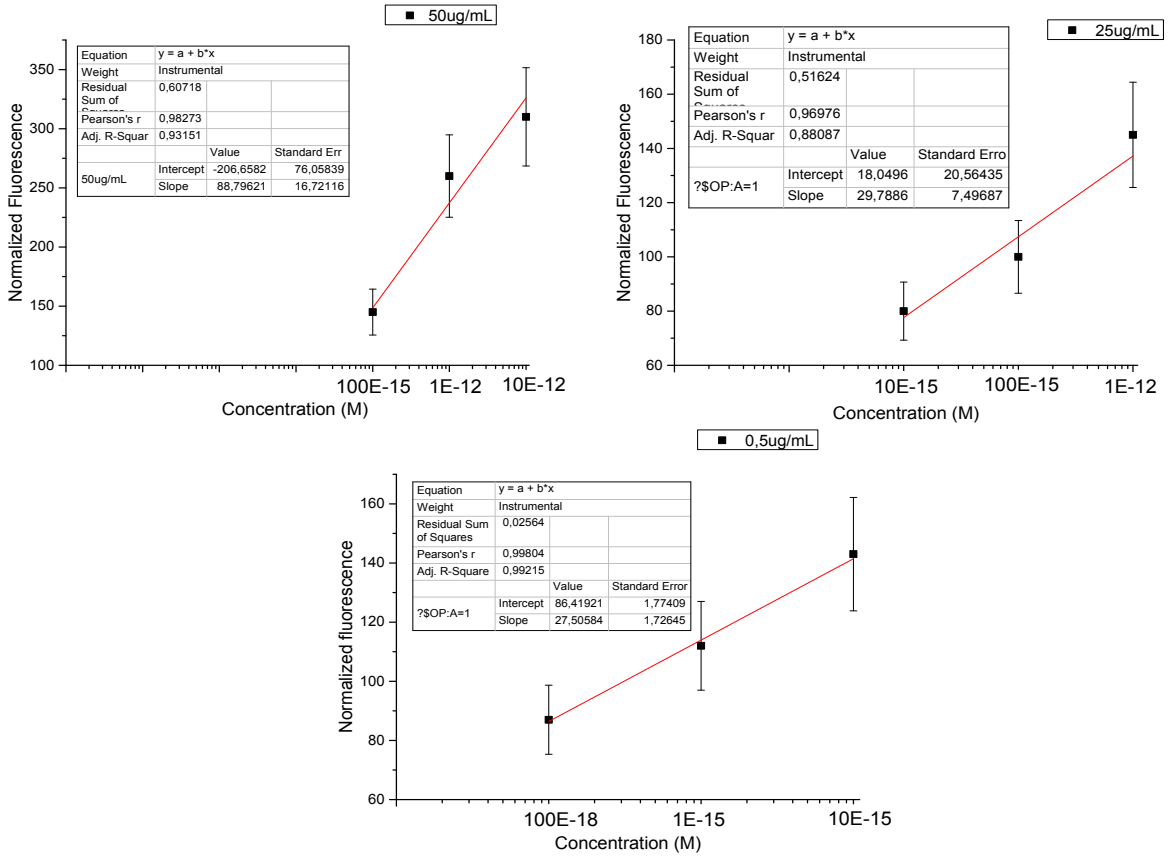


Fig 4 Calibration curve of the fluorescent tails

S5 Limit of detection analysis

All experiments were performed in triplicates and values are reported as mean ± standard deviation. Data are analyzed applying a non-linear regression and R2 is above 0.9 in all experiments. The LOD



values are determined by the three standard deviations above the background level

Fig.5 LOD calculation based on CLSM images analysis

.**Table 5** Non-linear regression data analysis for ds displacement assay and microgel-based assay performed in presence of hcmv-miR-US4-5p target.by confocal laser scanner microscopy.

Microgel concentration (µg/mL)	slope	Standard error (slope)	intercept	Standard Error (intercept)	LOD (fM)	R-Sq
50	88.796	16.721	-206.65	76.053	37.12	0.93
25	29.788	7.496	18.049	20.564	11.7	0.88
0.5	27.505	1.726	86.419	1.774	0.156	0.99

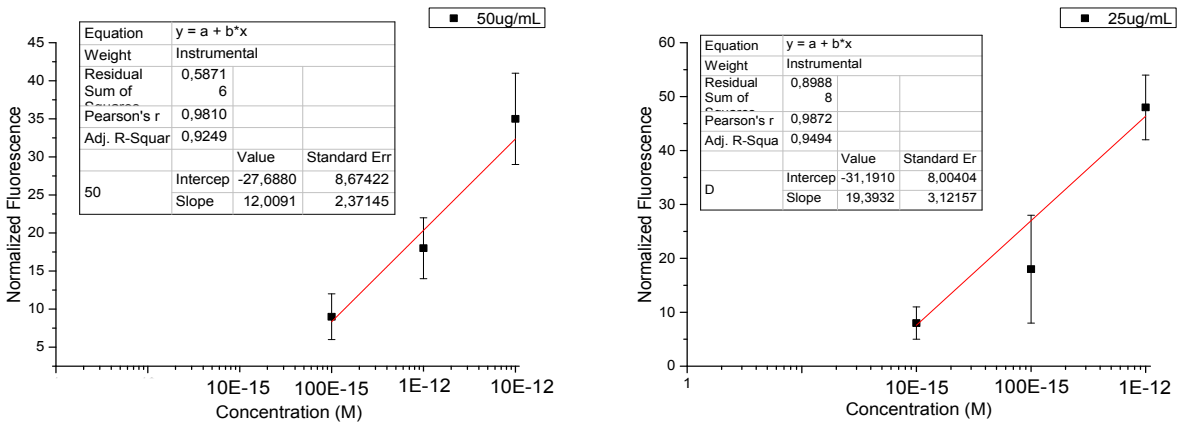


Fig. 6 LOD calculation based on spectrofluorometer data analysis

Table 6 Non-linear regression data analysis for ds displacement assay and microgel-based assay performed in presence of hcmv-miR-US4-5p target by Fluorimeter.

Microgel concentration (µg/mL)	Slope	Standard error (slope)	intercept	Standard Error (intercept)	LOD (fM)	R-Sq
50	8.339	2.201	12.009	2.37	354	0.924
25	26.407	3.03	20.079	3.19	283	0.95

Table 7 Microgel assay preformances measured by spectrofluorometer and CLSM

	Concentration	µg microgels	N microgels	Reaction Volume	Dilution	Analysis volume	LOD (fM)	Working range(fM)
Spectrofluorometer	50µg/mL	25	$1.45 \cdot 10^9$	500µL	-	500µL	354	10^6 -10^2
	25µg/mL	12.5	$7.25 \cdot 10^8$	500µL	-	500µL	283	10^6 -10^1
CLSM	50µg/mL	25	$1.45 \cdot 10^9$	500µL	1:10	30µL	39.1	10^6 -10^2
	25µg/mL	12.5	$7.25 \cdot 10^8$	500µL	1:5	30µL	11.7	10^6 -1
	0.5µg/mL	2.5	$1.45 \cdot 10^7$	500µL	-	30µL	0.156	10^6 -10^{-1}

S6 Assay stability

Microgel assay performances have been investigated after 1 years. The assay is carried on mixing 1nM of hcmv-miR-US4-5p with 25µg/mL of quenched microgels, in a final volume of 500 µL of hybridization buffer. As shown in figure 6 the assay is very stable also after long time and recovery of fluorescence is comparable to that observed after the quenching process.

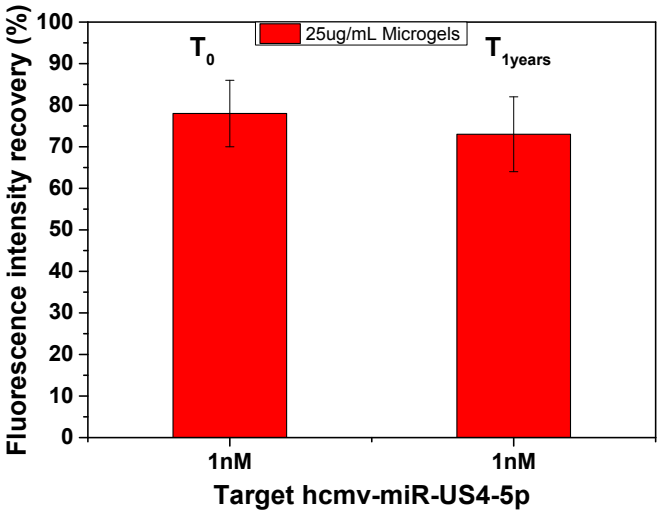


Fig 7 Microgel recovery of fluorescence measured after the quenching step (T_0) and after 1 years (T_1).