Supporting Information For

A Combination of DNA-peptide Probes and Liquid Chromatography-Tandem Mass Spectrometry: A Quasi-Targeted Proteomics Approach for Multiplexed MicroRNA Quantification

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Supplementary Tables

Table S1. Predicted mass response of reporter peptides using ESP Predictor.

Peptides	ESP Predictor Value
AVLGVDPFR	0.80
AVVGVDPFR	0.78
AVLGDPFR	0.76
AVQLGVDPFR	0.91
AVDLGVDPFR	0.86

Table S2. Internal standards and the corresponding MRM transitions.

Internal Peptide Standards	MRM Transitions
AV*LGV*DPFR	*m/z* 495.3 → 419.3
AV*VGV*DPFR	*m/z* 488.4 → 419.3
AV*LGDPFR	*m/z* 442.0 → 419.3
AV*QLGV*DPFR	*m/z* 559.4 → 698.3
AV*DLGV*DPFR	*m/z* 553.0 → 294.2

Table S3. Reporter peptides and the corresponding MRM transitions employed.

Reporter Peptides	MRM transitions
AVLGVDPFR	*m/z* 487.3 → 171.1 *m/z* 487.3 → 419.3 *m/z* 487.3 → 690.3
AVVGVDPFR	*m/z* 480.4 → 72.0 *m/z* 480.4 → 171.0 *m/z* 480.4 → 419.4
AVLGDPFR	*m/z* 438.0 → 72.0 *m/z* 438.0 → 175.0 *m/z* 438.0 → 419.3
AVQLGVDPFR	*m/z* 551.4 → 72.0 *m/z* 551.4 → 419.1 *m/z* 551.4 → 690.3
AVDLGVDPFR	*m/z* 545.0 → 72.2 *m/z* 545.0 → 170.8 *m/z* 545.0 → 286.2

Table S4. T_m values of miRNAs estimated using nearest-neighbor method.

miRNAs	Calculated Tm values
miR-21	62.5
miR-let 7a	63.8
miR-200c	68.8
miR-125a	72.8
miR-15b	66.0

Table S5. Accuracy and precision for QC samples.

Nominal Concentration	1.00 pM	3.00 pM	500 pM	80.0 nM
miR-21				
Mean	0.88	2.86	485	82.3
%Bias	-12.0	-4.7	-3.0	2.9
Intra-day Precision (%CV)	6.7	9.7	8.4	10.7
Inter-day Precision (%CV)	8.0	11.0	7.9	6.6
miR-15b				
Mean	0.89	2.77	478	78.1
%Bias	-11.0	-7.7	-4.4	-2.4
Intra-day Precision (%CV)	7.2	6.3	6.4	4.5
Inter-day Precision (%CV)	8.9	9.1	7.2	5.4
miR-Let 7a				
Mean	1.09	3.13	530	85.3
%Bias	9.0	4.3	6.0	6.6
Intra-day Precision (%CV)	5.3	5.1	4.3	6.7
Inter-day Precision (%CV)	4.6	4.6	4.9	3.7
miR-200c				
Mean	1.13	3.09	516	83.1
%Bias	13.0	3.0	3.2	3.9
Intra-day Precision (%CV)	7.9	6.4	5.6	6.5
Inter-day Precision (%CV)	5.8	5.3	4.9	4.2
miR-125a				
Mean	0.88	2.79	477	76.9
%Bias	-12.0	-7.0	-4.6	-3.9
Intra-day Precision (%CV)	6.3	7.6	4.6	5.7
Inter-day Precision (%CV)	6.1	5.9	5.8	6.1
n	18	18	18	18
Number of Runs	3	3	3	3

Table S6. A comparison of miRNA concentrations determined by quasi-targeted proteomics *w* and *w/o E. Coli* lysate.

Samples	miRNA concentrations (nM)				
	miR-21	miR-let7a	miR-200c	miR-125a	miR-15b
1 nM each miRNA	0.98±0.11	0.97±0.13	1.07±0.05	0.99±0.08	1.08±0.02
1 nM each miRNA + *E. coli* cell lysate	0.95±0.16	0.97±0.11	1.01±0.11	1.05±0.06	1.02±0.07

Table S7. Comparison of slopes of calibrations curves measured *w* and *w/o* other miRNAs in high concentration.

Samples	Calibration Curve Slopes				
	miR-21	**miR-let7a**	**miR-200c**	**miR-125a**	**miR-15b**
***w/o* other miRNAs**	0.906 ± 0.020	0.912 ± 0.020	0.908 ± 0.019	0.894 ± 0.012	0.887 ± 0.009
***w* other miRNAs**	0.918 ± 0.014	0.925 ± 0.010	0.914 ± 0.010	0.898 ± 0.018	0.893 ± 0.009
p value	0.42	0.38	0.67	0.77	0.47

Supplementary Figures

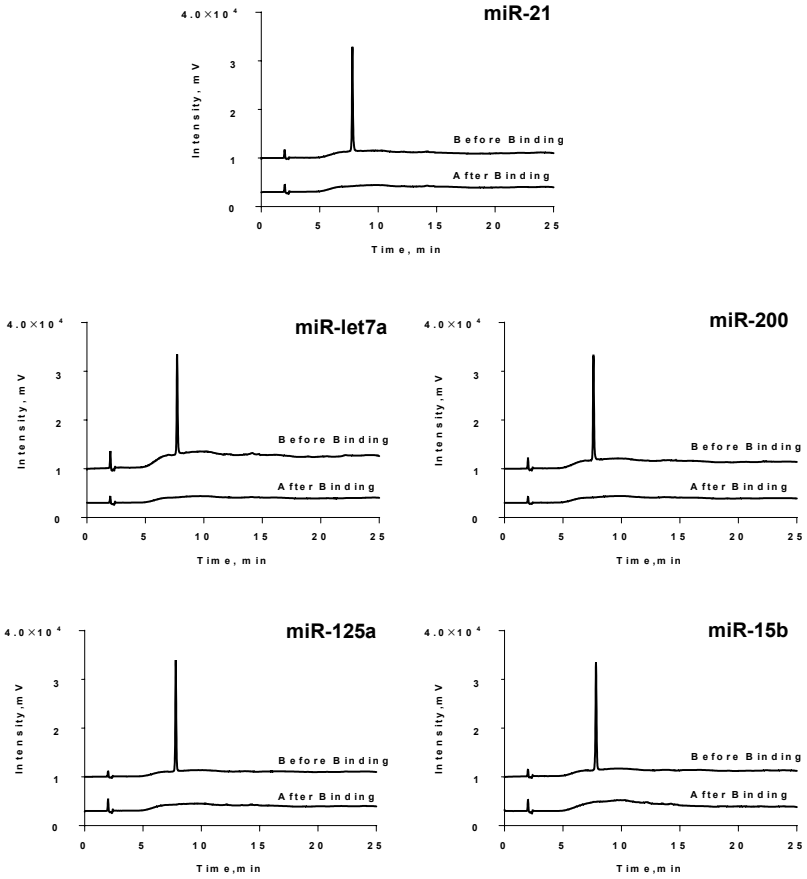


Figure S1. Chromatograms of biotinylated target miRNAs before and after binding with streptavidin agarose beads.

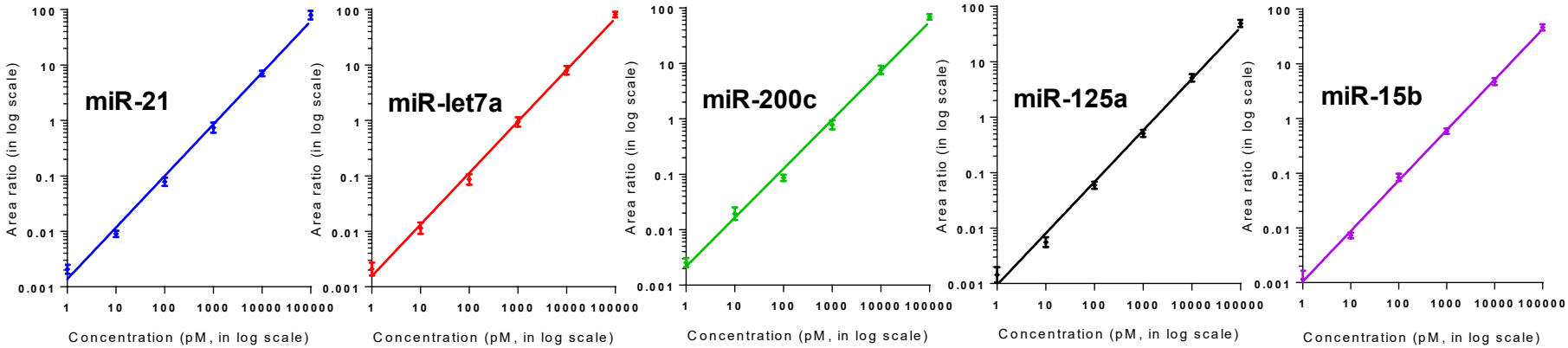
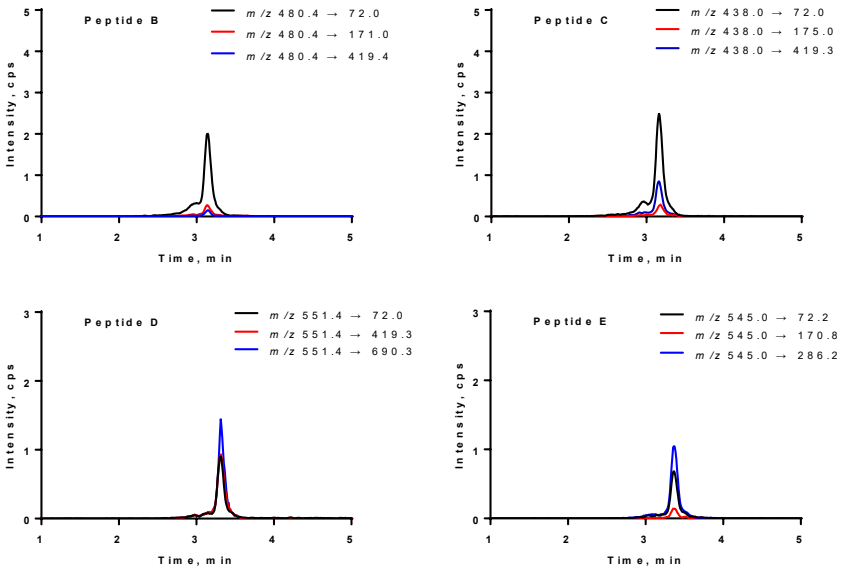


Figure S2. Representative calibration curves (1 pM to 100 nM) for target miRNAs. The relative peak area ratio of the reporter peptides and the corresponding stable isotope-labeled internal standards was plotted against concentration.

(A)



(B)

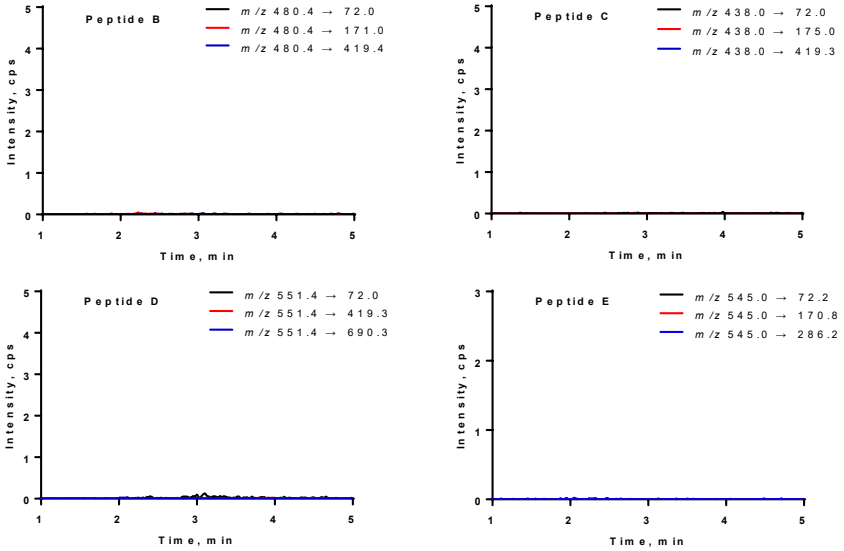


Figure S3. LC-MS/MS chromatograms for (A) the LLOQ of reporter peptides and (B) matrix blank. The internal standard is omitted for clarity.

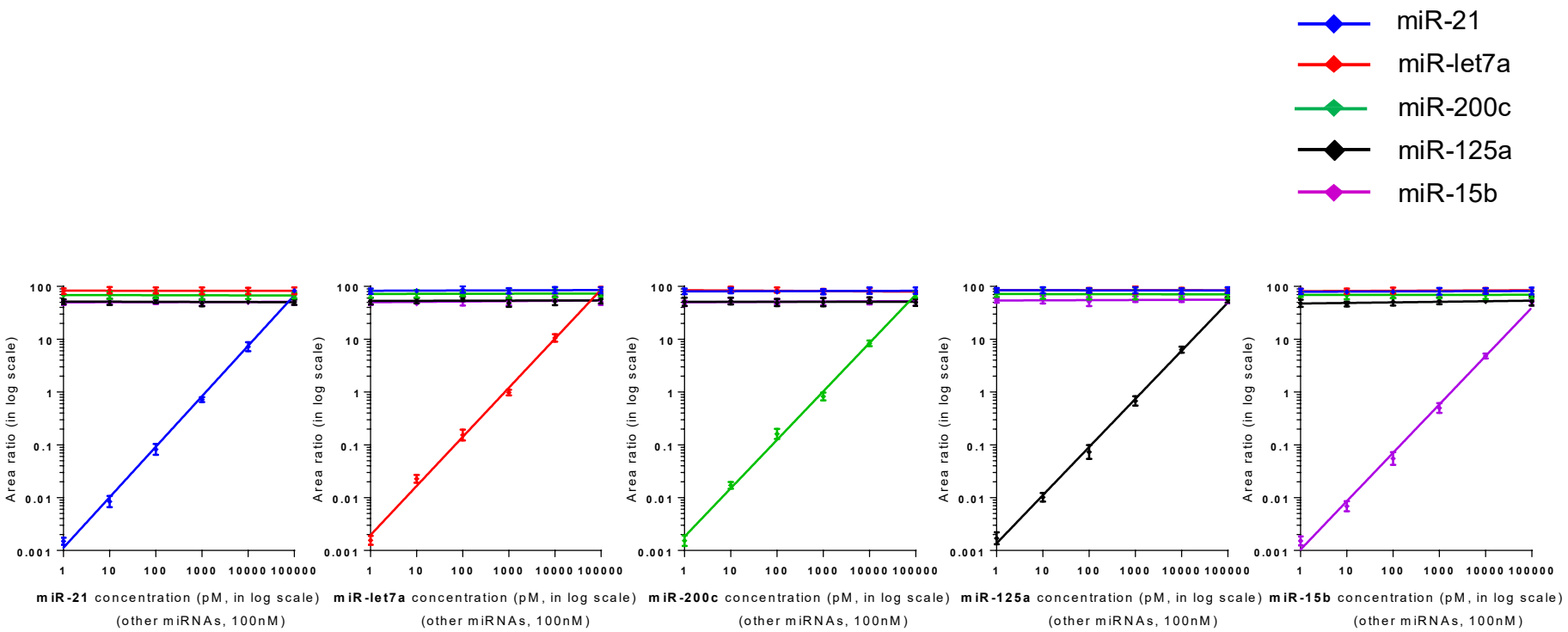
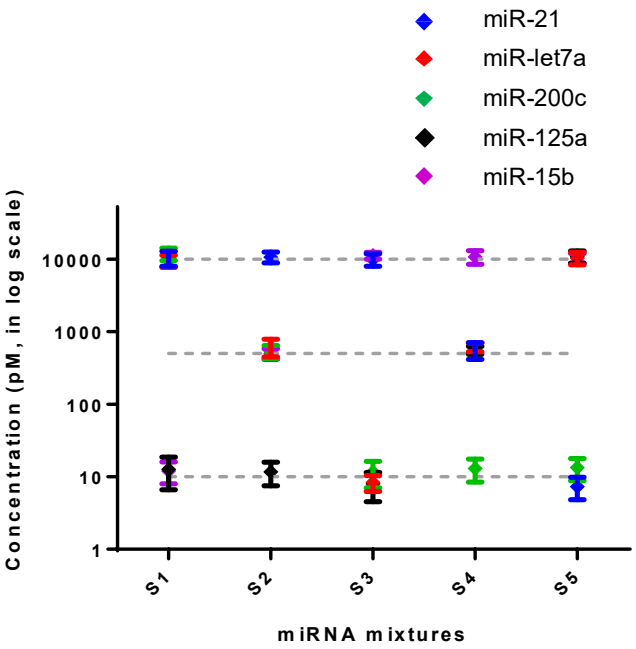


Figure S4. Calibration curves after crosstalk correction. The standards contain all five probes and five target miRNAs. One of the miRNAs has an increasing concentration. The other miRNAs have a constant concentration of 100 nM each. Even the presence of other miRNAs at high concentrations does not significantly alter the calibration curves.



miRNA mixtures	Concentrations (pM)				
	miR-21	miR-let7a	miR-200c	miR-125a	miR-15b
S1	10000	10000	10000	10	10
S2	10000	500	500	10	500
S3	10000	10	10	10	10000
S4	500	500	10	500	10000
S5	10	10000	10	10000	10000

Figure S5. Multiplexed detection of the target miRNAs with varying concentrations. Five samples (S1 to S5) containing miRNAs with concentrations between 10 pM and 10000 pM were measured and the calculated concentrations after crosstalk correction are shown as filled diamond and bar. Dotted line indicated the theoretical concentrations (i.e., 10 pM, 500 pM and 10000 pM).

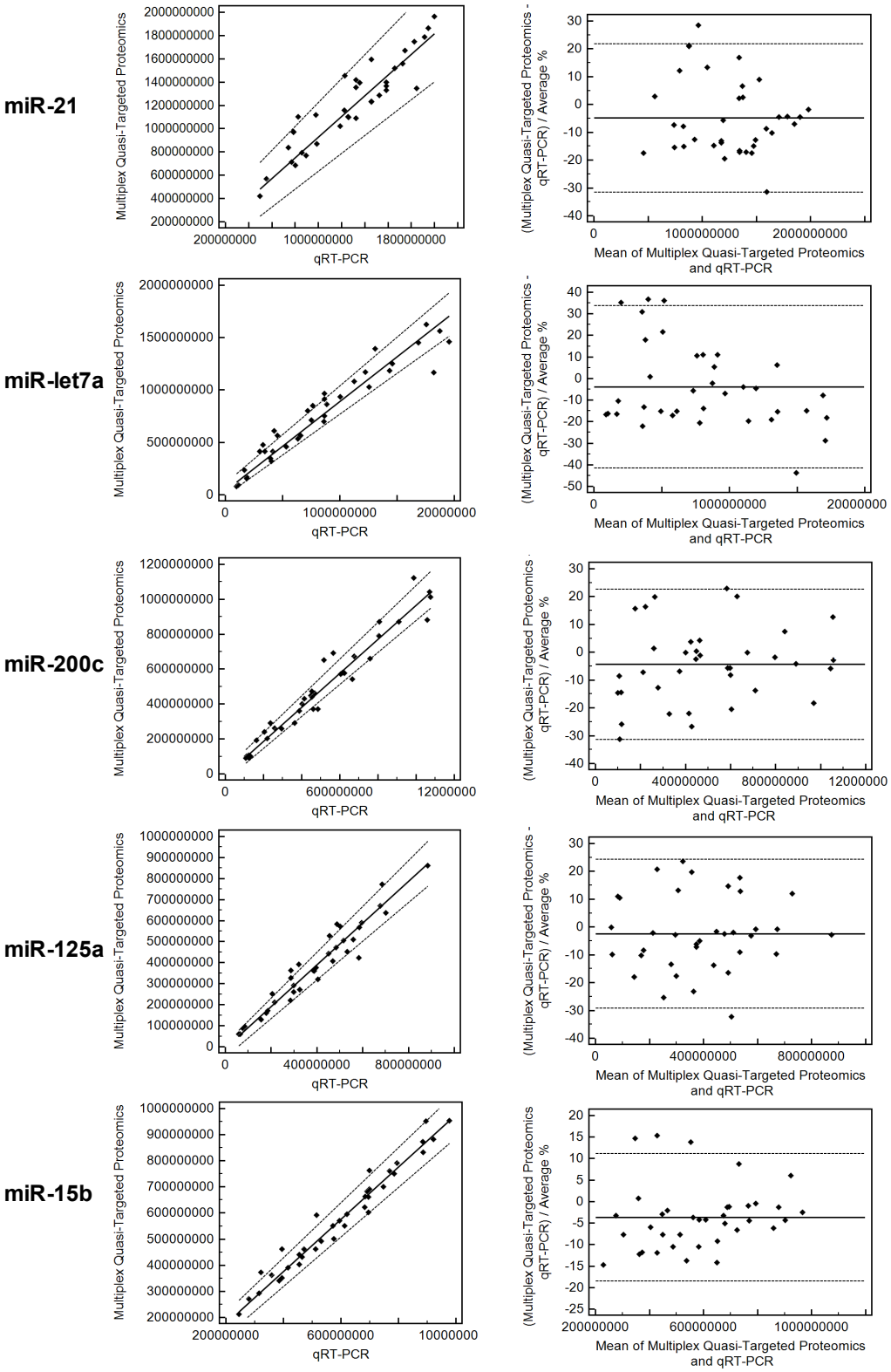


Figure S6. Passing-Bablok regression analysis and the corresponding Bland-Altman plot of breast tumor samples for multiplex quasi-targeted proteomics *vs.* qRT-PCR. The solid line corresponds to the regression line. Dashed lines represent the 95% confidence interval for the regression line in the Passing-Bablok regression plot and

the limits of agreement (± 1.96 × standard deviation (SD)) in the Bland-Altman plot.

Supplementary Material

S1. Formation of DNA-peptide probes

The synthetic DNA contained a disulfide modification on its 3' end that needed to be reduced prior to conjugation. Tris(2-carboxyethyl)-phosphine (TCEP) was used as the reducing agent. 20 μL of TCEP reducing beads (Thermo Fisher Scientific, IL, USA) was incubated with 100 μL of 2 μM DNA at 37°C for 2 h with vigorous shaking. The sample was then centrifuged at 1000 × g for 5 min, and the supernatant containing the reduced DNA was added to an equal volume of 20 μM maleimide-modified substrate peptide. The conjugation reaction was carried out at 37°C for 4 h with vigorous shaking, followed by immediate purification. The DNA-peptide conjugate was isolated from the excess of non-conjugated DNA and peptide by high performance liquid chromatography (HPLC). The HPLC condition was provided in the corresponding figure. Quantification was performed using external calibration peak area measurement.

S2. Biotinylation of miRNAs

Total RNA was isolated from cells and tissue homogenates using TRIzol® Reagent and quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., MA, USA). Following the manufacturer's protocol, 100 μg of total RNA in a 30 μL reaction volume was biotinylated using Pierce™ RNA 3' End Biotinylation Kit (Thermo Fisher Scientific Inc., MA, USA). Then, 20 μL biotinylated RNA was added with an equal volume of streptavidin agarose (Life Technologies, MD, USA) and incubated at 37°C on a shaker for 2 h, followed by washing and centrifugation. Streptavidin agarose was in advance coated with RNase-free BSA and yeast tRNA to prevent non-specific binding of RNA and protein complexes. For each 100 μL of beads, 10 μL tRNA (10 mg/mL) and 10 μL BSA (10 mg/mL) were added.

S3. In-solution Tryptic Digestion

The streptavidin agarose:biotinylated miRNAs:DNA-peptide complexes were mixed with 100 μL of 50 mM NH_4HCO_3. Subsequently, sequencing grade trypsin was added and the sample was incubated at 37°C for 24 h. The reaction was stopped by adding 10 μL of 0.1% TFA. Then, 100 μL of the internal standard solution was added to the tryptic peptide mixture before transferring it into an Oasis HLB cartridge (60 mg/3 mL; Waters, Milford, MA, USA) that was preconditioned with 3 mL ACN and 3 mL deionized water. After the sample was loaded, the cartridge was washed with 2 mL of water and 2 mL of ACN:water (50:50, v/v) and eluted with 1 mL of 100% ACN. Finally, the eluent was evaporated to dryness, and the sample was resuspended in 100 μL of ACN:water (50:50, v/v) containing 0.1% FA.

S4. Nearest-neighbor method

$$\mathrm{Tm} = \frac{H}{(A+S)+R\ln(Ct/4)} \times 273.15 + 16.6\log_{10}[\mathrm{salt}]$$

Where:

□ H (cal $mole^{-1}$) is the sum of the nearest-neighbor enthalpy changes for hybrid formation (< 0).

□ A (cal K^{-1} $mole^{-1}$) is a constant for helix initiation which is equal to -10.8 cal K^{-1} $mole^{-1}$ for non self-complementary sequences and -12.4 cal K^{-1} $mole^{-1}$ for self-complementary sequences).

□ S (cal K^{-1} $mole^{-1}$) is the sum of the nearest-neighbor entropy changes for hybrid formation (< 0).

□ R is the molar gas constant (1.987 cal K^{-1} $mole^{-1}$).

□ Ct is the total molar concentration of strands when oligonucleotides are not self complementary or it is equal to 4 times this concentration in the case of self-complementary sequences.

□ H and S values for nearest neighbor interactions of DNA and RNA have been presented previously

(http://www.sigmaaldrich.com/technical-documents/articles/biology/oligos-melting-temp.html).

S5. Crosstalk correction

The relation between the absolute intensities arising from all MRM transitions (I_1–I_5) and the real intensities for each single reporter peptide (A_1–A_5) can be expressed by Eq. 1, where M is the 5×5 crosstalk correction matrix consisting of five columns and rows of Fig. 5. Matrix M can be inverted numerically in order to calculate A_1–A_5 from the measured absolute intensities I_1–I_5 *via* Eq. 2.

$$\begin{bmatrix} I_1 \\ I_2 \\ I_3 \\ I_4 \\ I_5 \end{bmatrix} = M \bullet \begin{bmatrix} A_1 \\ A_2 \\ A_3 \\ A_4 \\ A_5 \end{bmatrix} \quad \text{Eq.1}$$

$$\begin{bmatrix} A_1 \\ A_2 \\ A_3 \\ A_4 \\ A_5 \end{bmatrix} = M^{-1} \bullet \begin{bmatrix} I_1 \\ I_2 \\ I_3 \\ I_4 \\ I_5 \end{bmatrix} \quad \text{Eq.2}$$

In this study,

$$M = \begin{bmatrix} 1 & 0 & 0 & 0.0131 & 0.0157 \\ 0 & 1 & 0 & 0.0172 & 0.0123 \\ 0 & 0.0152 & 1 & 0.0140 & 0 \\ 0.0133 & 0.0174 & 0.0151 & 1 & 0 \\ 0 & 0.0130 & 0.0156 & 0 & 1 \end{bmatrix}$$

$$M^{-1} = \begin{bmatrix} 1.00 & 4.26\times10^{-4} & 4.43\times10^{-4} & -1.31\times10^{-2} & -1.57\times10^{-2} \\ 2.29\times10^{-4} & 1.00 & 4.52\times10^{-4} & -1.72\times10^{-2} & -1.23\times10^{-2} \\ 1.83\times10^{-4} & -1.50\times10^{-2} & 1.00 & -1.37\times10^{-2} & 1.81\times10^{-4} \\ -1.33\times10^{-2} & -1.72\times10^{-2} & -1.51\times10^{-2} & 1.00 & 4.20\times10^{-4} \\ -5.83\times10^{-6} & -1.28\times10^{-2} & -1.56\times10^{-2} & 4.38\times10^{-4} & 1.00 \end{bmatrix}$$

S6. Result of backward logistic regression analysis

Logistic regression

Dependent Y	Breast_Cancer Breast Cancer

Method	Backward
Enter variable if P<	0.05
Remove variable if P>	0.1

Sample size	72
Cases with Y=0	36 (50.00%)
Cases with Y=1	36 (50.00%)

Overall Model Fit

Null model -2 Log Likelihood	99.813
Full model -2 Log Likelihood	27.280
Chi-squared	72.534
DF	3
Significance level	P < 0.0001

Coefficients and Standard Errors

Variable	Coefficient	Std. Error	P
miR_21	8.2105807197666E-009	2.6083428839936E-009	0.0016
miR_let7a	-2.1720429586 57E-009	1.0875124515593E-009	0.0458
miR_15b	6.6093465573157E-009	2.6732488230533E-009	0.0134
Constant	-7.4015		

Variables not included in the model
miR_200c
miR_125a

Odds Ratios and 95% Confidence Intervals

Variable	Odds ratio	95% CI
miR_21	1.0000	1.0000 to 1.0000
miR_let7a	1.0000	1.0000 to 1.0000
miR_15b	1.0000	1.0000 to 1.0000

Hosmer & Lemeshow test

Chi-squared	1.3189
DF	8
Significance level	P = 0.9953