

Supporting Information

Double inner standard plot model of electrophoresis titration chip for portable and green protein content assay of milk

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S-1. Brief Introduction of MRBET

Figure S1 shows the schematic diagram of moving reaction boundary (MRB) electrophoresis.^{S1,S2} In this system, two sides of electrophoretic tubes are filled with acid and alkali, respectively. Under an electric field, the hydrogen and hydroxyl ions move in opposite direction and react with each other. There is always an equal equivalent electromigration reaction between the hydrogen and hydroxyl ions. The boundary moves towards the cathode if the flux of hydrogen ions is more than that of hydroxyl ions, and vice versa.

Relied on the concept of MRB, the concept of electrophoresis titration (ET) was developed for protein content assay.^{S3,S4} In an alkaline MRB-ET system (**Figure S2**), an alkaline solution is added into the cathodic well, a neutral solution was injected into the anodic well, protein sample together with acid–base indicator is immobilized via highly cross-linked polyacrylamide gel (PAG). Under an electric field, the hydroxide ion electro-migrates toward to the anode, but the fixed protein molecules is stationary. The protein molecule carries acidic residue that can be titrated by the hydroxide ion. A neutralization boundary can be observed in **Figure S2** if an acid-base indicator exists in the MRB ET system. The higher protein content is, the more the acidic residues are, and the shorter MRB motion becomes.^{S3-S5}

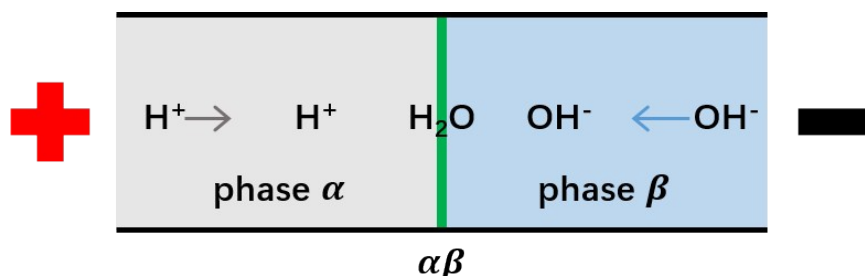


Figure S1. Schematic diagram of moving reaction boundary electrophoresis. The symbols of ‘phase α ’ and ‘phase β ’ represent the acidic and alkaline phases, respectively. The symbol of ‘ $\alpha\beta$ ’ indicates the reaction boundary of the two phases. The symbols of ‘+’ and ‘-’ imply the anode and cathode, respectively.

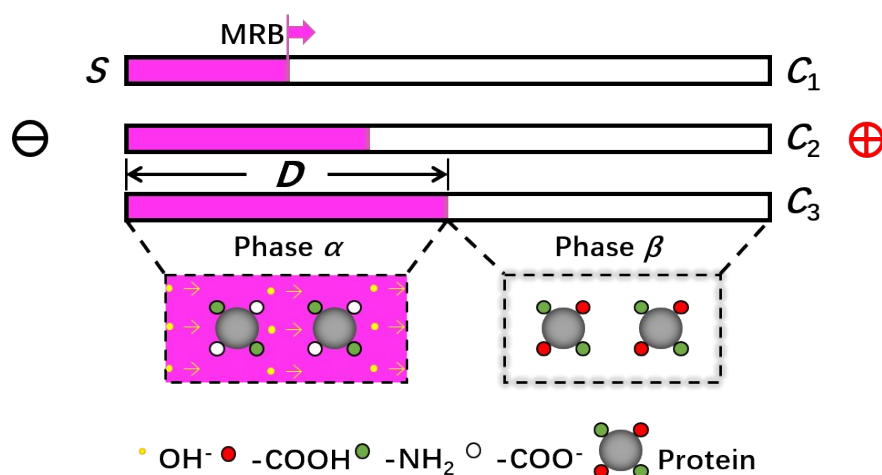


Figure S2. Schematic diagram of MRB-ET via a base in the cathode well for three protein contents in the same running time. The protein was immobilized in microchannel via PAG with an acid-base indicator. The symbols of ‘ \oplus ’ and ‘ \ominus ’ indicate the anode and cathode, respectively. The symbol of ‘ S ’ is the cross-sectional area of the microchannel. The symbol of ‘ D ’ represents MRB migration distance in the running time of t . The symbol of ‘ C ’ represents the content of protein ($C_1 > C_2 > C_3$). The symbols of ‘phase α ’ and ‘phase β ’ represent the titrated and un-titrated phases, respectively.

S-2. Experimental Section

Chemicals. Ammonium persulfate (APS), NaOH, NaCl, HCl, Na_2HPO_4 and NaH_2PO_4 were analytical reagent grade. Phenolphthalein was chemical reagent grade. They were all bought from Shanghai Chemical Reagent Co. (Shanghai, China). Acryl-amide, bis-acrylamide, N,N,N',N'-tetramethyl-ethylenediamine (TEMED), bovine serum albumin (BSA), hemoglobin (Hb) and avidin were bought from Sigma-Aldrich Co. (Shanghai, China). Milk samples were purchased from Inner Mongolia Mengniu Dairy Group Co. (Inner Mongolia, China), Inner Mongolia Yili Industrial Group Co., Ltd. (Inner Mongolia, China), Bright Dairy & Food Co., Ltd. (Shanghai, China) and Shanghai Yinan Dairy Farming Co., Ltd. (Shanghai, China). Ultrapure water yielded via a Millipore water purification system was used for the whole project.

Apparatus. An ultrasonic cleaning machine was purchased from Kunshan Ultrasonic Instruments Co., Ltd. (Kunshan, China). A cuft mechanical convection oven was from Shanghai Yiheng Scientific Instrument Co., Ltd. (Shanghai, China). An iPhone 6 was obtained from Apple Inc. (USA). A CCD camera was from Canon Co., Ltd. (Tokyo, Japan). An Automatic nitrogen determinator with Digest stove for Kjeldahl method was from Shanghai Qianjian Instrument Co., Ltd. (Shanghai, China). A protein electrophoresis titrator (MRB ET Instrument) was from Shanghai BioChemAn Biotechnology Co., Ltd. (Shanghai, China).

Solutions and Samples. For the DISP-ET chip ET, the cathodic well had 55 μL of cathodic buffer (20

mM NaOH, 80 mM KCl and 4 mM Na₂HPO₄, pH 12.3), the anodic reservoir had 55 μL of disodium hydrogen phosphate sodium dihydrogen phosphate buffer solution (15 mM pH 7.0 PB), and the channel of chip had 10 μL of mixed gel solution. Three protein solutions of 10 mg/mL were prepared with ultrapure water and stored at 4°C. Milk samples were stored at 4°C. The contents of model proteins (BSA, Hb and avidin) in gel samples ranged from 2.8 to 3.6 mg/mL. The 100 mL of PAG solution (20% total acrylamide, T and 3% cross-linker, C) had 19.4 g acrylamide and 0.6 g bis-acrylamide dissolved in ultrapure water and mixed uniformly. APS (10%, w/v) and TEMED were used for gel improving and catalyzing polymerization of gel, respectively.

Procedure of DISP-ET Chip. We prepared ET chips for DISP-ET experiments with the minor modifications.

1. Rinse with pure water for 1 min
2. Ultrasonic cleaning for 20 min
3. Rinse with 1 M NaOH for 2 min
4. Rinse with pure water for 1 min
5. Empty reservoirs and apply drying oven to dry for 20 min
6. Rinse with 30% acetic acid for 1 min
7. Rinse with pure water for 5 min
8. Empty reservoirs and apply drying oven to dry for 20 min

After those process, using adhesive film to smoothly glue the chip according to **Figure 2A**. The assembled chips are placed for experimental use. After ET experiments, the used chip can be reused by clean. Peel off adhesive film and take out the chip. After rinsing with pure water for 20 *sec*, we can gently pick out the gel in the channel with a fine needle. Then, using pure water to rinse the chip channel about 2 min, and using ultrasonic to clean it for 20 min. The treated chips put in a beaker with pure water and set aside to reserve.

Procedure of electrophoresis titrator. Glass tube was filled by gel and sealed at both ends of tube with anodic solution. Then the gel-filled tube was set between two chambers and fastened tightly by the springs. Cathodic and anodic solutions were pumped into two chambers, respectively, and then allowed a rapid fluid flow to eliminate residual air in the branches of chambers. The supply power was set at 80 V to yield a direct electric field. A digital camera was used to record the boundary movement.

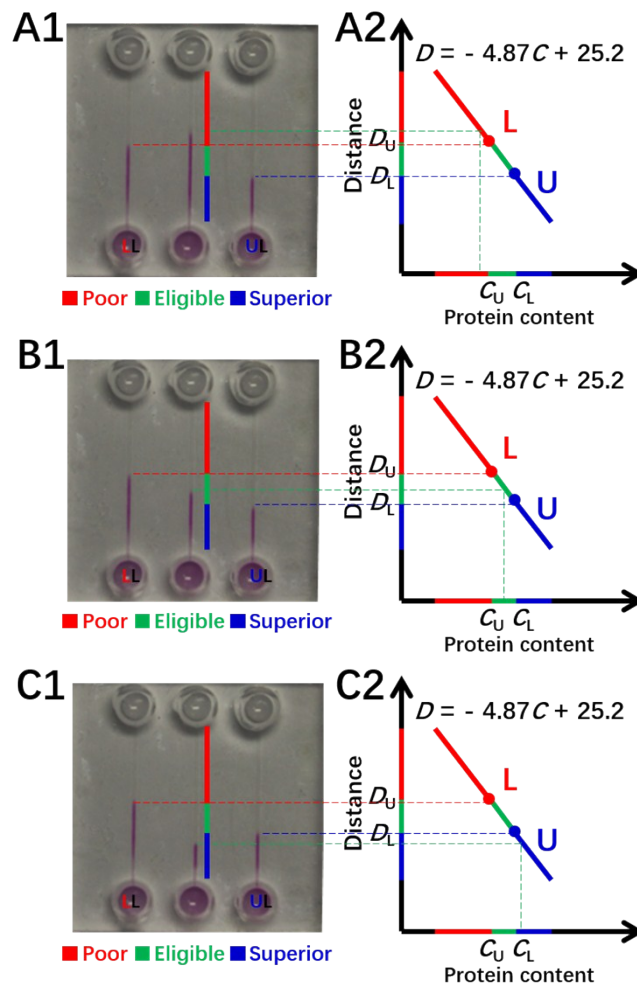


Figure S3. Quantitative assay of Hb content by DISP model. The direct protein quality readouts of poor (A1-A2), eligible (B1-B2) and superior (C1-C2) protein samples via DISP-ET chip. The other conditions are the same as those in Figure 4.

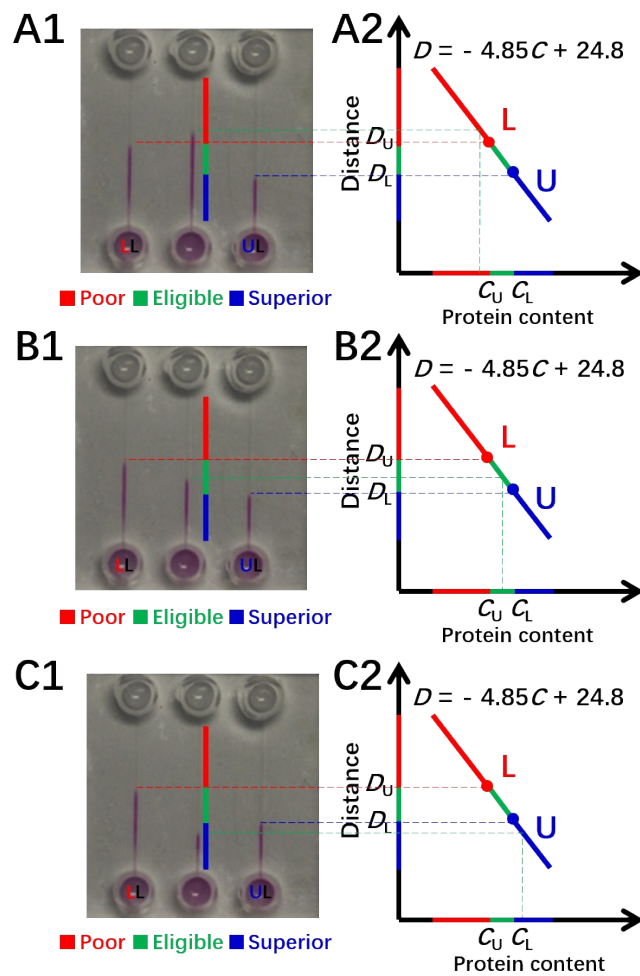


Figure S4. Quantitative assay of Avidin content by DISP model. The direct protein quality readouts of poor (A1-A2), eligible (B1-B2) and superior (C1-C2) protein samples via DISP-ET chip. The other conditions are the same as those in **Figure 4**.

S-3. Result Tables

Table S1. Linear regression equations between boundary motion distance (D) and running time (t) in Figures 4A, 4B and 4C.

Protein	Content	D vs t	R^2
BSA	C_{U1} (3.6 mg/mL)	$D=0.06t + 1.03 \times 10^{-3}$	0.9986
	C_{T3} (3.4 mg/mL)	$D=0.07t + 1.74 \times 10^{-2}$	0.9988
	C_{T2} (3.2 mg/mL)	$D=0.08t + 1.07 \times 10^{-2}$	0.9987
	C_{T1} (3.0 mg/mL)	$D=0.09t + 1.25 \times 10^{-2}$	0.9989
	C_{L1} (2.8 mg/mL)	$D=0.09t + 1.45 \times 10^{-2}$	0.9995
Hb	C_{U1} (3.6 mg/mL)	$D=0.06t + 2.09 \times 10^{-3}$	0.9997
	C_{T3} (3.4 mg/mL)	$D=0.07t + 1.61 \times 10^{-2}$	0.9986
	C_{T2} (3.2 mg/mL)	$D=0.08t + 1.48 \times 10^{-2}$	0.9989
	C_{T1} (3.0 mg/mL)	$D=0.09t + 1.82 \times 10^{-2}$	0.9995
	C_{L1} (2.8 mg/mL)	$D=0.10t + 1.09 \times 10^{-3}$	0.9996
Avidin	C_{U1} (3.6 mg/mL)	$D=0.07t + 8.62 \times 10^{-2}$	0.9993
	C_{T3} (3.4 mg/mL)	$D=0.08t + 3.10 \times 10^{-2}$	0.9989
	C_{T2} (3.2 mg/mL)	$D=0.08t + 6.15 \times 10^{-2}$	0.9995
	C_{T1} (3.0 mg/mL)	$D=0.09t + 8.43 \times 10^{-2}$	0.9996
	C_{L1} (2.8 mg/mL)	$D=0.10t + 1.14 \times 10^{-2}$	0.9997

Table S2. Linear regression equations between boundary motion distance (D) and protein content (C) in Figure 4D.

Sample	D vs C	R^2
BSA	$D= - 4.84C + 25.5$	0.9985
Hb	$D= - 4.87C + 25.2$	0.9989
Avidin	$D= - 4.85C + 24.8$	0.9998

Table S3. Comparisons of the protein content values between spiked and detected via DISP-ET model and those recovery, RSD and relative difference in Figures 4 and 5.

Sample	Spiked (mg/mL)	Detected(mg/mL)	Recovery (%)	RSD (%)	Relative difference (%)
BSA	3.0	2.98	99.3	3.41	0.67
	3.2	3.19	99.7	2.93	0.31
	3.4	3.38	99.4	4.17	0.59
Hb	3.0	2.97	99.0	4.36	1.00
	3.2	3.18	99.4	5.11	0.63
	3.4	3.42	101	4.92	0.59
Avidin	3.0	3.03	101	6.03	1.00
	3.2	3.22	101	5.86	0.63
	3.4	3.43	101	6.34	0.88
Milk	3.0	3.09	103	8.76	3.00

3.2	3.25	102	9.64	1.56
3.4	3.46	102	9.33	1.76

Table S4. Comparisons of the milk protein content values between spiked and detected via DISP-ET model and those recovery, RSD and relative difference at different time.

Time (sec)	Spiked (mg/mL)	Detected (mg/mL)	Recovery (%)	RSD (%)	Relative difference (%)
120	3.0	3.09	103	8.76	3.00
	3.2	3.25	102	9.64	1.56
	3.4	3.46	102	9.33	1.76
210	3.0	3.08	103	9.16	2.67
	3.2	3.23	101	8.67	0.94
	3.4	3.45	102	6.08	1.47

Table S5. Comparisons of milk protein content assay via MRB ET instrument and the portable device in **Figure 3**.

	MRB ET Instrument	Portable Device
UL Level	3.58 mg/mL	3.60 mg/mL
Test	3.65 mg/mL (Superior)	3.63 mg/mL (Superior)
LL Level	2.81 mg/mL	2.80 mg/mL

S-4. Data Treatment of Boundary

The chip ET experiments was conducted on the motions of MRB created with hydroxide ions and 2.8 mg/mL BSA in the running time of 0-120 *sec* and the relevant data treatment (**Figure S5**). **Panel A** directly shown the existence and stability of the MRB motion. To get a more accurate analysis, the images of **Panel A** was imported into the software of ImageJ, and the gray value was got via the software processing. Then the relation of the gray value versus channel location was obtained for different running times (**Panel B**). After a differential treatment, the derivative peak of boundary location was observed at different channel locations in **Panel C**. Thus, the relation of boundary motion versus channel location could be constructed by observing the peak location along the channel distance for different running times (**Panel C**). Finally, the calibration curve of boundary motion versus channel distance was obtained in **Panel D**.

Figure S5 shown the chip ET experiments on the motions of MRB created with hydroxide ions and 2.8 mg/mL BSA during the running time from 0 to 120 *sec*. In Panel A, all red MRB moved toward the anode because of high flux of hydroxide ions from the cathode electrolyte. The phenolphthalein in channel could indicate the movement of the red MRB. As the run continued, the red MRB became longer gradually. Panel A directly shown the existence and stability of the MRB motion. Panel D further revealed that the calibration

curve depicted the linear relationship between the boundary motion and ET running time. The linear regression equation was $D = 0.086t + 0.201$ ($R^2 = 0.9985$), indicating a good linearity. Evidently, the experiments of **Figure S5** demonstrated that the MRB motion distance was proportional to the running time in the chip of DISP-ET.

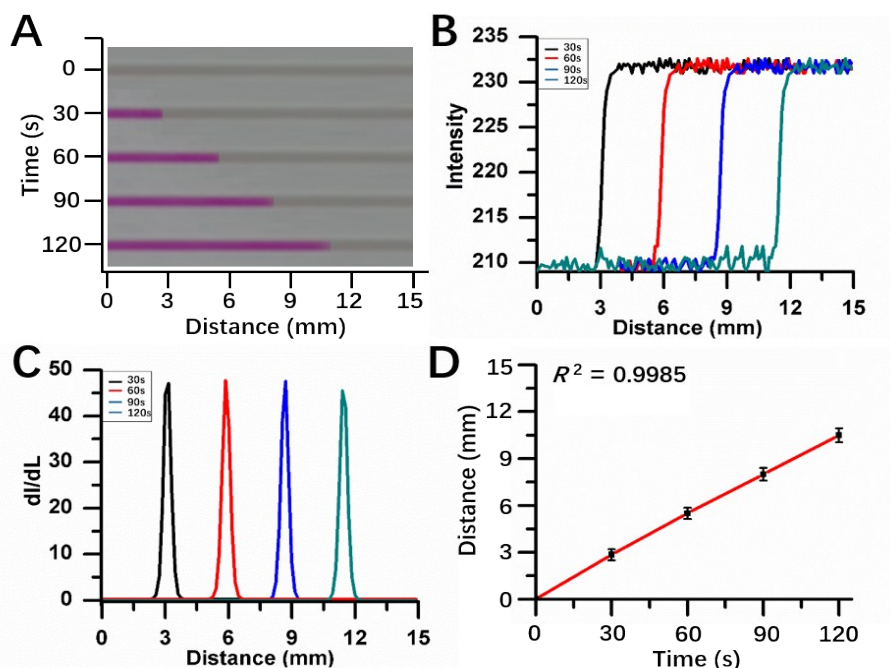


Figure S5. (A) Photograph of boundary motion distances during 0-120 s run of MRB created with 2.8 mg/mL BSA in microchannel; (B) raw intensity data vs distance in **Panel A** via ImageJ and Origin Pro 8 software; (C) derivatives of sigmoidal curves (dI/dL vs distance) transformed from **Panel B** via Origin Pro 8; (D) the calibration curve of boundary motion distance vs ET running time. The other conditions are the same as those in **Figure 4**. Each point was measured by three times.

S-5. Optimization and Selectivity

The high selectivity, good sensitivity and anti-non-protein nitrogen (NPN) have been repeatedly demonstrated in the previous works,^{S3,S4} we briefly introduced the optimizations of gel concentration, anti-NPN and selection of indicator herein.

Optimization of gel concentration. We used 1 mg/mL BSA to test five gel concentration levels (1) 30%T and 4%C; (2)30%T and 3%C; (3) 20%T and 4%C; (4) 20%T and 3%C and (5) 15%T and 4%C in **Figure S6** to optimize the gel concentration. The gel concentration has a great influence on MRB motion velocity. As the gel concentration increases, MRB motion velocity would be slower. High gel concentration with 20 V voltage was difficult to form a visual recognition boundary gap between different contents of

protein in a short time by naked eyes. In contrast, MRB moved too fast and retardation signal was not stable with low gel T and C. We test the time of the MRB to across the entire channel. 20%T and 3%C with the best stability via comparing the five levels. Considering the running time and stability, we finally used 20%T and 3%C as the experimental condition.

Anti-NPN. To investigate the selectivity of chip ET, we conducted the parallel group runs of milk samples added with glucose, urea and melamine at three concentration levels of 0.1, 0.2 and 0.3 mg/mL. Melamine and urea were NPN reagents, usually adulterated into milk. The milk without adding reagents are used as the control. The results in **Figure S7** shown that the values of MRB motions added with different compounds were much close to each other, indicating that proteins in milk were the only component related with the MRB motion.^{S3-S5} The developed method with high selectivity was due to the reason that MRB was created with the reaction of hydroxide ions and the acidic residues of immobilized milk proteins, rather than the reagents added.^{S3-S5} Thus, the added reagents would not change the motion of MRB. In contrast, little NPN reagents added would lead to great measure deviation in the Kjeldahl method or Dumas method, and it led to false increase of the protein content in adulterated milk and food.

Selection of indicator. It should be noted that phenolphthalein (PP) will migrate to the anode when the electric field is given. However, the weak influence and selection of indicators had discussed in the previous work,^{18,S6} and the different indicators had no evident influence on the MRB.^{S6} Herein the OH⁻ moved much faster than the colored-PP. Therefore, the PP migrate was very weak influence to the MRB result^{18,S6}, and the weak effects were the same in three channels of single chip and it can be automatically eliminated by DISP method. Particularly, the electric field intensity of this device was 16 V/cm and lower than previous work.^{18,S6} It suggested that the colored-PP migration was more weak influence to the quantitative accuracy.

In addition, the cationic pH indicator can be used to the method, and many cationic pH indicators were used in our previous work.^{18,S6} However, we had used acid (H⁺) and bromophenol blue (as indicator) to present this work. The red boundary of PP was easier to recognize with naked eye than the light green boundary of bromophenol blue. Therefore, we finally used PP as indicator to present this work, due to the colorful boundary was pretty better than the one of bromophenol blue.

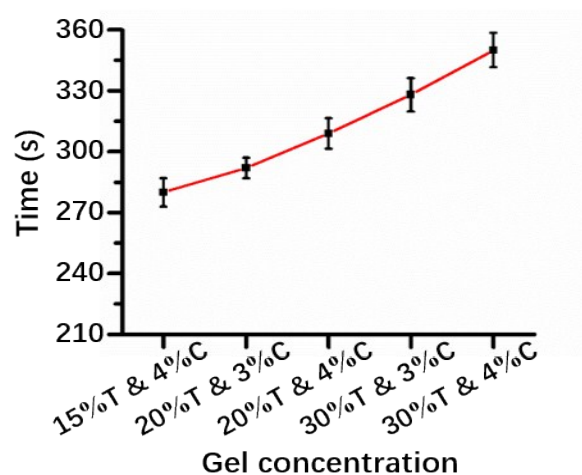


Figure S6. The vertical axis is the time that boundaries across the entire channel in the five gel concentration levels. The horizontal axis was 15%T and 4%C, 20%T and 3%C, 20%T and 4%C, 30%T and 3%C, 30%T and 4%C five gel concentration levels. The other conditions are the same as those in **Figure 4**. Each point was measured by three times.

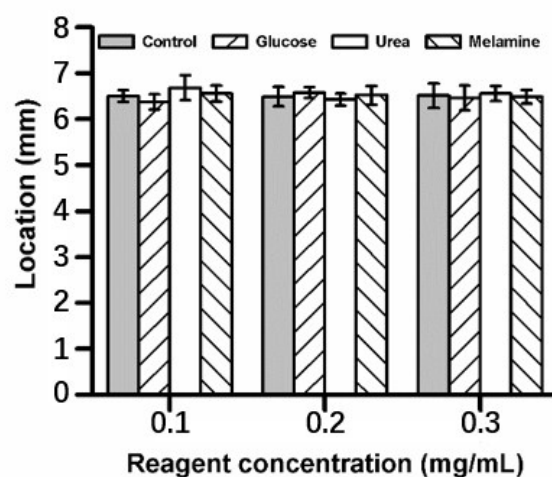


Figure S7. Influence of different reagents added to milk samples on the MRB motion distance in 120 sec. 0.1, 0.2 and 0.3 mg/mL glucose, urea and melamine are added to milk samples, respectively. The milk without adding reagents are used as control. Error bars represent the standard deviations for three replicates. The other conditions are the same as those in **Figure 4**.

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