Electronic Supplementary Information (ESI)

A novel template repairing-PCR (TR-PCR) reaction platform for microRNA detection using translesional synthesis on DNA templates containing abasic site

Dianming Zhou,‡[a] Xiaohui Lin,‡[a] Weichen Gao,[b] Jiafang Piao,[b] Shufei Li,[a] Ning He,[a] Zhiyong Qian,[a,*] Miao Zhao, [a,*] and Xiaoqun Gong[b,*]

[a]*Department of Toxicology, Tianjin Centers for Disease Control and Prevention, Tianjin 300011, China*

**Corresponding authors. E-mail:13323360260@163.com; zmxb129@163.com*

[b]*School of Life Sciences, Tianjin University and Tianjin Engineering Center of Micro-Nano Biomaterials and Detection-Treatment Technology (Tianjin), Tianjin 300072, China*

**Corresponding authors. E-mail:gongxiaoqun@tju.edu.cn*

Experimental Section

Materials and Reagents. Klenow Fragment DNA polymerase(3'-5'exo$^-$), *Bst* DNA polymerase (large fragment), uracil-DNA glycosylase (UDG), Taq DNA polymerase were purchased from New England Biolabs. Ltd. (Ipswich, MA, USA). RNase inhibitor, SYBR® Premix ExTM Taq II and dNTPs were provided by Takara biotechnology Co (Dalian, China). MiRcute miRNA isolation kit and DNase/RNase-free deionized water which used all the reaction solution were purchased from TIANGEN Biotechnology Co., Ltd. (Beijing, China). 20×EvaGreen was purchased from US Everbright® Inc.(Suzhou, China). Fumonisin B1 (FB1) was purchased from Santa Cruz Biotechnology, Inc. (USA). Sequences of the miRNA were obtained from the miRBase (www.mirbase.org). All synthesized DNA probes and RNA sequences were HPLC-purified and obtained from Sangon Biotech Co., Ltd. (Shanghai, China). The sequences of the synthesized probes and RNA sequences are listed in Table S1. All other chemicals were of analytical grade and obtained from Sinopharm Chemical Reagent Co. Ltd. All solutions used in the experiments were prepared using RNase-free water.

Table S1. Sequences (5'-3') of synthetic oligonucleotides used in this study[a]:

Synthetic oligonucleotide	Abbreviation	Sequence(5'-3')
microRNA-122	miR-122	UGG AGU GUG ACA AUG GUG UUU G
mismatch miR-122	mmiR-122	UGG AGU GUG ACA AUG CUC UUA G
microRNA-21	miR-21	UAG CUU AUC AGA CUG AUG UUG A
microRNA-141	miR-141	UAA CAC UGU CUG GUA AAG AUG G
Non-specific RNA	NC	UUG UAC UAC ACA AAA GUA CUG
DNA-122	DNA122	TGG AGT GTG ACA ATG GTG TTT G
Template122	T01	ACGGTTGGCCTTAGGGTTCACCTCTTTCC**X**CTTTGC CCTCTCGTCACCCACATAGGAGTCCTTTCAAACAC CATTGTCACTTTTT**ddC**
Template21	T02	ACGGTTGGCCTTAGGGTTCATCC**X**CTTTTCGTCACC CACATAGGAGTCCTTTTCAACATCAGTCT**ddC**
Template dU	T03	ACGGTTGGCCTTAGGGTTCACCd**U**CTd**U**TCCd**U**CTd **U**TGCCCd**U**Cd**U**CGTCACCCACATAGGAGTCCTTTCA AACACCATTGTCACTTTTT**ddC**
Forward primer	FP	GGA CTC CTA TGT GGG TGA CGA
Reverse primer	RP	ACG GTT GGC CTT AGG GTT CA

[a]X represents tetrahydrofuran abasic site mimic (THF), ddC represents 2',3'-dideoxycytidine, dU represents uracil deoxyribonucleotide residues.

Animal model and miRNA extraction. All experimental mice were Specfic Pathogen Free grade Blab/c mice, purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. The animal experimental protocol was approved by the Committee on the Ethics of Animal Experiments of Tianjin Centers for Disease Control and Prevention (permit number: TJCDCLL2018001). This study was carried out in strict accordance with The People's Republic of China Laboratory Animal Regulations.

A total of 30 mice, half males and half females, weighing 16-20g, were selected. All the mice were randomly divided into 3 groups, control group (sterile deionized water) and two FB1-treated groups (0.75 and 7.5 mg FB1 /kg/day). The treatment was conducted through gavage once a day at 0.04 ml/g for 28 consecutive days. At the end of the study, all the animals were euthanized. Liver samples were collected and stored at -80℃ until use. Small RNAs including miRNAs were extracted from liver tissue using miRcute Kit (Tiangen) according to the protocol.

Standard TR-PCR procedures. The volume of 20 μL reaction mixture including 1× NEB buffer 2, 8 U of RNase inhibitor, 250 μM dNTPs, 2.5 U of Klenow Fragment DNA polymerase(3'-5' exo-), 1 pM T01 and an appropriate amount of target miRNA was first incubated at 37℃ for 1h and inactivated at 85 ℃ for 30 min.

The qPCR experiment was conducted in a LightCycler® 480 Instrument II(Roche, Germany) with a 20 μL reaction system, including 1× SYBR Premix ExTM Taq II, 500 nM reverse primer, 500 nM forward primer and 1μL products from previous polymerization reaction. qPCR reaction was performed in a 96-well optical PCR plate at 95 ℃ for 30 s, followed by 40 cycles of 95 ℃ for 5 s and 63 ℃ for 30 s.

In fact, there are many other polymerases which can be used for translesional synthesis test besides Bst DNA polymerase and Klenow DNA polymerase, such as vent DNA polymerase, sulfolobus DNA polymerase IV, Therminator DNA polymerase, etc. The key factors for the selection are polymerase activity, exonuclease activity, and other special activities. Polymerase used in the assay need to retain polymerase activity, but lost the 5'→3'exonuclease activity and the 3'→5'exonuclease activity. These functions are necessary for primer extension and reducing non-specific reactions. Moreover, the polymerase also needs to extend RNA primer for miRNA detection, and could be inactivated by heating. If the PCR primers are extended by the translesional polymerase, the synthesis products will produce an obvious background in the PCR procedure. Heat inactivation can effectively inhibit primer extension in the following PCR procedures. Thereby, we prefer Klenow DNA polymerase in the detection system, which has

maximum activity at 37℃.

Modified TR-PCR (NTR-PCR) procedures. The volume of 18 μL reaction mixture including 1× NEB buffer 2, 8 U of RNase inhibitor, 250 μM dNTPs, 2.5 U of Klenow Fragment DNA polymerase(3'-5' exo-), 10 pM T03 and an appropriate amount of target miRNA was first incubated at 37℃ for 30 min and inactivated at 85 ℃ for 30 min. Then, 10 U of UDG was added to mixture and incubated at 37℃ for 2 h.

The qPCR experiment was conducted in a LightCycler® 480 Instrument II(Roche, Germany) with a 20 μL reaction system, including 1× SYBR Premix ExTM Taq II, 500 nM reverse primer, 500 nM forward primer and 1μL products from previous polymerization reaction. qPCR reaction was performed in a 96-well optical PCR plate at 95 ℃ for 30 s, followed by 40 cycles of 95 ℃ for 5 s and 63 ℃ for 30 s.

Melting temperature and Electrophoresis separation. To demonstrate the different translesional activity of the Klenow Fragment DNA polymerase(3'-5' exo-) and the Taq DNA polymerase at THF site, the melting curves of extension products from each DNA polymerase were recorded with a LightCycler® 480 Instrument II, and also the capillary electrophoresis separation (CE) of extension products from each DNA polymerase were conducted and analyzed by Fragment Analyzer Automated CE System. The volume of 20 μL reaction mixture both for melting curve and CE including 1× NEB buffer, 500 nM DNA122, 250 μM dNTPs, 200 nM T01 and 2.5 U of DNA polymerase was incubated at 37 ℃ for 1h. The reaction products were characterized by CE without any treatment. But for melting curve, the products were mixed with 1× EvaGreen, and then heated from 50 ℃ to 95 ℃ with an increment rate of 0.03 ℃ /s. The fluorescent signals were obtained throughout the melting analysis process, and the data was analyzed through LightCycler® 480 Instrument II software.

To assess the multiplexing ability of TR-PCR for miRNA detection, two miRNAs, miR-122 and miR-21 were chosen as the model targets, the T01 and T02 were chosen as templates respectively. The PCR products for polyacrylamide gel electrophoresis were analyzed using 1.0 mm thick 12% polyacrylamide gel in 1 × TBE buffer at room temperature. Electrophoresis was performed at a constant voltage of 90 V for 90 min by loading a mixture of 10 μL sample and 5 μL 6 × loading dye in each channel. After electrophoresis, the gel was visualized using Azure Biosystems C600(USA).

Fig.S1. Dependency of the TR-PCR strategy on the polymerase extension time (min). (C_{T01}=1pM, Δ Ct = Ct (control) - Ct ($_{miR\text{-}122}$=2 fmol))

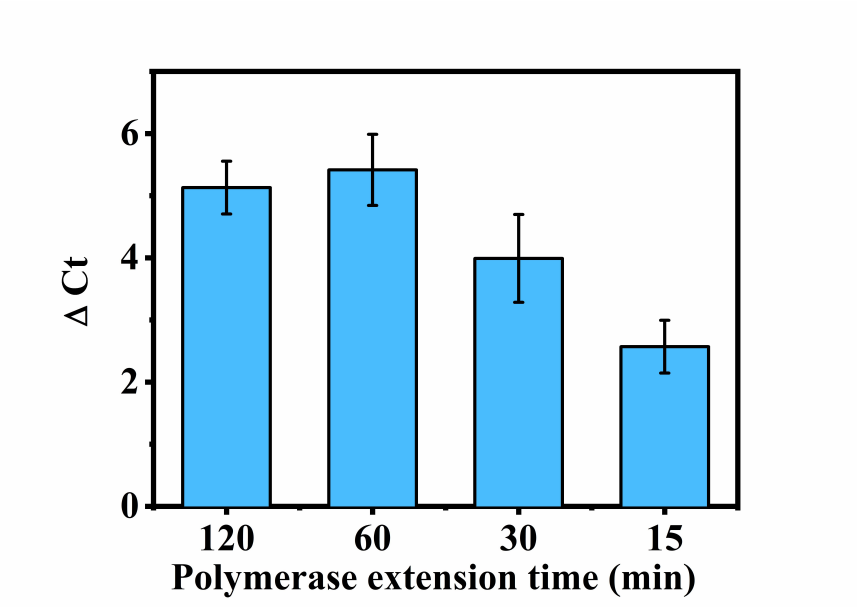


Fig.S2. Dependency of the TR-PCR strategy on the template concentration. (Δ Ct = Ct (control) - Ct ($_{miR-122}$=20 amol))

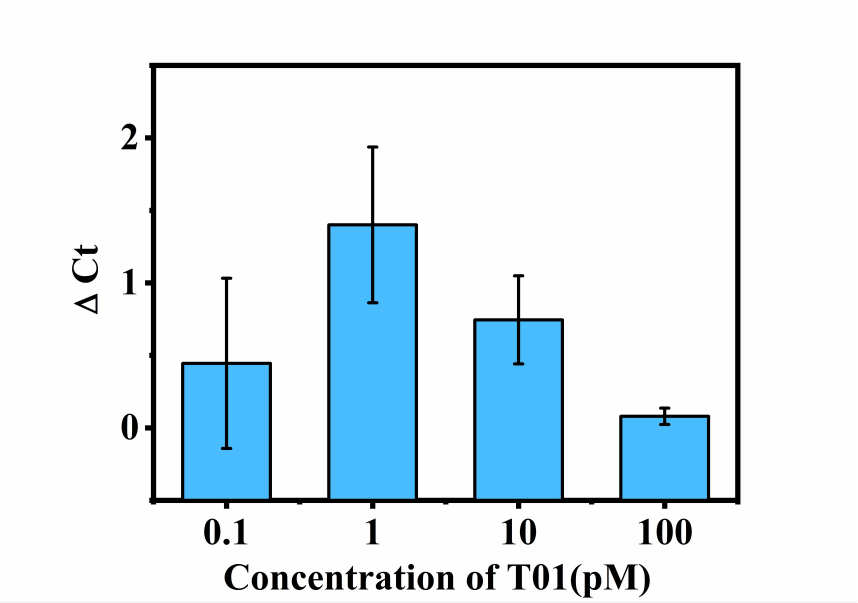
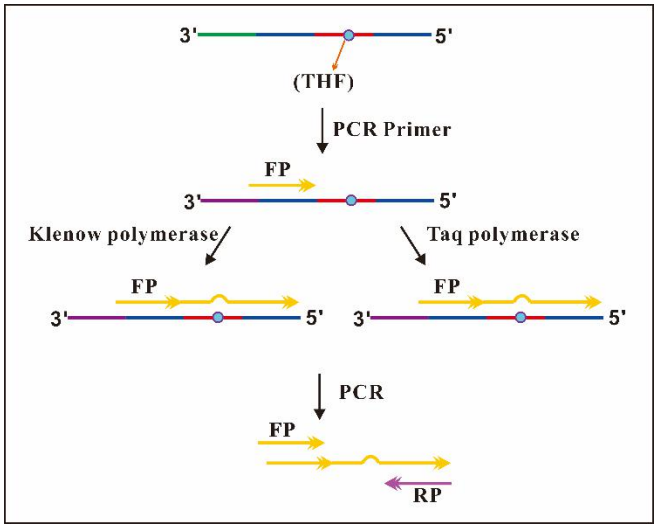


Fig.S3. Schematic representation of non-specific PCR amplification from lesion template-mediated PCR reaction.



According to our strategy design, the background comes from lesion template-mediated PCR reaction which is illustrated in Fig. S3. In our opinion, PCR primer-primed bypass synthesis on the THF/AP site involved PCR template is the main reason for producing non-specific amplification. This bypass synthesis may include two pathways, one from Klenow DNA polymerase which is not heat inactivated, and another from weak translesional synthesis activities of Taq DNA polymerase. In Fig.1B, we can find that Taq DNA polymerase-mediated DNA replication stopped at the THF site, and no bypass synthesis products were detected. However, according to the sensitivity of capillary electrophoresis, the possibility of bypass synthesis is not ruled out. In consideration of these reasons, the optimization of the template concentration was performed in our manuscript. At the same time, to suppress the possible background signal from Klenow DNA polymerase, the heat inactivation temperature was increased from 75℃ (product instruction) to 85℃, incubation time for heat inactivation was increased from 20 min (product instruction) to 30 min. Hence, under the optimum conditions, non-specific amplification is not quite significant for our test.

Fig.S4. Melting temperature test for assessing the multiplexing ability of TR-PCR for miRNA detection. PCR products for melting curves from (a) 1 pM T01+1 pM T02+20 fmol miR-122, (b) 1 pM T01+1 pM T02+20 fmol miR-21, (c) 1 pM T01+1 pM T02+20 fmol miR-122+20 fmol miR-21, (d) 1 pM T01+1 pM T02 no target.

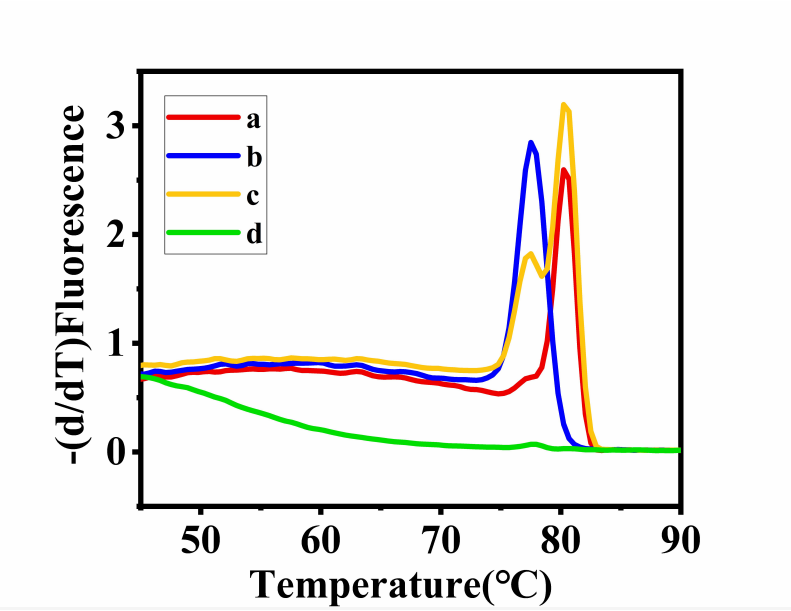


Fig.S5. Polyacrylamide gel electrophoresis test for assessing the multiplexing ability of TR-PCR for miRNA detection. PCR products for gel electrophoresis from (lane a) 1 pM T01+1 pM T02+20 fmol miR-122+20 fmol miR-21, (lane b) 1 pM T01+1 pM T02+20 fmol miR-122, (lane c) 1 pM T01+1 pM T02+20 fmol miR-21, (lane d) 1 pM T01+1 pM T02 no target.

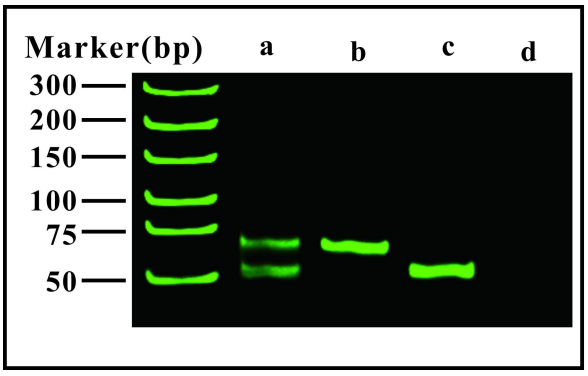


Fig.S6. Dependency of the novel design TR-PCR strategy (NTR-PCR) on the template concentration. (Δ Ct = Ct (control) - Ct ($_{miR-122}$=2 amol))

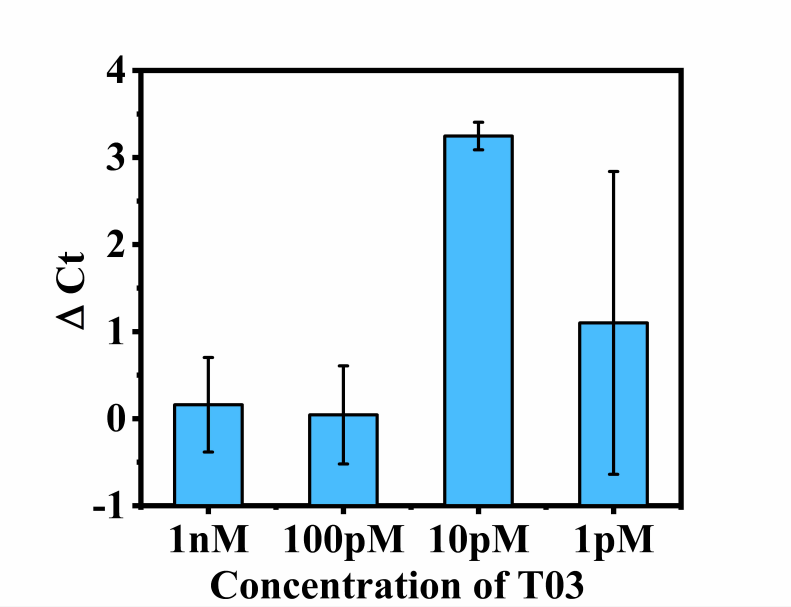
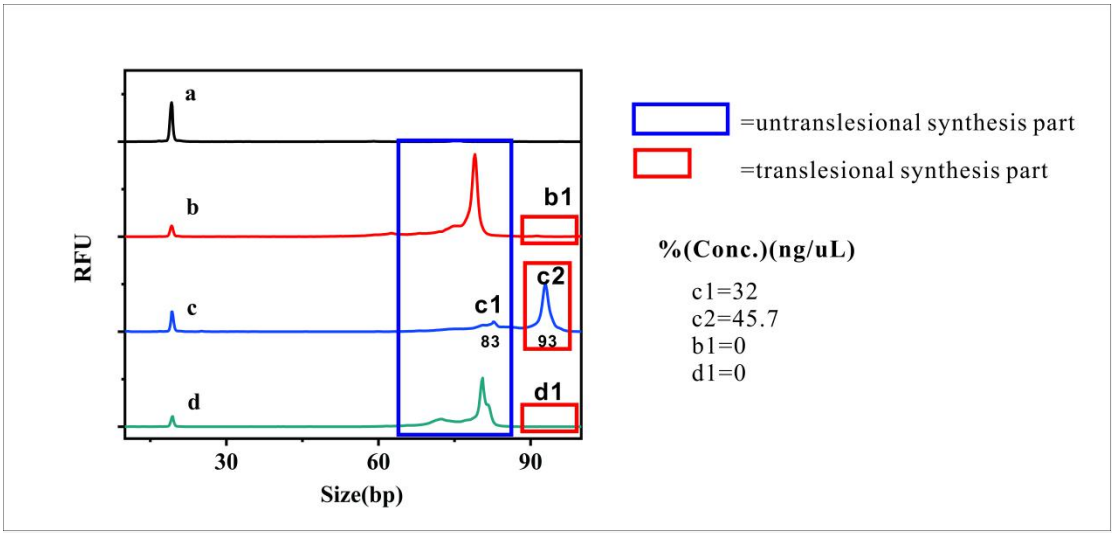


Fig.S7. Numerical analysis for the translesional synthesis activity of Klenow fragment DNA polymerase (c) and Taq DNA polymerase (d).



As shown in Fig.S6, capillary electrophoresis for discriminating the extent of polymerase translesional synthesis, Klenow DNA polymerase could bypass the THF site, producing a long duplex (curve c), and Taq DNA polymerase stopped at the THF site (curve d). These results can be analyzed numerically according to the software of capillary electrophoresis. From curve d, no translesional synthesis product was observed (d1=0%). We can calculate the translesional synthesis activity of Taq DNA polymerase about 0%. From curve c, peak c1 stands for untranslesional synthesis part of Klenow DNA polymerase, concentration (percentage ng/uL) is 32%, molecular weight is 83bp*2*M (M=average molecular weight of base). Peak c2 stands for translesional synthesis part of Klenow DNA polymerase, concentration (percentage ng/uL) is 45.7%, molecular weight is 93bp*2*M. We can calculate concentration (percentage mol/uL) of the translesional synthesis part and untranslesional synthesis part of Klenow DNA polymerase, and also calculate the the translesional synthesis activity of Klenow DNA polymerase about 56%.

Table S2. Comparison of the proposed method with other conventional methods for miRNA analysis that rely on either ligation or non-PCR amplification (miRNA-primed target recognition).

Methods	Enzyme	Linear/Exponenti-al amplification	Operation complexity	Design complexity	Ref.
stem-loop probes ligation qPCR	ligase, polymerase	expo	simple, ligation and PCR	medium, stem-loop probes design	1
ribonucleotide-modified probes ligation qPCR	ligase, polymerase	expo	simple, ligation and PCR	medium, modified base, special ligase, short probes hybridization design	2
padlock probes and RCA	ligase, polymerase	linear	quite complex, ligation and RCA, PAGE detection	medium, padlock probes design	3
toehold-initiated RCA	ligase, polymerase	linear	medium, ligation and exonuclease digestion and RCA	complex, toehold length optimization, dumbbell-shaped probe design	4
branched RCA	ligase, polymerase	linear	medium, ligation and BRCA (>6 hours)	medium, padlock probes design	5
EXPAR	polymerase, nicking enzyme	expo	simple, extension	medium, careful template design	6
EXPAR and quantum-dot QD	polymerase, nicking	expo	medium, multi-step	medium, careful template design	7

detection	enzyme		reactions		
TR-PCR	polymerase (two)	expo	simple, extension and PCR	simple, AP site,without other special design for template and primers	This method

1. J. Li, B. Yao, H. Huang, Z. Wang, C. H. Sun, Y. Fan, Q. Chang, S. L. Li, X. Wang and J. Z. Xi, *Anal. Chem.*, 2009, **81**, 5446-5451.
2. J. Y. Zhang, Z. P. Li, H. Wang, Y. C. Wang, H. X. Jia and J. L. Yan, *Chem. Commun.*, 2011, **47**, 9465-9467.
3. S. P. Jonstrup, J. Koch and J. Kjems, *RNA*, 2006, **12**, 1747-1752.
4. R. Deng, L. H. Tang, Q. Q. Tian, Y. Wang, L. Lin and J. H. Li, *Angew. Chem. Int. Ed.*, 2014, **53**, 2389-2393.
5. Y. Q. Cheng, X. Zhang, Z. P. Li, X. X. Jiao, Y. C. Wang and Y. L. Zhang, *Angew. Chem. Int. Ed.*, 2009, **121**, 3318-3322.
6. H. X. Jia, Z. P. Li, C. H. Liu and Y. Q. Cheng, *Angew. Chem. Int. Ed.,* 2010, **49**, 5498-5501.
7. Y. Zhang and C.Y. Zhang, *Anal. Chem.*, 2012, **84**, 224-231.