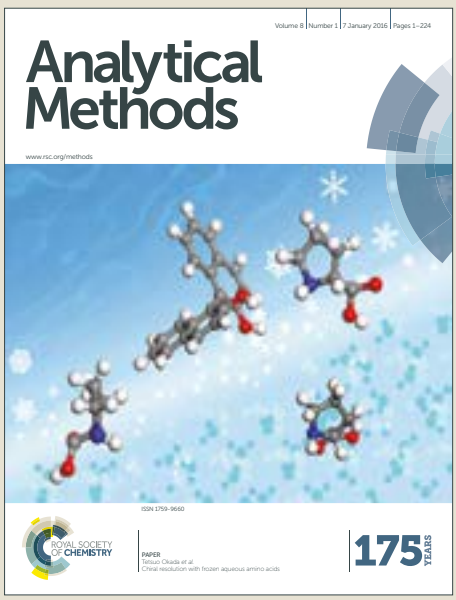
Analytical Methods

Accepted Manuscript

This article can be cited before page numbers have been issued, to do this please use: J. Wang, H. Li, T. Li, J. Zhang and L. Ling, *Anal. Methods*, 2018, DOI: 10.1039/C8AY01625A.



ROYAL SOCIETY OF CHEMISTRY

rsc.li/methods

View Article Online
DOI: 10.1039/C8AY01625A

Journal Name

COMMUNICATION

An efficient template-independent polymerase chain displacement reaction for the detection of *Salmonella Typhimurium*

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Jing Wang,[†1] Haigang Li[†1], Tingting Li[1], Ji Zhang*[2] and Liansheng Ling *[1]

Here, we incorporated the tailed tandem repeat gene-specific primer technique into PCDR (TTR-PCDR), developing a template-independent PCDR which allows a rapid, one-tube reaction. Moreover, this TTR-PCDR was applied to the efficient detection of *Salmonella Typhimurium*.

DNA are carriers of genetic information and encoders of proteins, playing a key role in life processes that translate the genetic code into proteins to regulate diverse cellular functions[1-3]. The methods to sensitively detect DNA in a convenient and low-cost manner are highly desirable in gene expression analysis, and biomedical studies and clinical diagnosis[4, 5]. Amplification assays, including polymerase chain reaction (PCR)[6, 7], hybridization chain reaction (HCR)[8, 9], rolling-circle amplification (RCA)[10, 11], strand displacement amplification (SDA)[12, 13], loop-mediated amplification (LAMP)[14, 15], have been developed to amplify very low levels of DNA and detect them in high sensitivity. In particular, PCR is an exponential nucleic acid amplification technique, and capable of amplifying longer DNA targets such as kilobase sequence, which is required in the food industry, disease diagnosis and other basic research[16, 17].

Although PCR has become a golden standard technique for DNA amplification due to its high sensitivity[18, 19], the higher efficiency of the PCR-based method is required to yield sufficient products for measurement in a range of applications, such as low-abundance drug-resistant viruses[20], genomic targets in complex samples[21]. To improve the efficiency of the PCR, considerable efforts have been made for producing a detectable product, including nested PCR[22], co-amplification at lower denaturation temperature-PCR (COLD-PCR)[23] and locked nucleic acids technique in PCR primers[24]. However, these PCR methods suffer from some drawbacks, such as tedious operating procedures and the risk of cross-contamination.

Theoretically, conventional PCR-based methods only produce a maximal two-fold increase in amplicon per each amplification cycle. To address this limitation, a unique PCR technique termed "polymerase chain displacement reaction" (PCDR) is first described by Harris et al[25]. PCDR employs multiply nested primers and a DNA polymerase with strand displacement activity[26, 27], enabling a more than two-fold increase of amplification product per each cycle. PCDR has then evolved to be a tandem repeat-based method for sensitive detection of *Candidatus Liberibacter asiaticus*[28]. However, the primers employed in these PCDR-based methods are template-dependent, thereby increasing the difficulty of primer design. Moreover, the total concentration of primers employed in PCDR is restricted to avoid the formation of primer-dimers, like other PCR methods which use multiple primers[29].

Tailed primer strategy is widely used in PCR to increase the specificity, and/or to achieve the multiple step amplification[30-32]. In this study, we incorporated a tailed tandem repeat to PCDR, developing a template-independent and tailed tandem repeat-based polymerase chain displacement reaction (TTR-PCDR). This system contains two pairs of primers: tailed tandem repeat gene-specific primers for initiating PCR at lower concentrations, and universal primers for driving PCR at relatively higher concentrations. In principle, this TTR-PCDR can enable four amplicons after each amplification cycle to improve sensitivity and reduce detection time. Moreover, its feasibility, sensitivity, specificity were further demonstrated by both a template DNA (59 bp) and *Salmonella Typhimurium* (670 bp).

Conventional PCR has a limitation that only maximum two-fold of target DNA is produced per each PCR cycle. Although recent reported PCDR enables more than two amplicons for target DNA per each amplification cycle, it employed template-dependent primers. Hence, we herein introduced a template-independent TTR-PCDR. The system contains two pairs of primers: tailed tandem repeat gene-specific primers (forward

[1]*School of Chemistry, Sun Yat-Sen University, Guangzhou 510275, P. R. China*
[2]*Department of Neurosurgery, State Key Laboratory of Oncology in South China, Sun Yat-sen University Cancer Center, Collaborative Innovation Center for Cancer Medicine, Guangzhou, 510060, P. R. China.*
† Equally contribution to the work.
** Corresponding author. E-mail: cesllsh@mail.sysu.edu.cn; zhangji@sysucc.org.cn.*
Electronic Supplementary Information (ESI) available: See DOI: 10.1039/x0xx00000x

View Article Online
DOI: 10.1039/C8AY01625A

primer F2 and reverse primer R2) and universal primers (forward primer Fu and reverse primer Ru). The tailed tandem repeat primers were designed to contain two repeat binding sites for universal primer, which were attached to the 5' end of the gene-specific sequence, enabling the system to be template-independent. While universal primers were designed to be non-homologous sequences of the template with the similar melting temperature with these of gene-specific sequences. As shown in Fig. 1, the method is based on a two-step PCR process with the same annealing temperature in the reaction. The PCR is first launched by tailed tandem repeat primers at lower concentrations at the initial stage, generating products with tailed tandem repeat primers sequences as the template for next exponential amplification. The universal primers are then complementary to the tailed tandem repeat regions in the template sequences produced in the initial step, for the exponential amplification. The extended strand of the outer site primer leads to the displacement of the extended strand produced from the inner site primer with the help of the thermostable SD DNA polymerase that possesses 5'→3'-strand displacement activity instead of 5'→3'-exonuclease activity[26]. Due to the same annealing temperature for two steps, this TTR-PCDR allows two steps to be performed in one tube. In theory, this PCR can allow four amplicons to be produced after each amplification cycle, thus largely improving sensitivity and reducing the detection time, while the conventional PCR only produce one after each amplification cycle (Fig. S3).

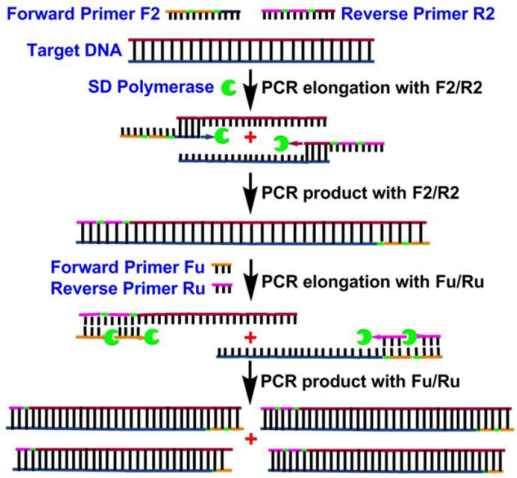


Fig. 1 Schematic diagram of the TTR-PCDR. The TTR-PCDR uses two pairs of primers: one pair of tailed tandem repeat primers (F2 and R2) at low concentrations for the initial PCR cycles, and one pair of universal primers (Fu and Ru) at relatively high concentrations to drive PCR. By utilizing SD DNA polymerase with strand displacement activity, it will yield four PCR products when the universal primers are extended. The amplification is allowed to proceed in one tube including four primers at the same annealing temperature.

The feasibility was verified through the comparison between the TTR-PCDR and conventional PCR with a designed 59 bp DNA as a model (Table 1). The amplification products of the TTR-PCDR and conventional PCR were first analyzed with gel electrophoresis. The TTR-PCDR was performed with two primer pairs F2/R2 and Fu/Ru, where the amplification products are prolonging with at least one Fu sequence and one Ru sequence. As amplification products are prolonged through Fu/Ru with two different binding sites (outer and inner), respectively. PCDR can generate four fragments: one long fragment (135 bp); two middle fragments (116 bp); and one short fragment (97 bp). The gel electrophoresis results confirmed that three bands (one 135 bp fragment, two 116 bp fragments, and one 97 bp fragment) were observed for the TTR-PCDR (Fig. 2A). As a comparison, only one band was observed, when conventional PCR was carried out with primers of F2 and R2 (Fig. 2B). These results showed that the TTR-PCDR allowed to amplifying more targets in one cycle than conventional PCR, displaying its capability of higher amplification efficiency.

The sensitivity of the TTR-PCDR was also examined compared with conventional PCR. 1.0 fM of the model DNA were employed. The TTR-PCDR exhibited a Ct (cycle threshold) value of 20.39, while 27.08 for the conventional PCR, where Ct value means the number of cycles required for the fluorescent signal to achieve 1.0 fM of target DNA (Fig. 2C). This result indicated that the TTR-PCDR assay achieved lower Ct value than that of conventional PCR, revealing that the TTR-PCDR is more sensitive than that of conventional PCR, and it takes less time to amplify the same concentration of a target DNA.

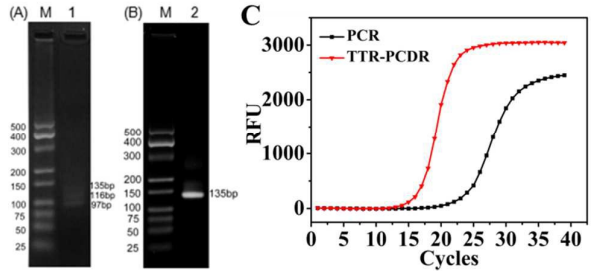


Fig. 2 Comparison of the TTR-PCDR with PCR by gel electrophoresis and real-time quantitative assay. (A) The TTR-PCDR amplification was carried out for 20 cycles using primer pairs F2/R2 and Fu/Ru. (B) PCR amplification was carried out for 35 cycles using primer pairs F2/R2. (C) Real-time quantitative TTR-PCDR was carried out using primer pairs F2/R2 and Fu/Ru, and PCR amplification was carried out using primer F0 and R0. The concentration of target DNA was 1.0 fM.

To achieve higher sensitivity, the conditions involved in the TTR-PCDR were optimized. Firstly, we investigated the different length of connecting bases between the repeat regions for the tailed tandem repeat primers. The results showed that there were no significant differences in sensitivity using the multi-fold primers with different length of connecting bases (Fig. S1). This enables the sequence of the first connecting bases to be designed to hybridize with the detection probe, showing potential in the development of real-time quantitative TTR-PCDR[33]. In this TTR-PCDR, the multi-fold primers are primarily used to enrich the targets and extend the sequence, which is complementary to the universal primers

View Article Online
DOI: 10.1039/C8AY01625A

sequence at the initial PCR stage, while the universal primers are used for the exponential amplification. The concentration of multi-fold primers was set at 30 nM, when the optimization of universal primers was conducted. As shown in Fig. S2, the concentration of universal primers can be reached to 1.0 μM even higher to drive the TTR-PCDR. Therefore, the concentration of universal primers was 1.0 μM in the following experiment.

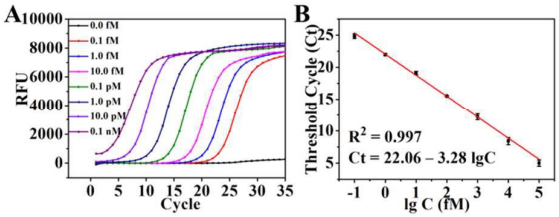


Fig. 3 (A) The TTR-PCDR curves for different copies of target DNA. (B) The linear relationship between Ct values and the logarithm values of target DNA. The TTR-PCDR was carried out using primer pairs F2/R2 and Fu/Ru with 0.1 fM to 0.1 nM of target DNA.

To further demonstrate the sensitivity of the TTR-PCDR assay, different concentrations of target DNA were tested under optimal experimental conditions. As shown in Fig. 3A, the TTR-PCDR curves for target DNA showed excellent regularity over the range from 0.1 fM to 0.1 nM. The Ct values were linearly related to the logarithmic value of the amount of target DNA, the linear regression equation was: Ct = 22.06 – 3.28lg C (R^2 = 0.997) (Fig. 3B). The detection limit for the model DNA reached as low as 0.07 fM. And the TTR-PCDR assay showed an amplification efficiency of 101.7%, according to the equation: Efficiency = $10^{(-1/slope)} - 1$[34, 35]. These results indicated the TTR-PCDR assay with primers F2/R2 and Fu/Ru showed the capability of achieving a more than 2-fold increase in amplicon per amplification cycle.

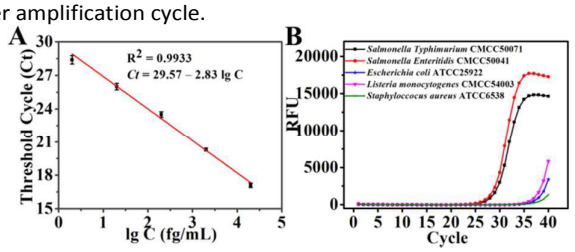


Fig. 4 (A) The standard curve for the detection of *Salmonella* genomic DNA by real-time quantitative TTR-PCDR. The Ct values were plotted against the log of five different ten-fold DNA dilutions (2, 20, 2×10^2, 2×10^3 and 2×10^4 fg/μL). (B) Specificity of the real-time quantitative TTR-PCDR graph for the selective recognition of *Salmonella*. The concentration of *Salmonella* genomic DNA was 2.0 fg/μL, the concentration for non-*Salmonella* strains genomic DNA was 2.0×10^5 fg/μL.

Salmonella leads to high incidences of infectious diseases and is one of the major causes of foodborne disease[36], thus it is significantly important to develop a highly sensitive method for monitoring its levels. We herein employed *Salmonella Typhimurium* CMCC50071 genomic DNA to further evaluate the application of the TTR-PCDR. The real-time quantitative TTR-PCDR was performed with the different concentration of the target genomic DNA. As demonstrated in Fig. 4A, the Ct value decreased with the increase of the concentration of *Salmonella Typhimurium* CMCC50071 genomic DNA over the range from 2.0 to 2×10^4 fg/μL, the linear regression equation between Ct and lg C was Ct = 29.57 – 2.83lg C (C: fg/μL, R^2 = 0.9933). The detection limit for *Salmonella Typhimurium* CMCC50071 genomic DNA was 1.6 fg/μL. Moreover, the TTR-PCDR showed a higher efficiency of 125.6%, which was obtained from the slope of the standard curve (–2.83) according to the equation above.

To demonstrate the specificity of the TTR-PCDR, several genomic DNA from different strains were examined, including *Salmonella Typhimurium* CMCC50071, *Salmonella Enteritidis* CMCC50041, *Staphylococcus aureus* ATCC6538, *Listeria monocytogenes* CMCC54003 and *Escherichia coli* ATCC25922. The results demonstrated (Fig. 4B) that only the fluorescent signal for *Salmonella* strains (*Salmonella Typhimurium* CMCC50071 and *Salmonella Enteritidis* CMCC50041) increased along with increasing cycles, while the signals for 100000-fold non-*Salmonella* strains were negative. The results indicated that this TTR-PCDR is capable of sensitively and specifically detecting *Salmonella*. Taken together, our developed TTR-PCDR is a reliable, highly sensitive, and selective method for the detection of target DNA in one tube.

To demonstrate the feasibility of this TTR-PCDR assay in a real cellular environment, a recovery experiment was conducted in a sensing system containing 5% cell lysis[37]. As shown in Table 1, different concentrations ranging from 1.0 fM to 1.0 pM of *Salmonella Typhimurium* DNA were added into corresponding cell lysis spiked samples, the results displayed that the recovery was in the range of 99.3%–104.0%, with the relative standard deviation (RSD) of 1.2%–3.2%. This demonstrates the potential applicability of the assay for detecting *Salmonella Typhimurium* in the complex biological environment.

Table 1 Recoveries of Salmonella Typhimurium DNA from a spiked sensing system containing 5% cell lysis.

Sample	Added	Found	Recovery (%)	RSD (%)
1	1.0 fM	1.04 fM	104.0	3.2
2	10.0 fM	10.3 fM	103.0	1.4
3	100.0 fM	103.6 fM	103.6	2.7
4	1000.0 fM	993.0 fM	99.3	1.2

Conclusions

In conclusion, we developed a template-independent PCDR, which combined the tailed tandem repeat gene-specific primer technique to PCDR. In this TTR-PCDR, one pair of tailed tandem repeat primers both recognized the target DNA, and worked as the template of the universal primer; another pair of universal primers was employed for the exponential amplification. The TTR-PCDR enables a more than two-fold increase of amplification product in each amplification cycle. Employing an artificial 59 bp DNA as the model, the feasibility of the TTR-PCDR was confirmed by the gel electrophoresis and

View Article Online
DOI: 10.1039/C8AY01625A

conventional PCR. And the assay showed an amplification efficiency of 101.7%. The application of the TTR-PCDR was further verified by the detection of *Salmonella Typhimurium*, the results showed that the TTR-PCDR can detect *Salmonella Typhimurium* genomic DNA ranging from 2.0 to 2×10^4 fg/μL, with a detection limit of 1.6 fg/μL, with an amplification efficiency of 125.6%. These results revealed that our developed TTR-PCDR technique produced more than two copies of target DNA at each cycle. Moreover, the universal primers were independent and can be used for both 59 bp DNA template and *Salmonella Typhimurium*, which simplified the design of primers. We anticipate that the sensitivity of the TTR-PCDR can be further improved by increasing the number of repeat sequence in tailed tandem repeat primers from 2 to 3 or even higher.

Conflicts of interest

There are no conflicts to declare.

Acknowledgments

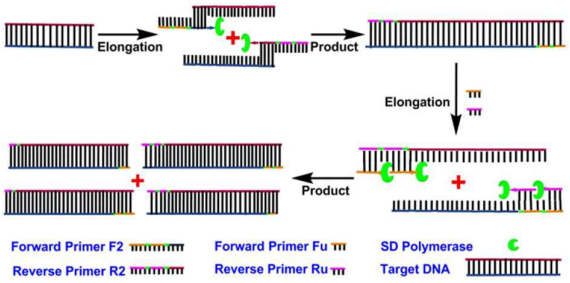
This work was supported by the National Natural Science Foundation of China (Grant No. 21375153). The bacteria used in this work were afforded by Guangdong Open Laboratory of Applied Microbiology from Guangdong Institute of Microbiology (Guangdong, China).

Notes and references

1. C. Zhao, L. Wu, J. Ren and X. Qu, *Chem. Commun.*, 2011, **47**, 5461–5463.
2. S. Lin, W. Gao, Z. Tian, C. Yang, L. Lu, J.-L. Mergny, C.-H. Leung and D.-L. Ma, *Chem. Sci.*, 2015, **6**, 4284–4290.
3. M. Wang, Z. Mao, T.-S. Kang, C.-Y. Wong, J.-L. Mergny, C.-H. Leung and D.-L. Ma, *Chem. Sci.*, 2016, **7**, 2516–2523.
4. X. Feng, L. Liu, Q. Yang and S. Wang, *Chem. Commun.*, 2011, **47**, 5783–5785.
5. Y. Hori, N. Otomura, A. Nishida, M. Nishiura, M. Umeno, I. Suetake and K. Kikuchi, *J. Am. Chem. Soc.*, 2018, **140**, 1686–1690.
6. F. Shen, B. Sun, J. E. Kreutz, E. K. Davydova, W. Du, P. L. Reddy, L. J. Joseph and R. F. Ismagilov, *J. Am. Chem. Soc.*, 2011, **133**, 17705–17712.
7. S. C. Sonkar, D. Sachdev, P. K. Mishra, A. Kumar, P. Mittal and D. Saluja, *Biosens. Bioelectron.*, 2016, **86**, 41–47.
8. J. Huang, Y. Wu, Y. Chen, Z. Zhu, X. Yang, C. J. Yang, K. Wang and W. Tan, *Angew. Chem. Int. Ed.*, 2011, **50**, 401–404.
9. Z. Li, X. Miao, Z. Cheng and P. Wang, *Sensor. Actuat. B-Chem.*, 2017, **243**, 731–737.
10. W. Zhao, M. M. Ali, M. A. Brook and Y. Li, *Angew. Chem. Int. Ed.*, 2008, **47**, 6330–6337.
11. Y. Zhu, H. Wang, L. Wang, J. Zhu and W. Jiang, *ACS Appl. Mater. Inter.*, 2016, **8**, 2573–2581.
12. Y. Zhao, F. Chen, Q. Li, L. Wang and C. Fan, *Chem. Rev.*, 2015, **115**, 12491–12545.
13. A. Chen, G. Gui, Y. Zhuo, Y. Chai, Y. Xiang and R. Yuan, *Anal. Chem.*, 2015, **87**, 6328–6334.
14. X. Fang, Y. Liu, J. Kong and X. Jiang, *Anal. Chem.*, 2010, **82**, 3002–3005.
15. W. Du, M. Lv, J. Li, R. Yu and J. Jiang, *Chem. Commun.*, 2016, **52**, 12721–12724.
16. L. Varadi, J. L. Luo, D. E. Hibbs, J. D. Perry, R. J. Anderson, S. Orenga and P. W. Groundwater, *Chem. Soc. Rev.*, 2017, **46**, 4818–4832.
17. M. Collot, T. K. Fam, P. Ashokkumar, O. Faklaris, T. Galli, L. Danglot and A. S. Klymchenko, *J. Am. Chem. Soc.*, 2018, **140**, 5401–5411.
18. D. R. Almassian, L. M. Cockrell and W. M. Nelson, *Chem. Soc. Rev.*, 2013, **42**, 8769–8798.
19. F. Barragán, P. López-Senín, L. Salassa, S. Betanzos-Lara, A. Habtemariam, V. Moreno, P. J. Sadler and V. Marchán, *J. Am. Chem. Soc.*, 2011, **133**, 14098–14108.
20. J. A. Johnson, J.-F. Li, X. Wei, J. Lipscomb, D. Bennett, A. Brant, M.-e. Cong, T. Spira, R. W. Shafer and W. Heneine, *PLoS One*, 2007, **2**, e638.
21. P. Saingam, B. Li and T. Yan, *J. Microbiol. Method.*, 2018, **149**, 73–79.
22. P. Perrott, G. Smith, Z. Ristovski, R. Harding and M. Hargreaves, *J. Appl. Microbiol.*, 2009, **106**, 1438–1447.
23. C. A. Milbury, J. Li, P. Liu and G. M. Makrigiorgos, *Expert Rev. Mol. Diagn.*, 2011, **11**, 159–169.
24. K. N. Ballantyne, R. A. H. van Oorschot and R. J. Mitchell, *Genomics*, 2008, **91**, 301–305.
25. C. L. Harris, I. J. Sanchez-Vargas, K. E. Olson, L. Alphey and G. Fu, *Biotechniques*, 2013, **54**, 95–99.
26. K. B. Ignatov, E. V. Barsova, A. F. Fradkov, K. A. Blagodatskikh, T. V. Kramarova and V. M. Kramarov, *Biotechniques*, 2014, **57**, 81–87.
27. C. L. Harris, I. J. Sanchez-Vargas, K. E. Olson, L. Alphey and G. Fu, *Biotechniques*, 2013, **54**, 93–97.
28. B. Lou, Y. Song, M. RoyChowdhury, C. Deng, Y. Niu, Q. Fan, Y. Tang and C. Zhou, *Phytopathology*, 2018, **108**, 292–298.
29. T. Stoeck, B. Hayward, G. T. Taylor, R. Varela and S. S. Epstein, *Protist*, 2006, **157**, 31–43.
30. V. Seitz, S. Schaper, A. Droege, D. Lenze, M. Hummel and S. Hennig, *Nucleic Acids Res.*, 2015, **43**, e135.
31. M. Gholami, W. A. Bekele, J. Schondelmaier and R. J. Snowdon, *Plant Biotechnol. J.*, 2012, **10**, 635–645.
32. J. Brownie, S. Shawcross, J. Theaker, D. Whitcombe, R. Ferrie, C. Newton and S. Little, *Nucleic Acids Res.*, 1997, **25**, 3235–3241.
33. X. Li, Y. Huang, Y. Guan, M. Zhao and Y. Li, *Anal. Chem.*, 2006, **78**, 7886–7890.
34. L. Gutierrez, M. Mauriat, S. Guenin, J. Pelloux, J.-F. Lefebvre, R. Louvet, C. Rusterucci, T. Moritz, F. Guerineau, C. Bellini and O. Van Wuytswinkel, *Plant Biotechnol. J.*, 2008, **6**, 609–618.
35. J. L. Yin, N. A. Shackel, A. Zekry, P. H. McGuinness, C. Richards, K. Van Der Putten, G. W. McCaughan, J. M. Eris and G. A. Bishop, *Immunol. Cell Biol.*, 2001, **79**, 213.
36. B. Malorny, E. Paccassoni, P. Fach, C. Bunge, A. Martin and R. Helmuth, *Appl. Environ. Microbiol.*, 2004, **70**, 7046–7052.
37. M. Yu, H. Wang, F. Fu, L. Li, J. Li, G. Li, Y. Song, M. T. Swihart and E. Song, *Anal. Chem.*, 2017, **89**, 4085–4090.

View Article Online
DOI: 10.1039/C8AY01625A

Graphical Abstract



A template-independent and tailed tandem repeat-based polymerase chain displacement reaction (TTR-PCDR) was developed for the efficient detection of *Salmonella Typhimurium*.