Electronic Supplementary Material (ESI) for Chemical Communications.

Supporting Information

Branch migration based selective PCR for DNA mutation enrichment and detection

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Experimental Section

Materials. ThermoPol Reaction Buffer was purchased from New England Biolabs (MA, USA). Taq DNA polymerase and dNTP was purchased from Tiangen Biotech Co. (Beijing, China). DNA strands were synthesized and purified by HPLC (Sangon Biotech Co., China). The sequences of all the DNA that have been used in this work are summarized in Table S1. DNase/RNase free deionized water from Tiangen Biotech Co. was used in all the experiments.

Taq DNA polymerase elongation. To a 200 μL PCR tube, 2 μL of the template (final concentration 250 nM), 2 μL of the primer (final concentration 250 nM), 2 μL of the blocker (final concentration 2 μM), 2 μL of 10× ThermoPol Reaction Buffer,1 μL of Eva Green Dye (20×, Biotium, USA) and 1 μL of Taq DNA polymerase (0.5 U) were added and brought up to a total volume of 20 μL by deionized water (Tiangen Biotech, Beijing). The tube was put into StepOne Plus Real-Time PCR Systems (Applied Biosystems, US) and the fluorescence intensity was measured from at 50 ºC every 10 s. All the standard deviations in this work were calculated by three dependent experiments.

BM PCR procedure for the synthesized target. To a 200 μL PCR tube, 2 μL of 10× ThermolPol Reaction Buffer, 1 μL of dNTPs (final concentration 1 mM), 1 μL of forward primer (final concentration 250 nM), 1 μL of reverse primer (final concentration 250 nM), 1 μL of blocker (final concentration 1 or 2 μM), 1 μL of template (final concentration 0.002 nM, the percentage of MT and WT varied depending on the purpose of the experiment), 0.5 units of Taq DNA Polymerase were added, and the total volume was brought up to 20 μL by deionized water then mixed well. PCR procedure (94 °C for 15 s, 58 °C for 30 s, 72°C for 15 s, 30 cycles) was performed on StepOne Plus Real-Time PCR System. For the two-round BM PCR, the solution after first-round BM PCR was diluted by 50 times with water. Then 1 μL of the diluted solution was taken as the template in the second round of BM PCR and other conditions kept the same as one-round BM PCR.

For High resolution melting (HRM) detection, additional 1 μL of Eva Green Dye (20×) was added before the PCR procedure. After PCR amplification, high resolution melting was conducted on LightCycler 96 Real-Time PCR System (Roche, Switzerland). For Sanger sequencing, after PCR amplification, the sequencing was done by Sangon Biotech Co. For fluorescent DNA probe-based detection, additional 1 μL of the probe (final concentration 500 nM) was added before the PCR procedure. The fluorescent signal was detected at the end of 72°C for 15 s step every cycle.

BM PCR procedure for the target from genomic DNA samples. The genomic DNA was extracted from the haemocytes of an ovarian cancer patient (for *BRCA2* K2729N (8187G>T)) or from the cancer tissue of

an oligoasthenospermia patient (for *MTHFR* A222V (665C>T)) and an endometrial carcinoma patient (for *PTEN* R130Q (389G>A)) using TIANamp Genomic DNA kit (Tiangen Biotech Co.). The extracted genomic sample was then PCR amplified, and the PCR products were sequenced to measure the abundance of *BRCA2* K2729N (8187G>T), *MTHFR* A222V (665C>T) and *PTEN* R130Q (389G>A), respectively. According to the abundance level, the sample was diluted by the specified amount of normal genomic DNAs brought from Promega (WI, USA) to prepare mixed genomic DNA samples with the abundance of *BRCA2* K2729N, *MTHFR* A222V or *PTEN* R130Q at different levels.

To a 200 μL PCR tube, 2 μL of 10× ThermolPol Reaction Buffer, 1 μL of dNTPs (final concentration 1 mM), 1 μL of forward primer (final concentration 250 nM), 1 μL of reverse primer (final concentration 250 nM), 1 μL of mixed genomic DNA samples (20 ng, with different mutation abundance), 0.5 units of Taq DNA Polymerase and 1 μL of Eva Green Dye (20×) were added, and the total volume was brought up to 20 μL by deionized water then mixed well. Conventional real-time PCR procedure (94 °C for 15 s, 58 °C for 30 s, 72°C for 15 s) was performed and stopped after cycle threshold (Ct) + 6 cycles. Then the blocker (final concentration 2 μM) and probe (final concentration 500 nM) was added and BM PCR was performed for 20 cycles.

Discussion

DNA strand design principles in BM PCR

To be more general and practical, we chose KRAS G13D (38G>A) as our modelling target and synthesized its mutant-type and wild-type template DNA, forward primer, reverse primer and blocker strands. The design of primers were the same as that in conventional PCR and the melting temperature (T_m) of the primers were 58.6 ℃ (forward primer-2 with WT or MT, predicted by IDT OligoAnalyzer) and 59.3 ℃ (reverse primer-2 with WT or MT). The mutation lied in the middle of the blocker when designing the sequence of blockers to make the Tm difference between WT and MT large enough. The T_m of blockers were 60.8 ℃ (blocker-2-1nt/WT), 49.1 ℃ (blocker-2-1nt/MT), 63.9 ℃ (blocker-2-4nt/WT) and 55.1 ℃ (blocker-2-4nt/MT). Thus, at the annealing temperature (58 ℃) in PCR procedure, primers will not dissociate from the targets, the blocker will not dissociate from WT and blocker will dissociate from MT.

The key differences or advantages of BM PCR toward competitive blocker PCR

i) The overlap region is quite short in BM PCR and the blocker sequence does not need to displace the primer from the template strand. Researchers do not have to balance the binding efficiencies of blocker/MT, blocker/WT and primer/MT to accomplish selective displacement which is involved with both thermodynamics and kinetics processes as in competitive Blocker PCR. In BM PCR, researchers only need to care about the thermodynamics process and the sequence design for BM PCR was quite simple. We

just followed the rules for conventional PCR to design the primers and thermal profiles. The design of BM blocker followed the rules that it overlaps 1~4 nt with the reverse primer, the Tm of blocker/MT is smaller than the annealing temperature in PCR process and Tm of blocker/WT is bigger than the annealing temperature, which is very simple and could deal with some tough conditions. For example, if the ΔTm of blocker/WT and blocker/MT is small (like ΔTm=2 ℃), we can change the sequence of BM blocker to make the Tm of blocker/MT around the annealing temperature. Though this design will lose about 50% of the elongation efficiency of MT, the efficiency of WT changes little. The Tm of primer can be set at 5 ℃ higher than the annealing temperature as in conventional PCR. Because even if the Tm of primer/WT is higher than the Tm of blocker/WT, the primer is not likely to dissociate the BM blocker off the WT. While for competitive blocker PCR, as it should follow the rule that Tm(blocker/WT) > Tm(primer/WT) > annealing temperature > Tm(blocker/MT) and Tm(blocker/WT)- Tm(blocker/MT)=2 ℃, the sequence design is hard, the annealing temperature should be carefully chosen and the PCR efficiency will be affected for both WT and MT. Thus, the mutation enrichment of competitive Blocker PCR in this condition would be a tough job.

ii) Due to the short length of the overlap region, there leaves a considerably larger space for designing a fluorescent probe to selectively hybridize with the MT rather than WT, which is not easy for competitive Block PCR. For example, Tm of a 16-nt length blocker with 50% GC content is about 60 ^{o}C (evaluated by OligoAnalyzer of Integrated DNA Technologies). The general overlap region between primer and blocker is around 8-nt in competitive Blocker PCR and there leaves 8-nt for the blocker to overlap with the probe. As some of the polymerases need 3-nt or larger gap between primer and probe to conduct primer elongation, the overlap region of blocker and probe will be less than 5-nt. As the mutation site should be within this region, even it is set in the middle, it would be the third nucleotide from the 3' end of the blocker and the third nucleotide from the 5' end of the probe and the mismatch recognition ability would be affected if the mutation site is by the end of blocker and probe. Also, the dissociation of primer, blocker and probe considering thermodynamics and kinetics processes should be carefully designed. As for BM PCR, the overlap region between primer and BM blocker is 4-nt and there leaves 9-nt for the blocker to overlap with the probe considering the gap for polymerase elongation. Thus, the mutation site could be set away from the ends of the blocker and probe and the mismatch recognition ability would hardly be affected. The additional probe would cooperate with BM blocker to further enhance the method's discrimination ability toward MT and WT and combine the enrichment step and subsequent analysis step into one. Actually, the blocker strand herein had two functions: it would compete with primer to enrich

the abundance of mutant-type DNA; it would also compete with the probe to enhance the discrimination power. Overall, the combination of three components enables the rapid and sensitive detection of low-abundance point mutations.

Table S1. DNA sequences used in this work.

Name	Sequences （5'->3'）
1. DNA sequences for Taq DNA polymerase elongation	
template-1-WT	AACTAGTAGTGCAGATACCCAAAAAGTGGCCATTATTGAACTTACAGATGGGTGGT ATGCTGTTAAGGCCCAGTTAGATCCTCCCCTCT
blocker-1-1nt a	***G***GGCCTTAACAGCATACCAATGT
blocker-1-2nt	***TG***GGCCTTAACAGCATACCAATGT
blocker-1-3nt	***CTG***GGCCTTAACAGCATACCAATGT
blocker-1-4nt	***ACTG***GGCCTTAACAGCATACCAATGT
primer-1	AGAGGGGAGGATCTA***ACTG***
2. DNA sequences for KRAS G13D (38G>A) enrichment and detection	
template-2-WT	CCTGCTGAAAATGACTGAATATAAACTTGTGGTAGTTGGAGCTGGTGGCGTAGGCA AGAGTGCCTTGACGATAC
template-2-MT [b]	CCTGCTGAAAATGACTGAATATAAACTTGTGGTAGTTGGAGCTGGTGACGTAGGCA AGAGTGCCTTGACGATAC
blocker-2-1nt	***T***GCCTACGCCACCAGTCTATGTC
blocker-2-4nt	***TCTT***GCCTACGCCACCAGTCTATGTC
forward primer-2	CCTGCTGAAAATGACTGAATA
reverse primer-2	GTATCGTCAAGGCAC***TCTT***
probe-2 [c]	FAM-CCTACGTCACCAGCTC-BHQ1
3. DNA sequences for *BRCA2* K2729N (8187G>T) enrichment and detection	
template-3-WT	AACTAGTAGTGCAGATACCCAAAAAGTGGCCATTATTGAACTTACAGATGGGTGGT ATGCTGTTAAGGCCCAGTTAGATCCTCCCCTCT
template-3-MT	AACTAGTAGTGCAGATACCCAAAAAGTGGCCATTATTGAACTTACAGATGGGTGGT

	ATGCTGTTAATGCCCAGTTAGATCCTCCCCTCT
blocker-3	***ACTG***GGCCTTAACAGCATACCAATGT
forward primer-3	AACTAGTAGTGCAGATACCCAA
reverse primer-3	AGAGGGGAGGATCTA***ACTG***
probe-3	FAM-GGCATTAACAGCATACCACC-BHQ1
4. DNA sequences for *MTHFR* A222V (665C>T) enrichment and detection	
template-4-WT	TTACCCCAAAGGCCACCCCGAAGCAGGGAGCTTTGAGGCTGACCTGAAGCACTTGA
	AGGAGAAGGTGTCTGCGGGAGCCGATTTCATCATCACGCAGCTTTTC
template-4-MT	TTACCCCAAAGGCCACCCCGAAGCAGGGAGCTTTGAGGCTGACCTGAAGCACTTGA
	AGGAGAAGGTGTCTGCGGGAGTCGATTTCATCATCACGCAGCTTTTC
blocker-4	***AATC***GGCTCCCGCAGACACAAAA
forward primer-4	TTACCCCAAAGGCCACCCCGAA
reverse primer-4	GAAAAGCTGCGTGATGATGA***AATC***
5. DNA sequences for *PTEN* R130Q (389G>A) enrichment and detection	
template-5-WT	CCTTTTGTGAAGATCTTGACCAATGGCTAAGTGAAGATGACAATCATGTTGCAGCAA
	TTCACTGTAAAGCTGGAAAGGGACGAACTGGTGTAATGATATGTGCATATTTATTA
	CAT
template-5-MT	CCTTTTGTGAAGATCTTGACCAATGGCTAAGTGAAGATGACAATCATGTTGCAGCAA
	TTCACTGTAAAGCTGGAAAGGGACAAACTGGTGTAATGATATGTGCATATTTATTAC
	AT
blocker-5	***TACA***CCAGTTCGTCCCTTTCCTAAA
forward primer-5	CCTTTTGTGAAGATCTTGACCAAT
reverse primer-5	ATGTAATAAATATGCACATATCAT***TACA***
probe-5	FAM-CAGTTTGTCCCTTTCCAGC-BHQ1

[a] The underlined bases showed the complementary region of the blocker and the template. The bases shown in bold and italic in the DNA sequences indicate that they are overlapped between the blocker and the primer.

[b] The point mutations in the sequences of KRAS, *BRCA2*, *MTHFR*, *PTEN* are indicated in red.

[c] The bases shown in blue in the probe for KRAS, *BRCA2* and *PTEN* indicate that they are complementary with the mutation site in the corresponding genes.

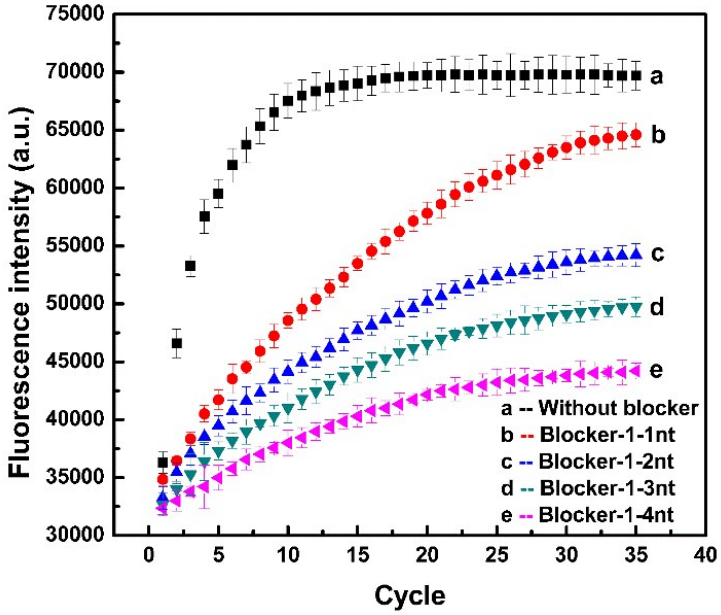

Figure S1 The fluorescence intensity response to Taq DNA polymerase elongation without or with branch migration blockers.

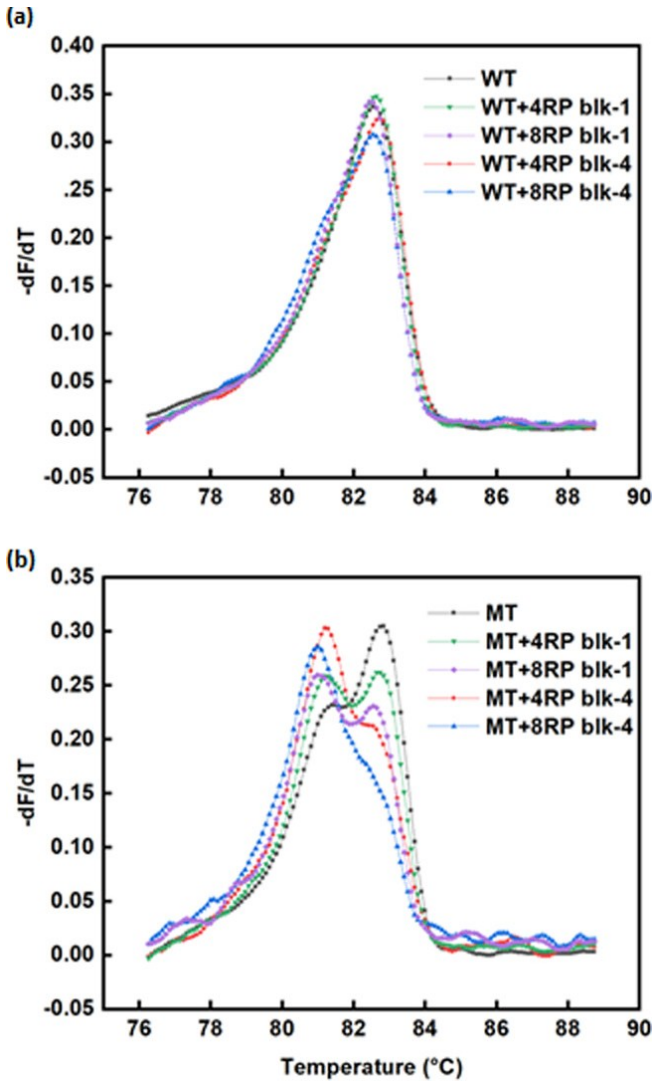

Figure S2. HRM curves of PCR with different blockers at different concentrations. (a) WT target (b) MT target. 4RP and 8RP mean that the concentration of the blocker is 4 times and 8 times to that of the reverse primer, respectively. Blk-1 is short for blocker-2-1nt and blk-4 is short for blocker-2-4nt.

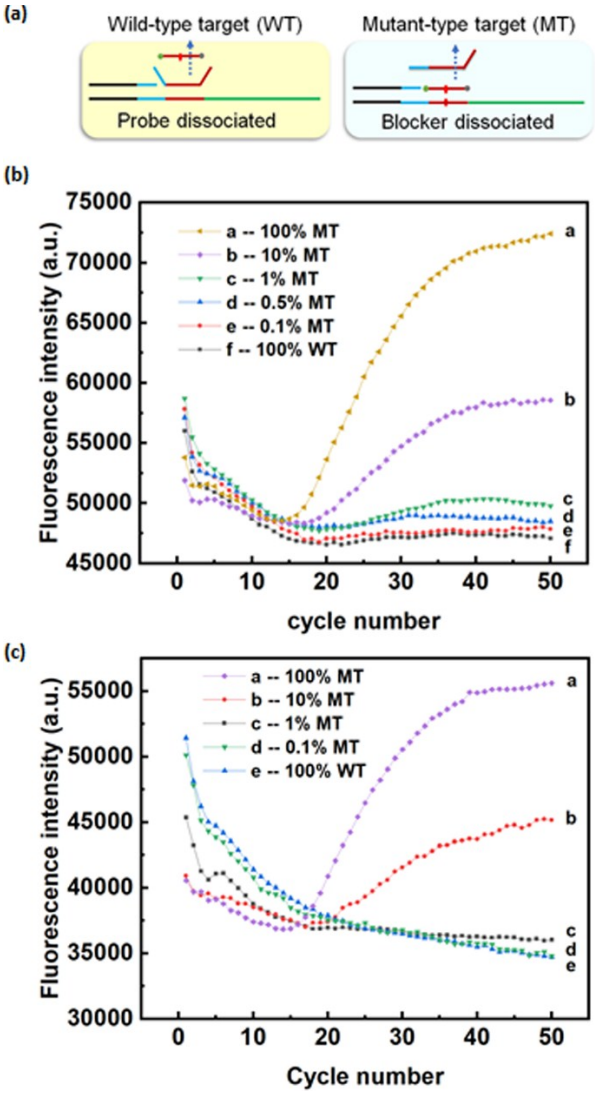

Figure S3. (a) The schematic depiction of the competition of probe and blocker for WT and MT target. (b-c) Fluorescence intensity response of *KRAS* G13D (38G>A) target at different mutation abundances in (b) BM PCR or (c) conventional PCR with fluorescent DNA probe.

Table S2. The Detection of *PTEN* R130Q (389G>A) mutation in Endometrial Carcinoma and Hyperplasia.

Case No.	Sanger Sequencing (%)	BM PCR (%)	Histologic Diagnosis
1	N.D.	N.D.	Endometrioid carcinoma
2	40% ± 2%	42% ± 2%	Endometrioid carcinoma
3	N.D.	N.D.	Endometrioid carcinoma
4	N.D.	N.D.	Simple hyperplasia without atypia
5	50% ± 3%	55% ± 3%	Endometrioid carcinoma
6	N.D.	N.D.	Endometrioid carcinoma
7	N.D.	N.D.	Complex hyperplasia with atypia
8	44% ± 2%	45% ± 2%	Endometrioid carcinoma
9	N.D.	N.D.	Endometrioid carcinoma
10	N.D.	N.D.	Endometrioid carcinoma

Table S3. Comparation of BM PCR with Blocker PCR.

PCR type	Reference [a]	Blocker type	Overlap region	Primer dissociate from WT	Blocker dissociate from MT	Limit of detection of mutation abundance
Conventional	1	PNA	0	No	Yes	0.1%[b]
Blocker PCR	2	LNA	0	No	Yes	1%
Competitive Blocker PCR	3	DNA, 3'-end modification	3~17 nt	Yes	Yes	1%~0.001%
	4	DNA, 3'-end overhang	9 nt	Yes	Yes	0.03%~0.005%[c]
	5	DNA, 3'-end overhang	6~14 nt	Yes	Yes	0.1%
BM PCR	This work	DNA, 3'-end overhang	1~4 nt	No	Yes	0.1%

[a] References

1. Behn M, Thiede C, Neubauer A, Pankow W, Schuermann M. Facilitated detection of oncogene mutations from exfoliated tissue material by a pna-mediated 'enriched pcr' protocol. J Pathol 2000;190:69-75.
2. Dominguez PL, Kolodney MS. Wild-type blocking polymerase chain reaction for detection of single nucleotide minority mutations from clinical specimens. Oncogene 2005;24:6830-4.
3. Lee ST, Kim JY, Kown MJ, Kim SW, Chun JH, Ahn MJ, et al. Mutant enrichment with 3 '-modified oligonucleotides a practical pcr method for detecting trace mutant dnas. J Mol Diagn 2011;13:657-68.

4. Xiao XJ, Xu AQ, Zhai JQ, Zhao MP. Combination of a modified block pcr and endonuclease iv-based signal amplification system for ultra-sensitive detection of low-abundance point mutations. Methods 2013;64:255-9.
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[b] LOD was achieved by Blocker PCR coupled with PCR-RFLP (restriction fragment length polymorphism).

[c] LOD was achieved by Blocker PCR coupled with Endo IV-based signal amplification system. The mutation enrichment fold is 4~6 times by Blocker PCR process.