Nucleic Acids Research, 2005, Vol. 33, No. 14 ***e127***
doi:10.1093/nar/gni120

Headloop suppression PCR and its application to selective amplification of methylated DNA sequences

Keith N. Rand, Thu Ho, Wenjia Qu[1], Susan M. Mitchell, Rose White, Susan J. Clark[1] and Peter L. Molloy*

CSIRO Molecular and Health Technologies, PO Box 184, North Ryde NSW 1670, Australia and [1]The Garvan Institute for Medical Research, 384 Victoria Street, Darlinghurst NSW 2010, Australia

Received May 19, 2005; Revised July 12, 2005; Accepted July 14, 2005

ABSTRACT

Selective amplification in PCR is principally determined by the sequence of the primers and the temperature of the annealing step. We have developed a new PCR technique for distinguishing related sequences in which additional selectivity is dependent on sequences within the amplicon. A 5′ extension is included in one (or both) primer(s) that corresponds to sequences within one of the related amplicons. After copying and incorporation into the PCR product this sequence is then able to loop back, anneal to the internal sequences and prime to form a hairpin structure—this structure is then refractory to further amplification. Thus, amplification of sequences containing a perfect match to the 5′ extension is suppressed while amplification of sequences containing mismatches or lacking the sequence is unaffected. We have applied Headloop PCR to DNA that had been bisulphite-treated for the selective amplification of methylated sequences of the human *GSTP1* gene in the presence of up to a 10^5-fold excess of unmethylated sequences. Headloop PCR has a potential for clinical application in the detection of differently methylated DNAs following bisulphite treatment as well as for selective amplification of sequence variants or mutants in the presence of an excess of closely related DNA sequences.

INTRODUCTION

Specificity in PCR amplification of DNA is principally determined by the sequence of the primers in combination with the temperature at which the annealing step is conducted. For closely related sequences, additional approaches targeted to sequences between the primers have been incorporated to increase the selectivity of amplification. For example, where a sequence difference corresponds to a restriction enzyme site, restriction enzyme digests can be used to cut an unwanted sequence and prevent its amplification. Another method of suppressing amplification is the use of oligonucleotides or peptide nucleic acid (PNA) molecules that anneal to one of the DNA strands, within the region to be amplified and/or overlapping the binding site of one of the primers; thus, preventing initiation or elongation of DNA synthesis (1–4). Such oligonucleotides are designed to preferentially anneal with and suppress amplification of one of two related sequences. This method has recently been applied to the selective amplification of methylated DNA sequences after treatment with bisulphite (5).

We describe below a novel method termed Headloop PCR for selectively suppressing the amplification of one or more closely related sequences while using PCR primers that can prime and extend on both the target and the suppressed sequences. In this method, amplification of selected sequences is prevented through a 5′ extension on one (or both) of the primers. After the 5′ extension is incorporated into the PCR product by being copied by polymerase, the new region ('head') has the potential of causing internal priming by looping back and hybridizing to an internal region of the unwanted product. The internal priming causes the production of a hairpin loop structure that is a poor substrate for further amplification, limiting amplification of the unwanted species.

Headloop PCR is well suited to situations in which the desired target for amplification is present as a rare sequence in a large excess of a closely related sequence. We have applied this technology for the selective amplification of methylated DNA sequences from bisulphite-treated DNA. Following bisulphite treatment, cytosines are converted to uracil and then to thymine during PCR, while methylated cytosines, predominantly present at CpG sites in mammalian DNA, are refractory to conversion and remain as cytosines following PCR (6). By designing Headloop primers that cause

*To whom correspondence should be addressed. Tel: +61 2 9490 5168; Fax: +61 2 9490 5010; Email: peter.molloy@csiro.au

looping back and extension on sequences derived from DNA not methylated at CpG sites it is possible to selectively suppress amplification of unmethylated sequences. Although emphasis here is on use in the methylation field, utility is not limited to this area and we show an example of how it can be used to improve specificity of the 16S rRNA gene detection of bacterial species.

MATERIALS AND METHODS

DNAs, oligonucleotide primers and probes

The sequences of primers are shown in Table 1; cartridge purified oligonucleotides were purchased from Sigma. Fully CpG-methylated genomic DNA (Chemicon) or white blood cell DNA (Roche Diagnostics) were treated with sodium bisulphite as described previously (7). For the *GSTP1* promoter region a pair of plasmids (plasmids U and M) containing inserts derived from bisulphite treatment and PCR amplification of the region between positions 854 and 1297 (GenBank accession no. M24485) were used as substrates for PCR. In Plasmid U, representing unmethylated DNA, all Cs in the original sequence had been converted to Ts. In plasmid M, representing methylated DNA, all Cs except those at CpG positions that correspond to methylated Cs had been converted to T. PCR amplification of the M + U plasmid mixture was performed with the base primer F2, or the Headloop primer LUH F2 or the control primer CLUR F2 in conjunction with the reverse primer R1T. Note that this primer has a short tail to provide a higher annealing temperature after initial incorporation—this is not relevant to the Headloop mechanism. Taqman probes for the promoter region were as follows: PBRM (specific for methylated bisulphite-converted DNA: FAM-TTGCGTATATTTCGTTGCGGTTTTTTTTT-TAMRA, where FAM = carboxyfluorescein and TAMRA = carboxytetramethylrhodamine) and PBRU (specific for unmethylated bisulphite-converted DNA: TET-TTGTGTATATTTTGTTG-TGGTTTTTTTTTTGTTG-TAMRA, where TET = tetrachlorofluorescein).

For the *GSTP1* intragenic region (top strand) Headloop primers HLint5-10 or HLint5-10Ni were used in conjunction with the forward primer F52A. PCRs were performed using plasmid clones of methylated and unmethylated top strand sequences as for the promoter region above. For the *GSTP1* intragenic region (bottom strand) Headloop primer Hlint5-10B1 was used in conjunction with reverse primer GSTBintR2 and Taqman probe GSTBMC3, 5′-VIC-TCGCCGCCGC-AAT-mgbnfg.

For 16S ribosomal RNA genes DNA isolated from *Escherichia coli*, *Sulfobacillus acidophilus* and *Sulfobacillus thermosulfidooxidans* was used in PCR (kindly provided by Dr Chun Liu). The reverse primer NR-R1 was used with the forward primers NR-F1i or NR-F1i with Headloop extensions SAHL, EHL48 or EHL2a. The amplicon covers the region starting at base 9609 of the *E.coli* 16S rRNA gene (GenBank accession no. AE000452).

Headloop PCRs

Real-time PCR was carried out using an ABI PRISM® 7700 Sequence Detection System. Standard conditions for hot start PCR (in 25 µl) were 1× platinum *Taq* buffer [20 mM Tris–HCl (pH 8.4) and 50 mM KCl], 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 200 nM primers and 0.75 U platinum *Taq* DNA polymerase from Invitrogen. Taqman probes were used at 50 nM (PRBM) or 100 nM (PBRU).

For the *GSTP1* promoter Headloop PCR, cycling conditions were as follows: 95°C for 120 s, then 50 cycles at 95°C for 15 s, 60°C for 60 s. For *GSTP1* intragenic (top strand) PCR cycling conditions were as follows: 95°C for 120 s, then 50 cycles at 95°C for 15 s, 60°C for 60 s. For *GSTP1* intragenic (bottom strand) PCR cycling conditions were as follows: 95°C for

Table 1. Primers

Target	Head sequence: 5′–3′	Priming sequence: 5′–3′
GST promoter		
F2		GGTTtTAGGGAATTTttttt
LUHF2	**a**C**a**CC**a**aaACATC**a**C**a**aaa	GGTTtTAGGGAATTTttttt
CLURF2	CCATCAACAAAAAACACACA	GGTTtTAGGGAATTTttttt
R1T		CACCTTTCCCAaaTCCCCAa
GST intragenic		
HLint5-10	**t**G**t**G**t**GGTT**t**G**t**GTTT**t**G	CCCCATaCTaaaAaCTCTaAaCCCCAT
HLint5-10Ni	**t**G**t**G**t**GGTT**t**G**t**GTTT**t**G	CTCTaAaCCCCATCCCCIaaa
F52A		GGGAttAtttTTATAAGGtTAGGAGGt
GST intragenic bottom strand		
HLBint5-10	**a**C**a**C**a**ACCC**a**C**a**TCCCC**a**AA	TGtTGGGAGtTtTGAGtttATttt
BintR2i		aAaaCCICIAaaCCTTCICTaaAaTTTC
MSP Intragenic		
Msp102		**C**GtAGTtTT**C**GttAttAGTGAGTA**C**G**C**
Msp104		**G**AaaTaaaC**G**AaaAaCCCTaCC**G**a
16S rRNA		
NR-F1i		GTAGTCCIIGCIITAAACGAT
SAHL	CGACACCTCGTATCCAT	GTAGTCCIIGCIITAAACGAT
EHL2a	ACAACCTCCAAGTCGACAT	GTAGTCCIIGCIITAAACGAT
EHL48	GACTTAACGCGTTAGCTC	GTAGTCCIIGCIITAAACGAT
NR-R1		GACTTAACGCGTTAGCTC

For all primers a lower case t or a corresponds to a U or T that results from the bisulphite conversion of a C in the original DNA, while boldface bases correspond to the positions of Cs at CpG sites. The underlined A in F52A is a mismatch corresponding to the T or C expected at CpG-4. I stands for inosine, introduced as a mismatch at some CpG sites in primers for bisulphite-treated DNA or at variable positions in the 16S rRNA genes.

120 s, 5 cycles (95°C for 15 s, 60°C for 60 s), then 60 cycles (88°C for 15 s, 60°C for 60 s).

For 16S rRNA PCR cycling conditions were as follows: 95°C for 60 s, then 40 cycles at 95°C for 30 s, 58°C for 30 s and 72°C for 30 s.

When SYBR Green was included either during the reaction or added subsequently for melting curve analysis, it was added at 1/125 000 dilution of the solution obtained from Molecular Probes, Inc. Variations in reaction conditions are indicated in the text.

Methylation-specific PCR

Methylation-specific PCR (MSP) of the *GSTP1* intragenic region was carried out using primers Msp102 and Msp104 (Table 1) and fluorescent probe PRBCS3, 5′-VIC-CCCATA-CTAAAAACTCAAACCCCATCCC-TAMRA, specific for bisulphite-converted DNA. After initial denaturation at 95°C for 120 s. cycling conditions were as follows: 5 cycles (95°C for 15 s, 65°C for 60 s), then 50 cycles (87°C for 15 s, 65°C for 60 s).

RESULTS

Principle of Headloop PCR

The mechanism of action of Headloop PCR is outlined in Figure 1. The two sequences A and B are closely related, but differ in the boxed regions. The reverse primer R matches both sequences exactly, as does the black arrow region of the forward primer, F. The Headloop primer is shown as the forward primer—it comprises a standard forward PCR primer with homology to the target sequences to be amplified with a 5′ extension (coloured red) that is complementary to a region within Sequence A. When the reverse primer is extended on the product of first round synthesis with the forward primer, this extension is incorporated into the second strand product. After denaturation the incorporated 3′ tail extension is able to loop back and anneal to its complementary region, and be extended to form a hairpin structure. Since intramolecular annealing is known to be very rapid this is expected to re-anneal after denaturation and no longer provide a template for further amplification. However, in the case of Sequence B, mismatch(es) to the equivalent region limit self-priming to form a hairpin and the DNA is able to undergo further amplification with the forward and reverse primers. If the forward primer is chosen as the base for a Headloop primer, the sequence of the 5′ extension on the primer is the reverse complement of the target top strand sequence. If the Headloop primer is based on the reverse primer the extension will comprise the sequence of the target region as directly read from the top strand.

We have applied the principle of Headloop suppression PCR for selective amplification of methylated DNA sequences following bisulphite conversion of DNA. Treatment with sodium bisulphite converts cytosines to uracils (thymines after PCR). Methylated cytosines are unreactive, however, and remain as cytosines following the bisulphite reaction

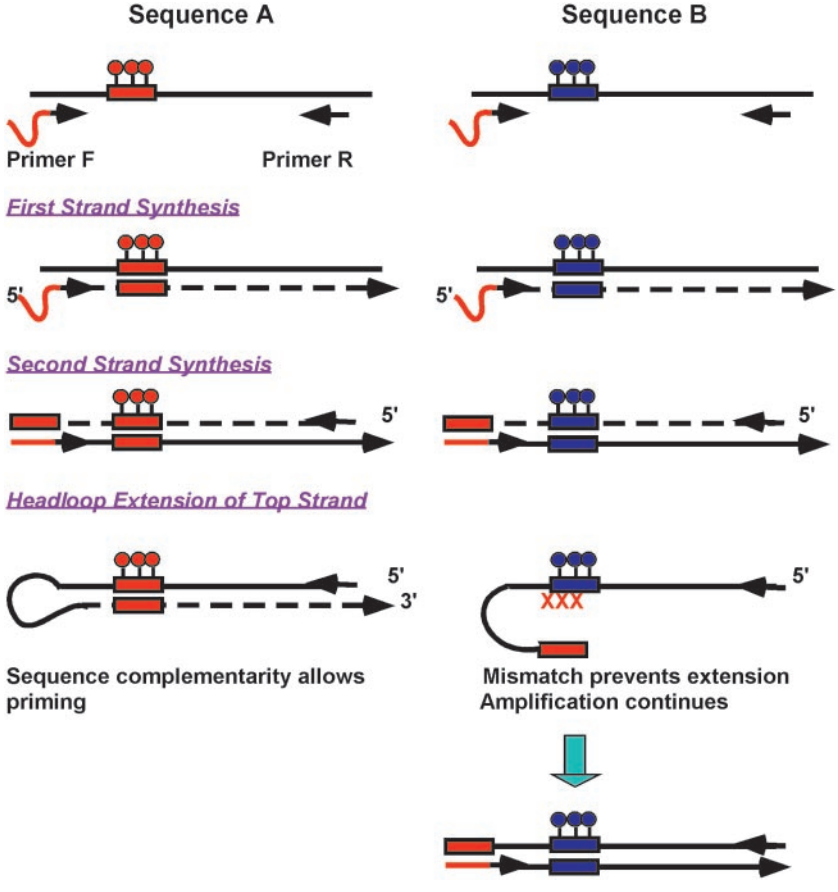


Figure 1. Principle of Headloop suppression PCR.

and subsequent PCR. Thus, the treatment of two DNA sequences that are identical except that one is methylated at specific cytosines while the other is not, leads to molecules that are identical except for the presence of Cs rather than Us at the specific sites that were methylated in the original DNA. It is therefore possible to design the head region of the primer to selectively suppress amplification of sequences derived from unmethylated DNA; thus, allowing the detectable amplification of low amounts of methylated DNA that would otherwise have been out competed. Application to selective amplification of methylated sequences of the *GSTP1* promoter is shown below.

Selective amplification of methylated *GSTP1* sequences

The human *GSTP1* gene promoter is commonly methylated at CpG sites in prostate cancer (8) and genomic sequencing has indicated extensive methylation across the promoter and intragenic regions (9,10). We have previously cloned from bisulphite-treated DNA *GSTP1* promoter sequences corresponding to DNA that was originally either fully methylated at all CpG sites from CpG sites −41 to +10 or fully unmethylated. A Headloop PCR was developed to selectively amplify methylated DNA sequences from within this region. The base primers F2 and R1T are specific for amplification of bisulphite-treated DNA from the *GSTP1* promoter region (both cover regions where there are a number of Cs, including those corresponding to the terminal, priming bases) but have no selectivity for differential priming on methylated or unmethylated DNA. A Headloop extension, 5′-**a**C**a**CC**a**aaA-CATC**a**C**a**aaa to the forward primer F2 was designed so that after its incorporation into the PCR product it would loop back, anneal to the target region of the unmethylated DNA as indicated in Figure 2 (primer LUH F2), priming to form an extended

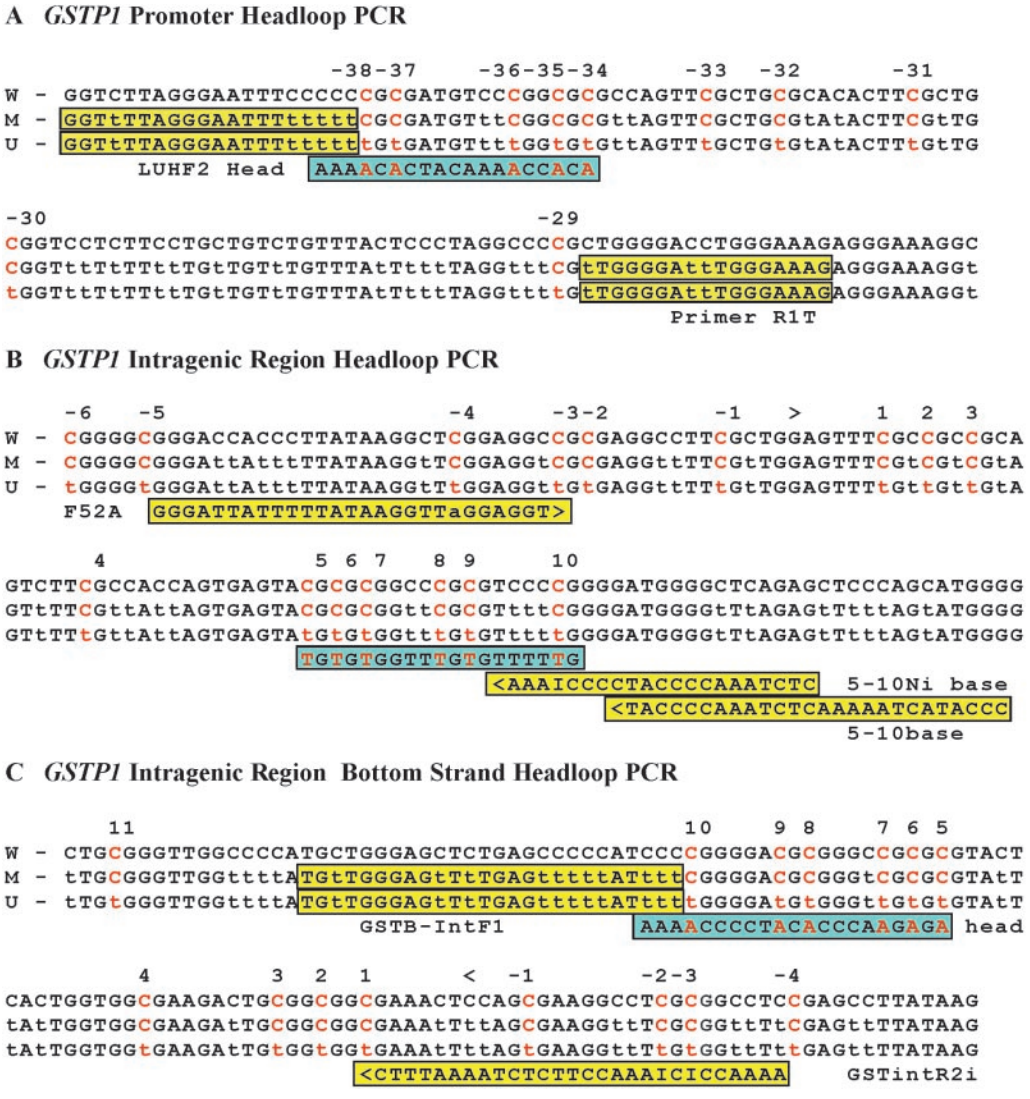


Figure 2. Target regions for Headloop PCR on *GSTP1* gene. Sequences are shown for the promoter region of the *GSTP1* gene (**A**) and the intragenic region, top strand (**B**) or bottom strand (**C**). For each the unmodified sequence is shown (W) and below it the expected sequences after bisulphite treatment if the DNA were methylated (M) or unmethylated (U). Numbering of CpG sites relative to the transcription start site is shown above the sequences. Primer regions are boxed and shaded yellow. Head regions are boxed and shaded blue. T residues resulting from conversion of a C are shown as lower case (t); I = inosine. Cs or Ts at the position of CpG sites and the discriminatory A bases in the head sequence are highlighted in red.

hairpin molecule. The target region includes five CpG sites, with the underlined base corresponding to CpG site −34 defining the 3′ priming base for Headloop extension to form a hairpin structure. The primer CLUR is a control primer in which the Headloop extension sequence has been jumbled.

Suppression of amplification of unmethylated sequences was tested by performing real-time PCR with separate probes for the detection of methylated and unmethylated sequences on plasmid mixtures containing 10^7 unmethylated molecules and 10^3 methylated molecules. In the absence of the Headloop extension, or with a control randomized extension, amplification yields only unmethylated PCR products (Figure 3). Presence of the Headloop (primer LUH F2) allows efficient amplification of methylated sequences, with essentially complete suppression of amplification of unmethylated sequences that are present in 10^4-fold excess.

Factors affecting Headloop PCR efficiency

We have evaluated a number of reaction parameters in order to identify those important in allowing selective amplification. Among factors evaluated, the level of free Mg^{2+} ions was particularly critical. The effect of varying the concentration of Mg^{2+} ions is shown in Figure 4 using two Headloop primers targeted to sequences just downstream from the *GSTP1* transcription start site (Figure 4). Amplifications using either HLint5-10Ni or HLint5-10 were carried out in the presence of 1.1, 1.3 or 1.5 mM $MgCl_2$. The proportion of methylated and unmethylated amplicons in the final product was estimated

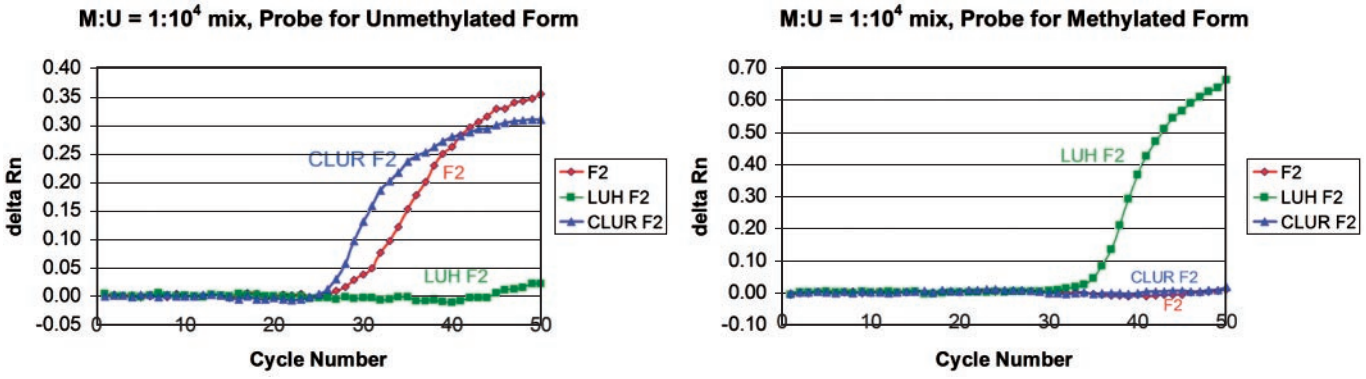


Figure 3. Selective amplification of methylated *GSTP1* promoter sequences. PCR amplification was done on a mixture of methylated (10^3 copies) and unmethylated (10^7 copies) DNA using base primer F2 (red line), Headloop primer LUH F2 (green line) or control primer CLUR F2 (blue line). The left panel shows detection with the probe specific for unmethylated DNA and the right panel the probe for methylated DNA.

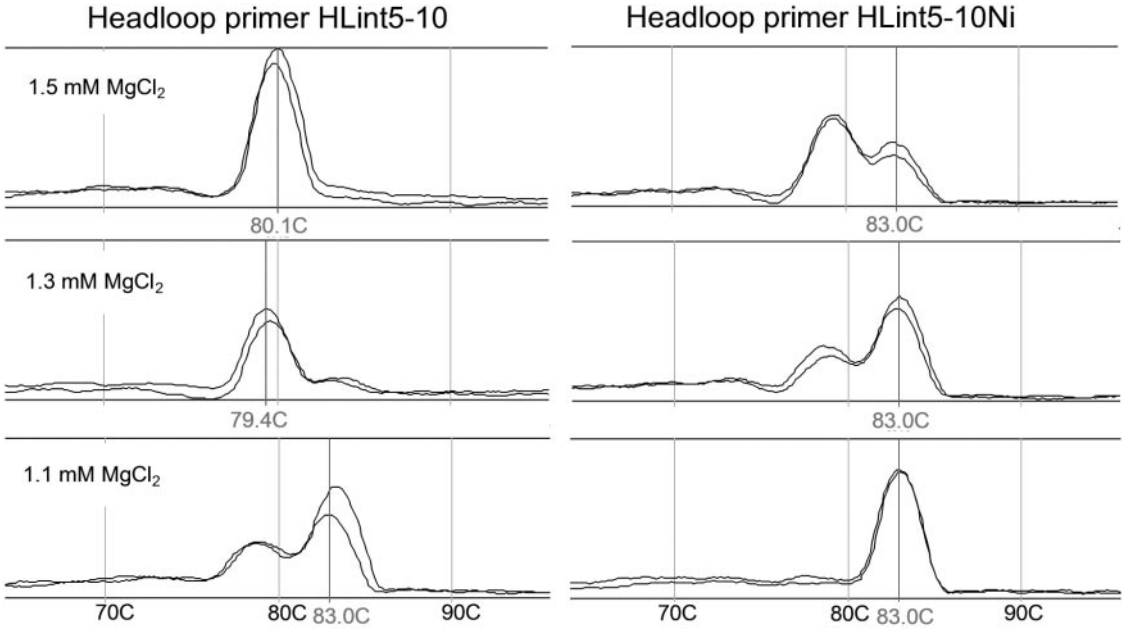


Figure 4. Effect of magnesium ion concentration on Headloop PCR. Headloop PCR on the intragenic region of the *GSTP1* gene was performed in a mixture of methylated (10^3 copies) and unmethylated (10^7 copies) plasmids using either the Hlint5-10 or the Hlint5-10Ni primer under standard conditions except that the concentration of $MgCl_2$ was varied as shown. Denaturation profiles of the amplification products are shown. The lower T_m peak, 79–80°C, corresponds to the unmethylated amplicon and that at 83–84°C to the methylated amplicon (verified previously using individual plasmids).

from melting curves. In both cases there was a concentration-dependent improvement in selectivity with decreasing levels of $MgCl_2$. This effect has been seen with a number of different Headloop primers (data not shown). Equivalent effects are seen when the concentration of free Mg^{2+} ions is lowered by the addition of EDTA or extra nucleotides. Optimal Headloop selectivity is consistently seen at the lowest Mg^{2+} levels used that allow PCR amplification of the target sequences. In most cases this has corresponded to a level of free Mg^{2+} ions of 0.3 mM.

The selectivity of Headloop PCR depends on competition between competing intermolecular and intramolecular processes. The rate and extent of intramolecular hybridization of the head region to its target site and subsequent priming to form hairpin structures is dependent on the degree of match with the target sequence and also on the annealing conditions, including temperature. Intermolecular hybridization of primer and template leading to PCR amplification is similarly dependent on the annealing temperature, but will be prevented or limited if a hairpin structure has already formed. The effect of annealing temperature on a Headloop PCR is shown in Figure 5. Also shown are the effects of the addition of betaine, a reagent that weakens the interaction of G–C base pairs (11). Mixtures of unmethylated (10^7) and methylated (10^3) plasmids were amplified using the Headloop primer HLint5-10 with the annealing step performed at a range of temperatures. The selectivity of the reaction was seen to be dependent on the annealing temperature, the maximum proportion of methylated amplicon being produced at 62.5°C for this Headloop PCR. Addition of betaine was seen to substantially improve the ratio of methylated product at lower annealing temperatures, but its effect was minimal at higher temperatures. It might also be expected that intramolecular priming would be favoured by lower concentrations of Headloop primer and lowering of a Headloop primer concentration to 20–60 nM was shown to enable selective amplification of

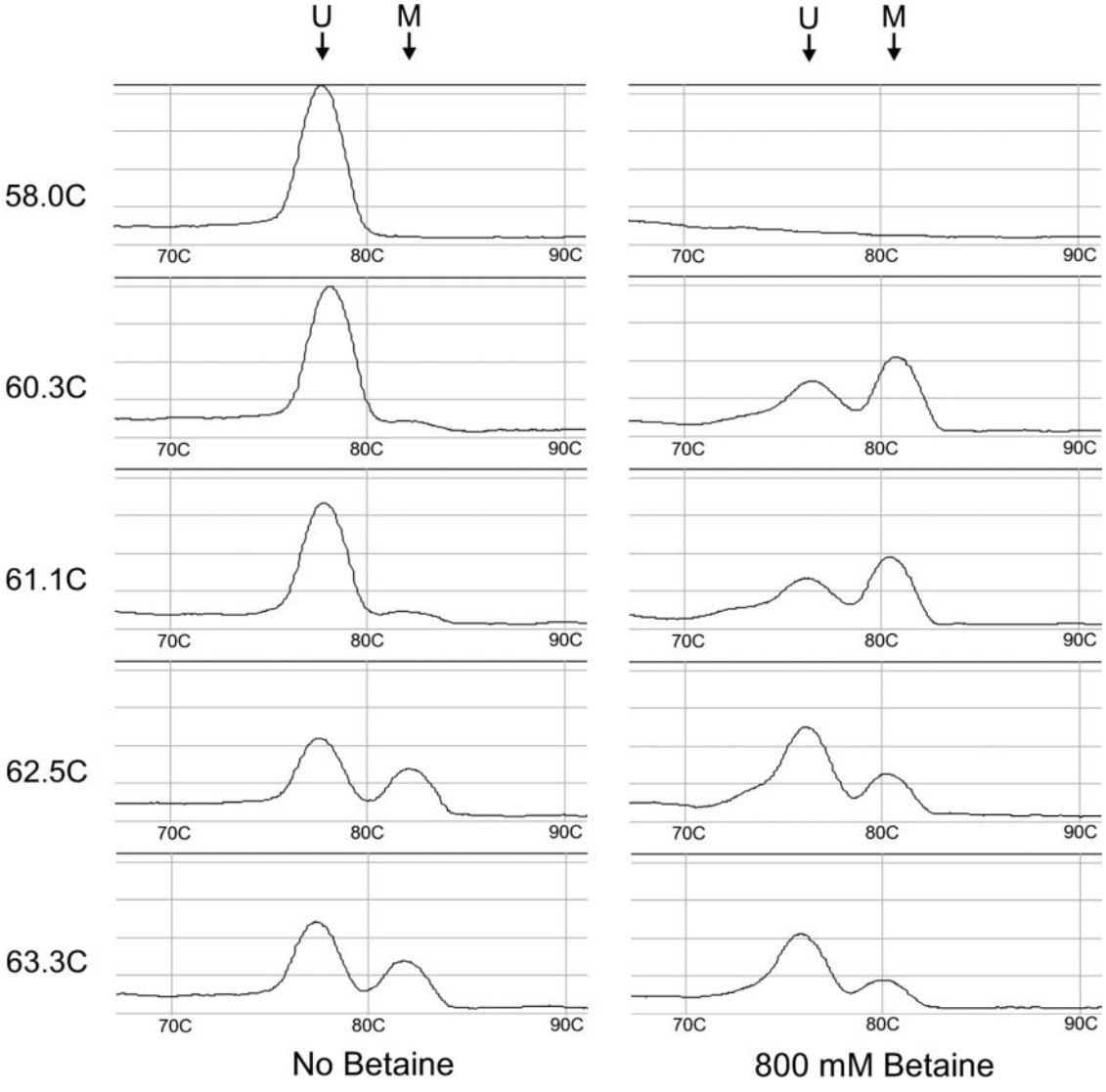
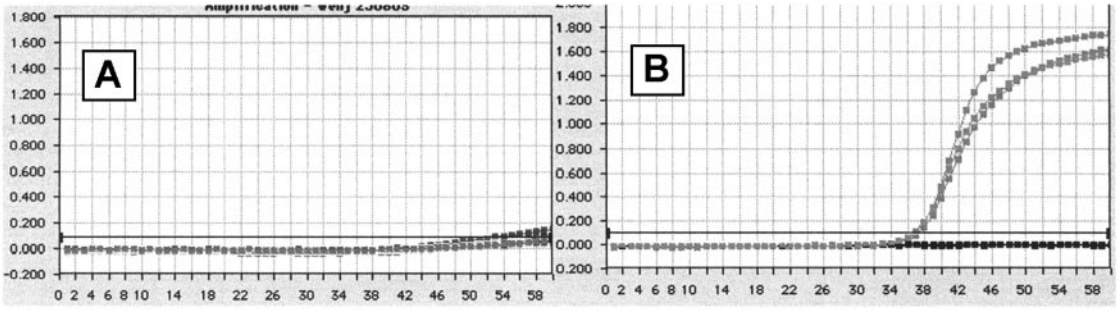


Figure 5. Effects of temperature and betaine on Headloop PCR. Headloop PCR on the *GSTP1* intragenic region was done under standard conditions using the Hlint5-10 primer except that the annealing/extension temperature of the reaction was varied as shown. In reactions in the right column, betaine was included at 800 mM. Denaturation profiles of PCR products are shown.

methylated DNA when present in a ratio of $1:10^5$ with unmethylated DNA (Supplementary Figure 1).

Selective amplification from genomic DNA

Amplification of small quantities of methylated gene sequences from an excess of unmethylated sequences in genomic DNA is most commonly performed using MSP (12) or its real-time derivatives, Methylight (13) or ConLight PCR (14), where specific fluorescent probes are used to detect the PCR product. Mixtures of genomic DNAs containing methylated and unmethylated *GSTP1* sequences were used to evaluate the capacity of Headloop PCR to amplify target sequences from mixed sequence DNA. A Headloop PCR assay designed to the bottom strand of the *GSTP1* gene and using CpG sites 5–10 for selectivity, was compared with MSP designed to the same DNA region. The sequence of the bisulphite-converted bottom strand and primer positions are shown in Figure 2C. The sensitivity of both assays was compared using limiting amounts of methylated DNA (Supplementary Figure 2); both assays showed a similar capacity to detect sequences down to single cell levels and reliable detection from levels >25 pg (~4 cell equivalents). The Headloop PCR amplification profiles on 100 pg of methylated DNA spiked into 10 ng of white blood cell DNA (in which there is minimal methylation of *GSTP1* gene) is compared with that from 400 ng of white blood cell DNA in Figure 6A and B.



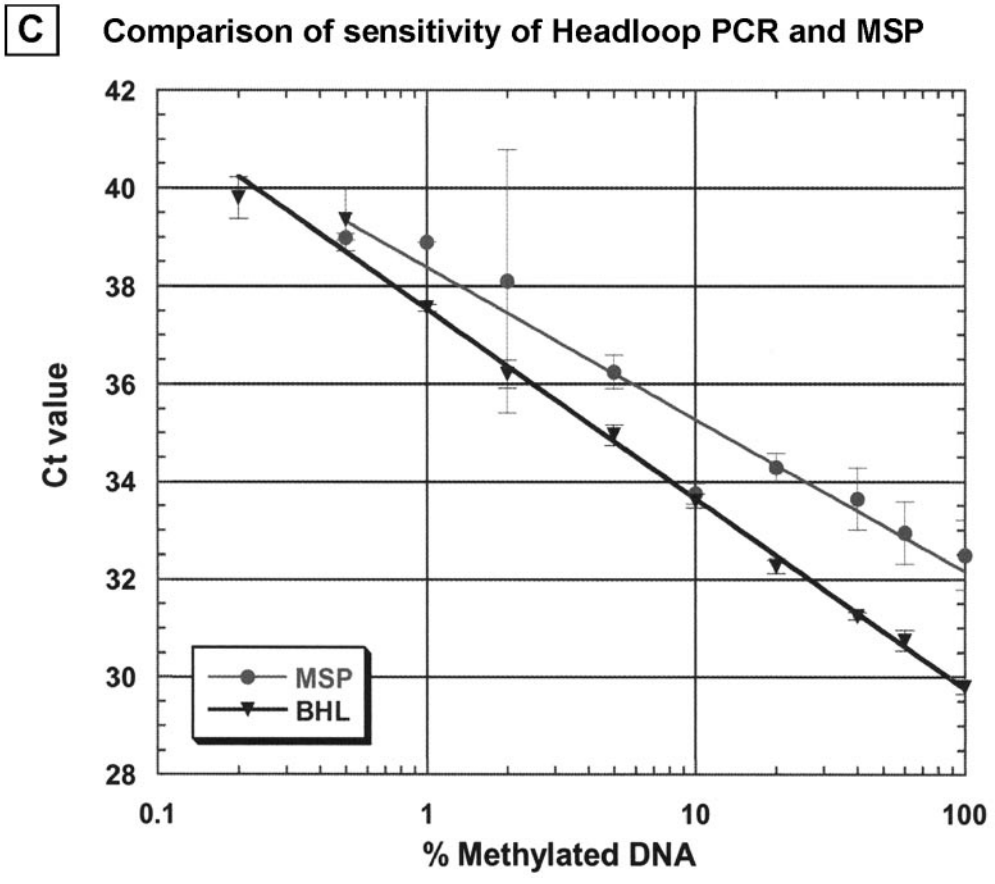


Figure 6. Headloop PCR on genomic DNA. Headloop PCR to the intragenic region (bottom strand) of the *GSTP1* gene was performed using 400 ng bisulphite-treated white blood cell DNA (**A**) or 100 pg of *in vitro* methylated white blood cell DNA spiked into 10 ng unmethylated DNA (**B**). (**C**) Amplification using Headloop PCR (BHL, triangles) is compared with MSP (circles). Total input DNA was 50 ng in each PCR with the amount of methylated DNA ranging from 100 pg to 50 ng. Ct values are plotted against % methylated DNA in starting mixture.

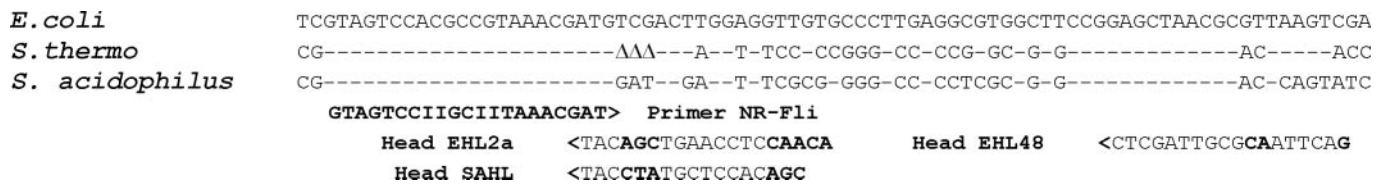


Figure 7. Headloop target regions on 16S rRNA. Sequences from the *S.thermosulfooxidans* and *S.acidophilus* 16S rRNA genes are shown below that from the *E.coli* gene. Dashes indicate identity to the *E.coli* sequence and 'Δ' deletions. The position of the forward base primer NR-Fli is shown as are the head sequences EHL2a and EHL48 targeted to suppress *E.coli* rDNA amplification, and SAHL targeted to suppress amplification of *S.acidophilus* rDNA. Mismatches to non-target sequences are shown in boldface.

A concentration range of 0.2–100% methylated DNA (in a total of 50 ng DNA) was used to compare selective amplification by Headloop PCR with that of MSP (Figure 6C). The data show the potential of Headloop PCR to be used to quantify levels of methylated DNA. In a separate experiment both of these assays and the Headloop assay to the top strand of the intragenic region were shown to detect the methylated *GSTP1* gene in 100 pg of methylated DNA mixed with 400 ng of unmethylated DNA in 8 of 8 replicates, equivalent to detection of methylated DNA from <20 cells (Supplementary Figure 3).

Selective amplification of bacterial 16S rRNA genes

To demonstrate that its application is not restricted to DNA methylation analysis, Headloop PCR was applied to the differential amplification of a region of the 16S rRNA genes from three bacterial species (Figure 7). The base primers we used were primers NR-R1i and NR-F1i that were designed to well-conserved regions to amplify the corresponding region from a wide range of bacterial species. Different 5′ extensions were added to the NR-R1i primer. Two of these, EHL48 and EHL2a were designed for looping back and priming on *E.coli* derived sequences. These were used for amplification from a mixture of 50:1 *E.coli* to *S.thermosulfooxidans* DNA (Figure 8). With both Headloop primers substantial selective amplification of *S.thermosulfooxidans* DNA was seen compared to the control non-Headloop primer. The EHL2a Headloop that targets a region immediately adjacent and overlapping the forward primer showed >50-fold enrichment, while EHL48 showed significant but lower enrichment. The EHL48 Headloop target is further away from the primer and has less mismatches with the *S.thermosulfooxidans* sequence.

A Headloop primer at the equivalent position to the EHL2a primer, but targeted to suppress amplification of the 16S rRNA amplicon from *S.acidophilus* was also designed and evaluated for its capacity to allow selective amplification of *E.coli* DNA. Under standard PCR conditions, with $MgCl_2$ at 1.5 mM an ~50-fold enrichment of *E.coli* sequences was seen. Lowering the $MgCl_2$ concentration to 1.3 mM caused a significant improvement in selectivity and *E.coli* sequences could clearly be detected when a 250-fold excess of *S.acidophilus* DNA was present in the starting mix.

DISCUSSION

We have developed a new, sensitive technology that allows the amplification of trace amounts of methylated DNA from bisulphite-treated DNA in the presence of a large excess of unmethylated DNA. Selectivity of at least 10^4-fold has been obtained with a number of Headloop primers targeted to different sequences. Headloop PCR uses three sequence regions to determine the final specificity of amplification. Even greater selectivity can potentially be achieved by incorporating heads on both forward and reverse primers and we have shown that this can work in one instance (data not shown). The two priming sites are used to provide selective amplification of the target gene from bisulphite-treated DNA, with a key criterion to their design being that they are selective for DNA that has been efficiently converted by bisulphite—annealing and extension should be dependent on T (or U) bases that have arisen through bisulphite conversion of Cs. The head is targeted to a region of differential methylation of CpG sites within the amplicon so that after incorporation into the PCR product it can fold back, anneal and prime to form a hairpin structure. In its application to bisulphite-treated DNA we have introduced 'heads' onto either the forward or reverse primer thus having either As or Ts, respectively, as the bases imparting selectivity in suppression of amplification. Difficulties in PCR caused by internal priming of short inverted repeats yielding inhibitory hairpin structures have been described previously (13) and in Headloop PCR this feature is used to provide a specific, selective suppression. We expect that the hairpin molecules formed are amplified very poorly because they will snap back rapidly after the denaturation step, thus preventing access of primers. Cloning of 'suppressed' PCR products indeed has indicated that hairpin structures were formed. Sequenced clones were truncated as though the loop had been cut off and only the anticipated double-strand region of the molecule maintained. We assume such products arose from nuclease action in the bacteria to remove the loop allowing ligation of the second end to the plasmid vector.

Comparison with other approaches to selective amplification of methylated DNA

Currently the most widely used technique for amplifying methylated DNA sequences from a large excess of unmethylated sequences is MSP (12) and real-time methods based on the same primer design principle (13,14), while an approach using blocking oligonucleotides, HeavyMethyl PCR, has been published more recently (5). We have demonstrated selectivity of Headloop PCR of $1:10^4$ to $1:10^5$ in a number of assays and its performance compares favourably with MSP [Figure 6, Supplementary Figure 3 and up to $1:10^5$, (13)] and with HeavyMethyl PCR [up to 1:8000 with genomic DNA, (5)].

In MSP the amplifying primers are targeted to include a number of CpG sites, particularly toward the 3′ end priming site in order to take advantage of the sequence differences after bisulphite conversion of DNAs methylated or unmethylated at specific CpG sites. While MSP is widely used and can allow very sensitive detection of methylated molecules, there can be

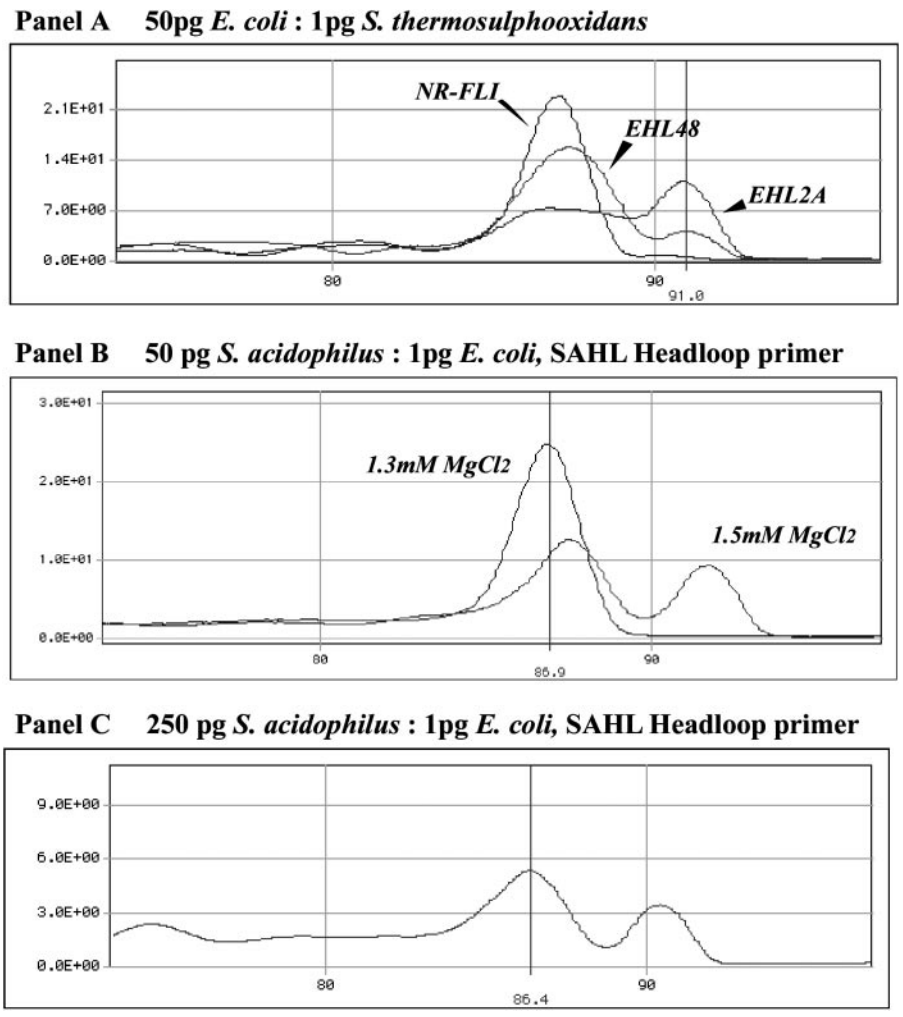


Figure 8. Headloop PCR of bacterial 16S rRNA genes. Headloop PCR with mixtures of bacterial DNAs as shown were performed using the control primer NR-Fli and primers EHL2a or EHL48 to suppress *E.coli* rDNA amplification (**A**), with primer SAHL to suppress *S.acidophilus* rDNA amplification (**B** and **C**). Melting profiles of PCR products were determined to distinguish amplicons from different species. The left peak (86–88°C) corresponds to the *E.coli* amplicon and the right peak (91–93°C) to the amplicons from the thermophilic bacteria *S.acidophilus* and *S.thermosulfooxidans*.

problems in its application. When primers have a number of CpG sites adjacent to the priming site there is a significant potential for mis-priming on DNA that has not been fully converted by bisulphite as the 3′ end of the primer will match well with the unconverted DNA. This can result in spurious false positive signals (14). With Headloop PCR it is possible and desirable to design the base primers with the maximum specificity for amplification of fully converted DNA by ensuring that the 3′ ends of the primers correspond to regions containing non-CpG cytosines. The methylation selectivity is achieved by preventing amplification of the unmethylated molecules. If there is partial or mosaic methylation its amplification should still be advantaged. Since suppression depends on the annealing of the head region with sequences within the amplicon, molecules derived from partially methylated DNA will contain different levels of mismatch and should be proportionately suppressed. The suppression of molecules methylated only at the terminal, priming base of the head should be most affected and least suppressed. Since the differentiating bases are internal to the amplicon selection will occur during each cycle of PCR. By using the same base primers but different head sequences it should also be possible to scan different CpG sites within a region to identify mosaic methylation. In MSP mosaic methylation similarly affects primer annealing, and lack of methylation, particularly at the 3′ position of the primer will severely impair amplification. Once priming has occurred and the MSP primer is incorporated into the PCR product amplification will continue independently of the initial methylation pattern.

Headloop PCR has a number of similarities with the recently published HeavyMethyl assay (5) for selective amplification of methylated DNA. Both utilize primers that are bisulphite conversion-specific, but not methylation-specific and both suppress amplification of the unmethylated DNA by targeting a CpG containing region between or overlapping the primers. In the case of HeavyMethyl suppression of amplification is achieved through inclusion of high concentrations of a blocker oligonucleotide that binds to the unmethylated template and inhibits primer binding and/or extension. In Headloop PCR the equivalent region causing suppression becomes incorporated in the PCR product. Both approaches

have the advantage over MSP in that selection against the unwanted sequence is applied in every round. With MSP, once a mis-priming event has occurred there is no further selection against its amplification. The most significant difference between Headloop PCR and HeavyMethyl PCR is the requirement for high concentrations of blocker oligonucleotide in the latter assay, increasing the possibility for inhibition of amplification and mis-priming events. In the HeavyMethyl assay the blocker is designed to overlap the priming site, placing some limits on primer site selection. For Headloop PCR we have used head regions both adjacent to, or overlapping the primer site or up to 30 bases away. This provides greater flexibility, though from the limited number of Headloop primers we have tested, overlapping the priming site by a few bases could be advantageous.

Parameters affecting Headloop PCR

The design of primers is clearly critical for optimal Headloop PCR performance. We aim to design base primers with similar calculated melting temperatures (T_ms) of ~60°C. Since shorter primers will cause less problems for later Headloop design owing to lower potential for the formation of internal hairpins or of primer dimers, we look at both strands in the planning phase to give optimum flexibility. For work with bisulphite-treated DNA most of our functional Headloop primers have contained head regions of ~20 bases; with a calculated T_m of at least 45°C; shorter heads, e.g. of 10 or 12 bases, have not worked. Lowering the concentration of the Headloop primer generally improves suppression, but this needs to be balanced with the overall efficiency of the PCR (Supplementary Figure 1). This effect is consistent with expectations as lower primer concentrations would favour the intramolecular Headloop priming and extension compared with the bimolecular annealing and extension of the primer. For any specific PCR the annealing temperature should be optimized since relatively small temperature changes can significantly affect selectivity. It would be expected that the relative T_ms of the primer portion and the head portion could have a significant influence, but we have made successful Headloop primers with the T_m of the head portion at least 5°C above or below the primer portion.

The most striking effect on specificity was its dependence on the concentration of free Mg^{2+}. Direct adjustment of the level of free Mg^{2+} by altering its overall concentration or indirectly through the addition of extra deoxynucleoside triphosphates or EDTA had similar effect. Lower levels of free Mg^{2+}, frequently as low as 0.3 mM, consistently provided improved selectivity. The low Mg^{2+} can significantly slow the amplification rate and impair detection with Taqman probes, for example, levels <0.3 mM lead to unacceptably slow or failed amplifications. The final choice of Mg^{2+} concentration is therefore a balance between these factors and the improved selectivity. In the presence of 0.8 mM total concentration of deoxynucleoside triphosphates successful Headloop PCRs have included Mg^{2+} at concentrations ranging from 1.1 to 1.8 mM.

Potential applications for Headloop PCR

The technique of Headloop PCR has principally been developed and evaluated for the selective amplification of methylated DNA sequences where they constitute a minor fraction of the DNA being analysed, such as in the detection of aberrantly methylated cancer-derived DNA. Selectivity of up to 1 in 10^5 with plasmid DNAs and 1:4000 with genomic DNAs has been demonstrated. This indicates the potential of the approach for clinical application and we have successfully applied Headloop PCR for the detection of methylated DNA sequences in tissue and bodily fluid samples (P.L. Molloy, T. Ho, D.S. Millar, W. Qu, M. Patel, C. Paul, K.N. Rand, P.J. Russell and S.J. Clark, manuscript in preparation).

Although emphasis here is on use in the methylation field, utility is not limited to this area and we have shown an example of how it can be used to improve specificity of the 16S RNA gene detect, detection of bacterial species. In principle, Headloop PCR can be used to suppress any sequence that would otherwise dominate in a PCR and allow amplification of sequence variants within the region targeted by the 'head'. This could include suppression of amplification of DNA from a dominant species in order to allow the detection of minor species (e.g. using 16S rRNA), detection of deletion or other mutations in populations or amplification of minor gene or splice variants.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

ACKNOWLEDGEMENTS

This work was supported in part by a grant from the National Health and Medical Research Council of Australia (Grant ID 293810) and in part by Epigenomics AG. Funding to pay the Open Access publication charges for this article was provided by CSIRO.

Conflict of interest statement. Susan Clarke is a member of the Scientific Advisory Board of Epigenomics that partly funded this work. No other conflicts are declared.

REFERENCES

1. Seyama,T., Ito,T., Hayashi,T., Mizuno,T., Nakamura,N. and Akiyama,M. (1992) A novel blocker-PCR method for detection of rare mutant alleles in the presence of an excess amount of normal DNA. *Nucleic Acids Res.*, **20**, 2493–2496.
2. Orum,H., Nielsen,P.E., Egholm,M., Berg,R.H., Buchardt,O. and Stanley,C. (1993) Single base pair mutation analysis by PNA directed PCR clamping. *Nucleic Acids Res.*, **21**, 5332–5336.
3. Orou,A., Fechner,B., Utermann,G. and Menzel,H.J. (1995) Allele-specific competitive blocker PCR: a one-step method with applicability to pool screening. *Hum. Mutat.*, **6**, 163–169.
4. Thiede,C., Bayerdorffer,E., Blasczyk,R., Wittig,B. and Neubauer,A. (1996) Simple and sensitive detection of mutations in the ras proto-oncogenes using PNA-mediated PCR clamping. *Nucleic Acids Res.*, **24**, 983–984.
5. Cottrell,S.E., Distler,J., Goodman,N.S., Mooney,S.H., Kluth,A., Olek,A., Schwope,I., Tetzner,R., Ziebarth,H. and Berlin,K. (2004) A real-time PCR assay for DNA-methylation using methylation-specific blockers. *Nucleic Acids Res.*, **32**, e10.
6. Frommer,M., McDonald,L.E., Millar,D.S., Collis,C.M., Watt,F., Grigg,G.W., Molloy,P.L. and Paul,C.L. (1992) A genomic sequencing protocol which yields a positive display of 5-methyl cytosine residues in individual DNA strands.. *Proc. Natl Acad. Sci. USA*, **89**, 1827–1831.
7. Clark,S.J., Harrison,J., Paul,C.L. and Frommer,M. (1994) High sensitivity mapping of methylated cytosines. *Nucleic Acids Res.*, **22**, 2990–2997.

8. Lee,W.H., Morton,R.A., Epstein,J.I., Brooks,J.D., Campbell,P.A., Bova,G.S., Hsieh,W.S., Isaacs,W.B. and Nelson,W.G. (1994) Cytidine methylation of regulatory sequences near the pi-class *glutathione S-transferase* gene accompanies human prostatic carcinogenesis. *Proc. Natl Acad. Sci. USA*, **91**, 11733–11737.
9. Millar,D.S., Ow,K.K., Paul,C.L., Russell,P.J., Molloy,P.L. and Clark,S.J. (1999) Detailed methylation analysis of the glutathione *S*-transferase π (*GSTP1*) gene in prostate cancer. *Oncogene*, **18**, 1313–1324.
10. Millar,D.S., Paul,C., Molloy,P.L. and Clark,S.J. (2000) A distinct sequence $(ATAAA)_n$ separates methylated and unmethylated domains at the 5′ end of the *GSTP1* CpG island. *J. Biol. Chem.*, **275**, 24893–24899.
11. Henke,W., Herdel,K., Jung,K., Schnorr,D. and Loening,S.A. (1997) Betaine improves the PCR amplification of GC-rich DNA sequences. *Nucleic Acids Res.*, **25**, 3957–3958.
12. Herman,J.G., Graff,J.R., Myohanen,S., Nelkin,B.D. and Baylin,S.B. (1996) Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc. Natl Acad. Sci. USA*, **93**, 9821–9826.
13. Eads,C.A., Danenberg,K.D., Kawakami, K, Saltz,L.B., Blake,C., Shibata,D., Danenberg,P.V. and Laird,P.W. (2000) MethyLight: a high-throughput assay to measure DNA methylation. *Nucleic Acids Res.*, **28**, E32.
14. Rand,K.N., Qu,W., Ho,T., Clark,S.J. and Molloy,P.L. (2002) Conversion-specific detection of DNA methylation using real-time polymerase chain reaction (ConLight-MSP) to avoid false positives. *Methods*, **27**, 114–120.
15. Potaman,V.N. (1999) Prevention of unexpectedly long PCR products primed at short inverted repeats. *Biotechniques*, **27**, 1110–1112.