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# Sensitive and selective amplification of methylated DNA sequences using helper-dependent chain reaction in combination with a methylation-dependent restriction enzymes

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## ABSTRACT

**We have developed a novel technique for specific amplification of rare methylated DNA fragments in a high background of unmethylated sequences that avoids the need of bisulphite conversion. The methylation-dependent restriction enzyme Glal is used to selectively cut methylated DNA. Then targeted fragments are tagged using specially designed 'helper' oligonucleotides that are also used to maintain selection in subsequent amplification cycles in a process called 'helper-dependent chain reaction'. The process uses disabled primers called 'drivers' that can only prime on each cycle if the helpers recognize specific sequences within the target amplicon. In this way, selection for the sequence of interest is maintained throughout the amplification, preventing amplification of unwanted sequences. Here we show how the method can be applied to methylated Septin 9, a promising biomarker for early diagnosis of colorectal cancer. The Glal digestion and subsequent amplification can all be done in a single tube. A detection sensitivity of 0.1% methylated DNA in a background of unmethylated DNA was achieved, which was similar to the well-established Heavy Methyl method that requires bisulphite-treated DNA.**

## INTRODUCTION

The fact that hypermethylation of tumour-associated genes frequently occurs in early stages of cancer development can be exploited for development of epigenetic biomarkers for early diagnosis, monitoring and prognostication of cancer (1–3). Indeed, the potential of several such biomarkers for early detection of cancer, for

predicting outcome and response to therapy and ultimately, for improved management of patients and the disease has been clearly shown in translational and clinical settings (3). Examples include detection of hypermethylated (i) glutathione *S*-transferase (GSTP1) in urine and serum samples to help distinguish benign lesions from prostate cancer (4–6), (ii) vimentin 1 (7,8) in faeces and Septin 9 genes in blood to detect colon cancer (9) and (iii) multiple genes (including P16) in sputum to mark early stages of lung cancer (10–13). Finally, clinical success of *O*<sup>6</sup>-methylguanine-DNA methyltransferase promoter hypermethylation as a strong prognostic factor for patients with newly diagnosed glioblastoma and as potent predictor of response to treatment with alkylating agents is a testament to the clinical feasibility of these concepts (14–19).

There is now much interest in the use of CpG island hypermethylation as a tool to detect cancer in the easily accessible body fluids and tissue specimens from cancer patients (20). The issue with such type of samples, however, is the difficulty of detection of the tumour-derived DNA as it is generally present in low quantities (<0.1%) (21) with most of the DNA being derived from healthy cells. Thus, translational success of these approaches hinges on use of excellent detection technology to detect minute amounts of rare methylated target DNA in the presence of a vast excess of non-target DNA that can include both related unmethylated and methylated sequences.

Several PCR-based assays have been developed, the majority of which depend upon the use of DNA treated with sodium bisulphite. Bisulphite treatment deaminates cytosine, but not 5-methyl cytosine, to uracil (22). This allows the preservation of the methylation information through PCR which otherwise would not distinguish between methylated and non-methylated residues. Methylation-specific PCR (MSP) is the most widely used approach and yields excellent analytical sensitivity,

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detecting up to 0.1% of methylated template in an excess of unmethylated DNA (23). Based on the differences in the DNA sequences after the bisulphite treatment, the primers are specially designed to amplify the methylated template only. The primary issue with this technology is the potential for false positive due to mispriming or due to incomplete bisulphite conversion of DNA (24,25). In an attempt to tackle these issues, heavy methyl and headloop suppression PCR methods, which also used bisulphite-treated DNA as the template, but maintain selection against unmethylated sequences during each PCR cycle, were developed. Yielding comparable sensitivity to MSP, the 'heavy methyl' method minimizes the frequency of false positives through use of oligonucleotide blockers that block amplification of unmethylated DNA (26). In the case of headloop suppression PCR, primers are designed to specifically suppress amplification of the unmethylated sequence (27).

A particular example demonstrating the clinical feasibility of such methods is the application of the heavy methyl PCR method to detect methylated Septin 9 for early diagnosis of colorectal cancer (CRC). Detection of methylation in the Septin 9 promoter region was found to effectively discriminate CRC from normal specimens (28). The ability to detect circulating methylated Septin 9 DNA in plasma has led to the development of a minimally invasive blood test for CRC (9,29). As mentioned earlier, bisulphite-treated DNA is most commonly used for most detection assays. However, these assays suffer from the drawbacks of this technology, for example (i) preparation of bisulphite-treated DNA requires multiple steps (denaturation with NaOH, incubation with Na-bisulphite, desulphonation and DNA purification) and hence, it is time-consuming; (ii) loss of material due to DNA degradation (30) and handling steps and (iii) suboptimal conversion rate based on technical variability, DNA sequence, quality and sample source which could sometimes lead to inconsistencies (31); this might be particularly relevant to clinical specimens.

As an alternative to bisulphite treatment, restriction enzyme (RE)-based methods have also been used for site-specific detection of DNA methylation (32); based on the type of RE, these enrich for either methylated or unmethylated DNA. Methylation-sensitive enzymes (e.g. HpaII, BstUI or AciI) are generally used and these cut only if the DNA is unmethylated; the lack of cutting is then detected by PCR of the methylated sequence using flanking primers. The main source of error here is the possibility of high background (false positives) arising from incomplete cutting of the target sites and the approach has not found wide acceptance. We have recently described a RE-based method, end-specific PCR (ESPCR), which was used for detection of hypomethylation of repeated DNA sequences following digestion with methylation-sensitive enzymes (33). ESPCR uses specially designed helper or facilitator oligonucleotides to specifically target the cut fragments and thus is much less affected by incomplete digestion.

The discovery of methylation-dependent enzymes that cut at specific target sequences only if those sequences are

methylated allows this approach to be extended to the detection of hypermethylation. Based on this premise, in this proof of principle study and using Septin 9 as an example, we describe a new methylation detection method that avoids the use of bisulphite. After digestion with the methylation-dependent enzyme *GlaI* (34,35), a particular methylated Septin 9 fragment is specifically amplified by using a new method called 'helper-dependent chain reaction' or 'HDCR'. This combines ESPCR (33) with a unique feature that allows maintenance of the selection for the target sequence throughout amplification and thus results in enhanced specificity. To our knowledge, this is the first report of a method that results in the continuous positive selection for a targeted sequence.

## MATERIALS AND METHODS

### DNAs and oligonucleotides

K562 DNA, purified from a subculture of the human chronic myelogenous leukaemia cell line, was obtained from Promega. CpGenome Universal Methylated DNA is fully CpG-methylated human male genomic DNA and was originally obtained from Chemicon (Temecula, CA, USA). It is now available from Millipore (<http://www.millipore.com/catalogue/item/s7821>). Tumour samples were obtained from patients undergoing surgery at Flinders Medical Centre (Adelaide, Australia) with consent having been obtained prior to surgery—Human Research Ethics Committee approval RGH 09/04. Following tissue disruption using the Retsch TissueLyser (Qiagen), DNA from cancer and matched normal tissue was isolated using a Wizard<sup>®</sup> Genomic DNA purification Kit (Promega).

Oligonucleotide sequences are shown in Table 1. The helper oligonucleotides (denoted as 'helper/s' from here on) were purchased from Biosearch Technologies (Novato, CA, USA) or from GeneWorks (Adelaide, Australia). Other oligonucleotides were purchased from GeneWorks or from Sigma-Aldrich (Sydney, Australia). All oligonucleotides were dissolved in TEX buffer (10 mM Tris-HCl, 0.1 mM EDTA, 0.01% Triton X-100).

### Heavy methyl PCR assay

Genomic DNAs were bisulphite-converted using the EZ DNA Methylation-Gold<sup>™</sup> kit (Zymo Research). The Septin 9 heavy methyl assay was done, using Quantitect PCR mix without ROX (Qiagen) as described by de Vos 2009 (9).

### *GlaI* digestion

*GlaI* RE was purchased from Sibenzyme (<http://www.sibenzyme.com/info627.php>). DNAs pre-cut with *GlaI* were prepared by digesting 1 µg in 'SE Buffer *GlaI*' (10 mM Tris-HCl, pH 8.5 at 25°C; 5 mM MgCl<sub>2</sub>; 10 mM NaCl; 1 mM 2-mercaptoethanol) plus 16 units of *GlaI* in a total volume of 50 µl for 2 h at 30°C followed by 15 min at 70°C. After adding 5 µl of 50 mM EDTA and 145 µl of TEX, the 5 ng/µl digested DNAs were stored at -20°C.

**Table 1.** Oligonucleotide sequences and final concentrations used in Septin 9 Glal-HDCR assay

Oligo name	Role	Sequence 5' to 3'	Purification and source	Final conc. (nM)
UDr2H	Driver F	CCCGTMGMMGMTMMMTMuuuMTAMAG	HPLC, GeneWorks	400
UDr3H	Driver R	CGCCTMMMGMGTGMTMuuuMATMGA	HPLC, GeneWorks	400
S9m3HF354	Helper F	GTCGCCGTCCCTCTTTCTACAGTGggaCCCGCTGCCuuuT	PCR grade, GeneWorks	25
S9m3HR355	Helper R	CTCCCGCTGCTCTTTCATCGATGuugACCGCGGGGTCCuuuT	PCR grade, GeneWorks	25
S9HEXPr	Probe	HEX- CAC[+C]AG[+C]C[+A]T[+C]AT[+G]TCG BHQ1	HPLC, Sigma	50

F = 'Forward', R = 'Reverse'; 2'-O-methyl nucleotides are shown in lower case; 5-methyl cytosines are shown as 'M'; locked nucleic acid residues are shown as [+C].

### Helper-dependent chain reaction

HDCR was carried out using a Corbett Rotor-Gene 3000 machine with a 72-tube rotor and 10 µl volumes. Conditions for hot start PCR were 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 7.5 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, oligonucleotides (as listed in Table 1), 1/50 000 dilution of SYBR Green, 0.01 mM dithiothreitol, 0.1% Triton X-100 and 0.04 U/µl Platinum Taq Polymerase. Glal was included at 0.04 units/µl when the restriction digest was carried out in the PCR tube.

Suitable cycling conditions for HDCR for 10 µl volumes and the Rotor-Gene 3000 using pre-cut DNA samples were five cycles (90°C 15 s, 55°C 20 s, 76°C 5 s, 67°C 20 s) followed by second stage of up to 85 cycles (90°C 15 s—read HEX channel, 67°C 40 s, 76°C 5 s, 67°C 20 s—read FAM/SYBR Green channel). For the results shown in Figure 3B, 50°C was used in place of 55°C in the first stage. When Glal digestion was done in the PCR mix, an initial incubation of 10 min at 30°C was carried out.

The SYBR Green is included to allow a more complete analysis of the amplification if necessary, for example by allowing a melt curve analysis to be carried out after the amplification. We have found that the addition of a low level of Triton X-100 in some situations improves the SYBR Green signal; possibly by preventing loss of the hydrophobic SYBR Green to surfaces. The dithiothreitol appears in our experience to be needed to stabilize some batches of Platinum Taq Polymerase, especially when high denaturation temperatures are used. For quantification, all samples were diluted such that 2 ng of each was assayed with a standard curve ranging from 100 pg to 2 ng of fully methylated CpGenome DNA (mixed with K562 DNA in which Septin 9 is unmethylated to make a total amount of 2 ng, where necessary). The standards were cut by Glal in the initial incubation of the PCR tubes at 30°C, along with the test samples. The correlation between the two assays was done by assessment of Spearman's rank correlation coefficient (Spearman's rho) using the Graph Pad Prism, version 5.

### RESULTS

We describe here a new one-step PCR-based HDCR method that can be used to detect cancer-associated hypermethylation in clinical samples. To show sensitivity and specificity of the assay, we used commercially available DNAs so that other laboratories will be able to make direct comparisons with other assays already in use.

### The concept and strategy

Overall, three primary steps are involved: (1) genomic DNA is cut with the methylation-dependent RE Glal, (2) the Glal-cut fragment from the target gene is specifically tagged with 3'-extensions at each cut end and (3) HDCR is used to specifically amplify and detect the tagged molecules that derive only from the methylated target. In these experiments, the Septin 9 gene that is hypermethylated in >90% of CRC patients (29) was targeted.

#### Generation of a methylation-dependent restriction digest

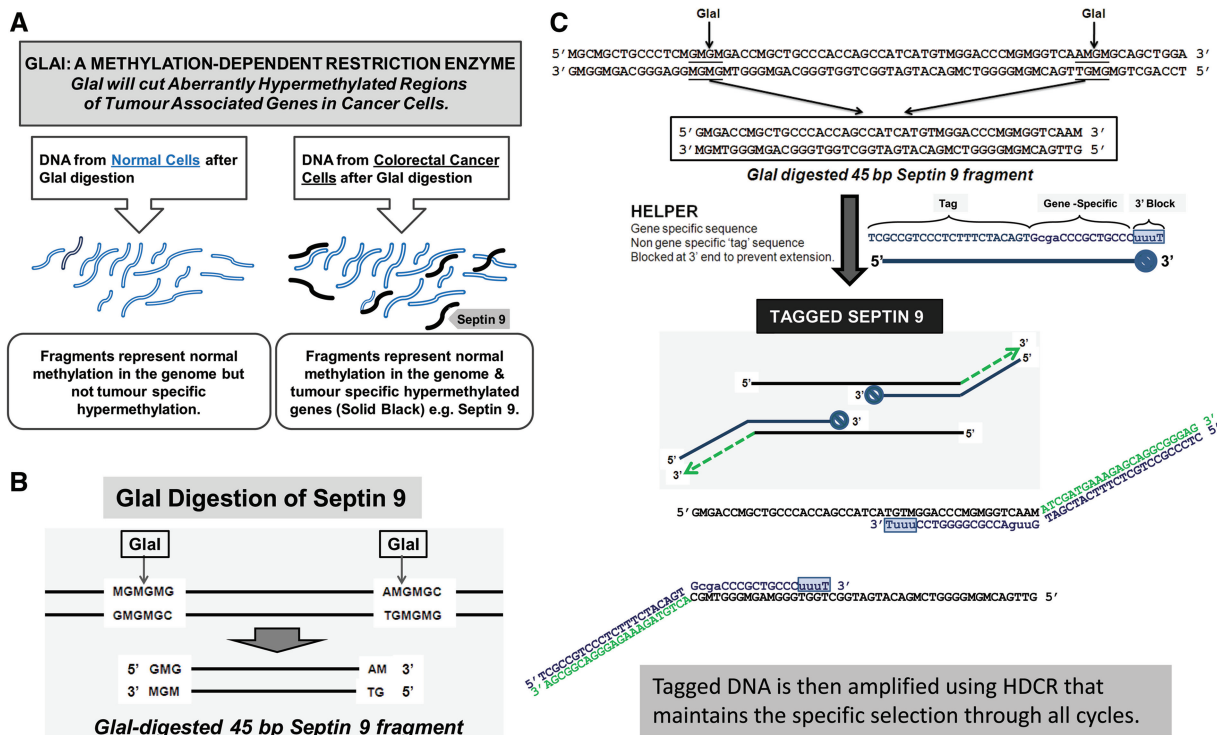
Genomic DNA is cut with the Glal RE; Glal cleaves the DNA sequence, 5'-GCGC-3'/3'-CGCG-5' if there are two, three or four 5-methylcytosines (5 meC, or 'M') (34,35) (Figure 1A). The efficiency of cleavage is variable (6%–100%) based on the quantity and location of 5meCs, as well as the composition of the enzyme recognition sequence. Fragments generated will be derived from DNA that is normally methylated as well as sequences that are specifically methylated in cancer cells [e.g. Glal-cut Septin 9 gene fragment (Figure 1A)]. The hypermethylated region in the Septin 9 gene includes two consensus Glal target sites that are expected to yield a fragment of 45 bp after Glal digestion (Figure 1B and C).

#### Tagging of Glal-cut candidate gene using gene-specific 'helper' oligonucleotide

In this step, the target hypermethylated gene is tagged using a specially designed oligonucleotide—called a 'helper', which can bind but cannot be extended thus preventing any copying of annealed genomic DNA. Specifically, the helper contains at its 5'-end a non-gene-specific tag sequence (TAG) followed by a gene-specific sequence and then a blocked 3'-end. The 3'-end comprises a stretch of three 2'-O-methyl residues adjacent to a 3'-nucleotide that is mispaired with the target sequence. The gene-specific elements of the helper lead to specific hybridization to the target. If the target has been cut by Glal, then it becomes tagged by copying of the non-gene-specific 5'-helper sequences (Figure 1C).

#### HDCR detects the hypermethylated candidate genes in Glal-cut DNA mix

HDCR is designed to increase the specificity of detection through use of a specially designed primer—called the 'driver' together with the 'helper' (Figure 2).



**Figure 1.** Methylation-dependent restriction of genomic DNA and sequence-specific 3'-0 tagging of the cut fragment using the helper. Panel (A): a schematic describing the methylation-dependent enzyme (*GlaI*)-based restriction of normal genomic DNA (fragments shown in blue) or that from the cancer patients (fragments shown in solid black). The fragment representing methylated Septin 9 is shown. Panel (B): the cartoon shows the *GlaI* consensus sites and a schematic of Septin 9 digestion using *GlaI* generating a 45-bp sequence; M: 5-methylcytosine. Panel (C): after *GlaI* digestion the cut fragment (example of Septin 9 sequences is shown) is then tagged at its 3'-ends using two 'helpers' (shown in blue). The helper shown in the upper part of the figure contains a non-gene-specific 'Tag' sequence followed by a gene-specific sequence and a 3'-sequence uuuT (u = 2'-O-methyl uracil and T is a mismatch with the target) designed to prevent extension. A second helper with similar features is used to tag the other 3'-end. The resulting tags on the 3'-ends of both strands of the target Septin 9 target are shown in green.

The driver is complementary to the extended TAG sequence and acts as a primer to copy the target *GlaI* fragment of the Septin 9 sequence and extension of the target strand occurs (Figure 2, Step 1). However, in the second strand synthesis, the block in the driver causes premature termination and prevents the regeneration of the full binding site for the driver (Figure 2, Step 2). Here the block to extension is caused by the presence of three 2'-O-methyl uracil bases in the driver that cause the polymerase to stall.

Amplification then becomes dependent upon the 'helper' that is required to allow completion of strand synthesis. As in the initial tagging reaction (Figure 1), the helper anneals to the 3'-end of the incomplete strand, the Septin 9 sequences and first six (underlined in Steps 2 and 3 in Figure 2) bases of the TAG sequence. Specific binding leads to the copying of the 5' tail of the helper, thus regenerating the binding site for the driver (Figure 2, Step 3).

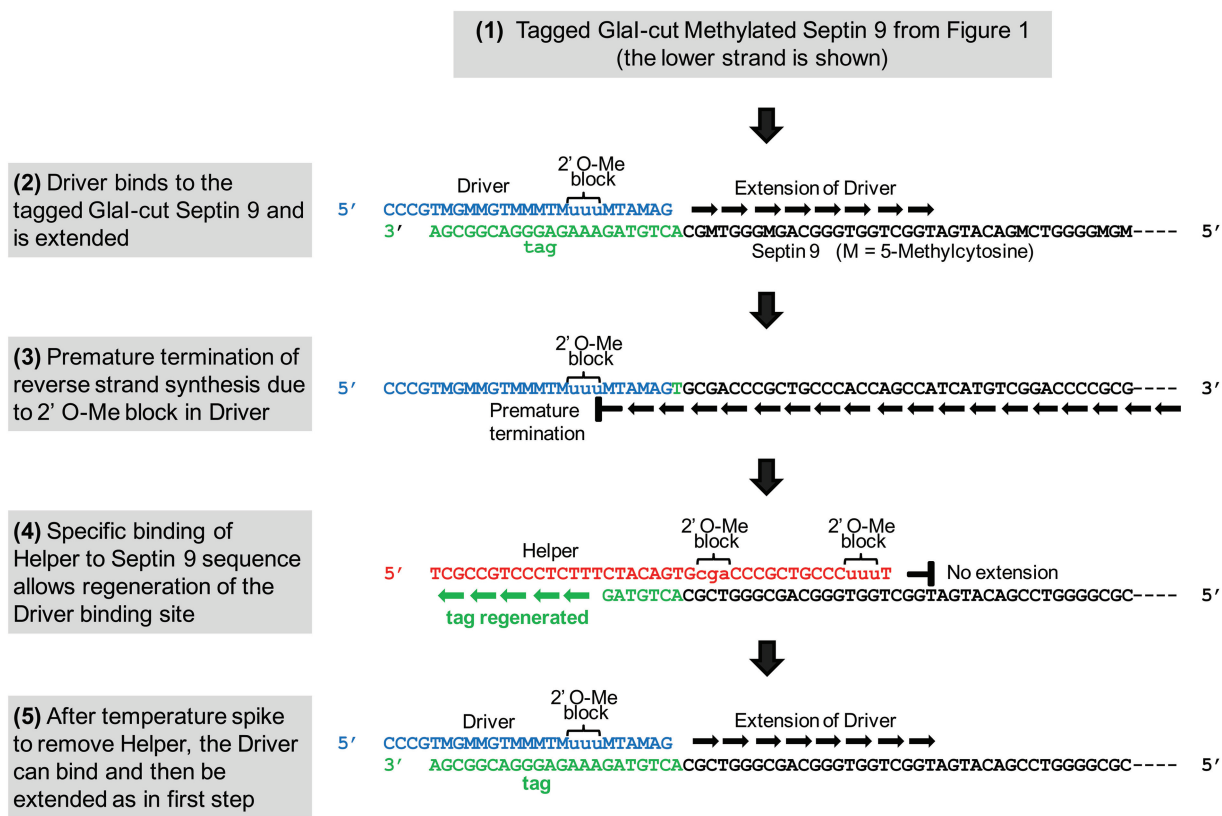
After denaturation, the driver binds to the regenerated site and the amplification cycle continues (Figure 2, Step 4). Because continued specific binding of the helper to the target gene sequence is required through the entire amplification, very high specificity is potentially achieved.

As is evident in Figure 2, the sequence of the driver primer is included within that of the helper

oligonucleotide and both will compete for hybridization to the tagged Septin 9 strand. In order to improve amplification efficiency, sequences and reaction conditions need to be optimized to favour the driver binding and priming. Here we have increased the driver's hybridization stability (i.e.  $T_m$  value) through replacement of some of the cytosines by 5MeC in its sequence. This has been shown to increase stability of binding in other systems (36,37). Additional bias towards driver binding was obtained through keeping the concentration of the helper oligonucleotide much lower (40 nM) relative to the driver (400 nM). Furthermore, after Step 2 (Figure 2) a temperature spike to 76°C is included to facilitate replacement of the helper with the driver and permit priming of the next round of amplification.

### HDCR and detection of hypermethylated Septin 9

The Septin 9 HDCR assay uses a helper/driver combination at both ends of the 45 bp *GlaI* Septin 9 fragment (Table 1). In Figure 2, for clarity, the scheme only shows reactions from one end. Successful amplification of Septin 9 was shown by using a specific HEX-labelled TaqMan<sup>®</sup> probe (Table 1). The performance of the assay was initially demonstrated using CpGenome fully methylated DNA and K562 DNA as a control in which the Septin 9 gene is unmethylated. To investigate the specificity of the



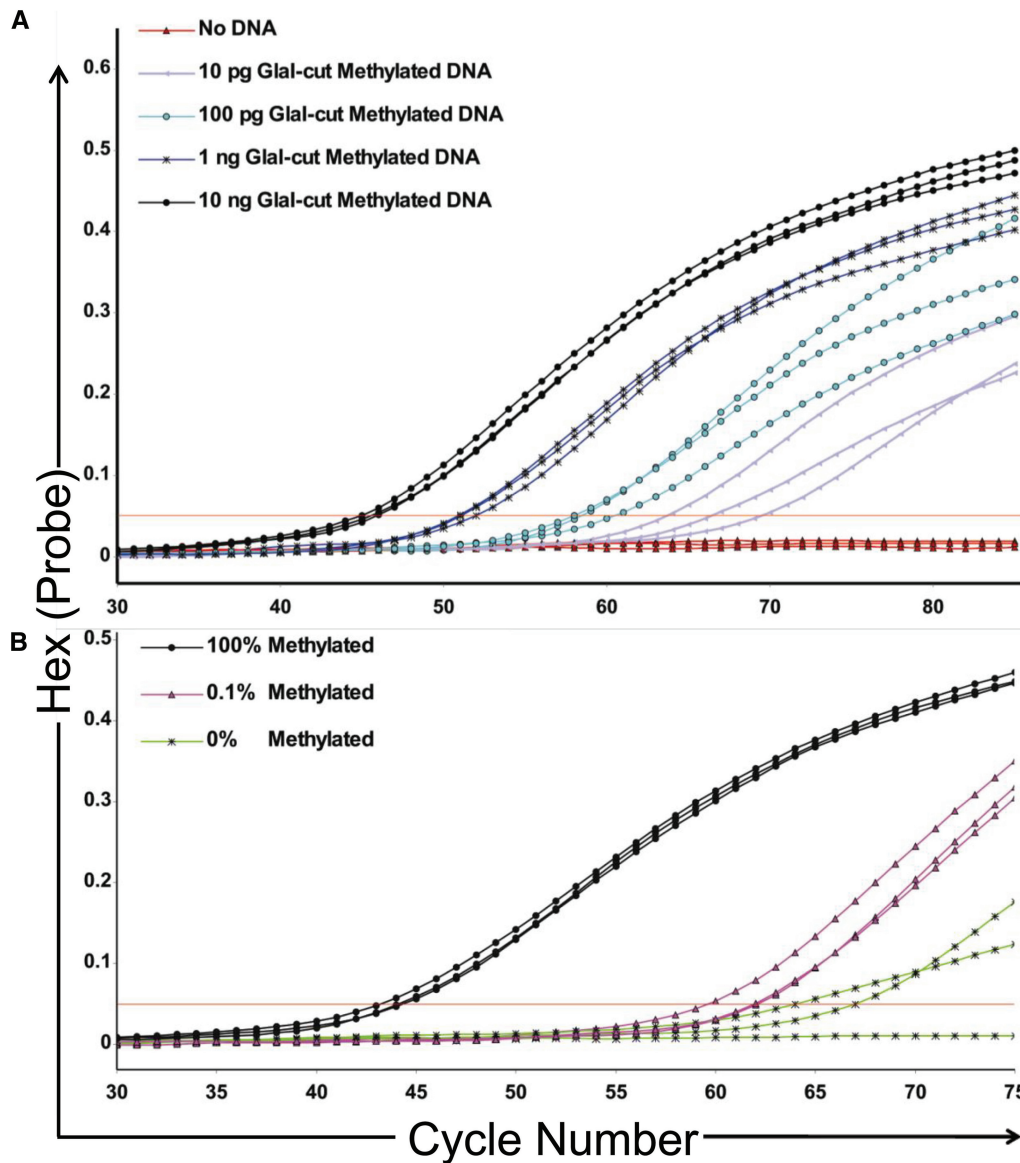
**Figure 2.** HDCR: quantitative PCR-based selective amplification of tagged Septin 9. This figure describes the main features of HDCR that are needed to maintain selection for the correct target throughout amplification. (1) For the sake of clarity, reactions are shown for one strand only. Only the lower, 3'- to 5'-tagged Septin 9 strand from Figure 1 is followed here. (2) A specially designed oligonucleotide, the 'driver' (blue sequence), anneals to the tag (green). The driver contains three 2'-O-methyl nucleotides but because they are located 6 nt from the 3'-end, they do not prevent extension and thus the copying of the Septin 9 fragment. (3) In the next round of replication, the new strand formation (copying) stops at the 'block' sequence (uuu) in the driver, thus preventing the regeneration of the driver-binding site. (4) After denaturation, an oligonucleotide called the 'helper' (shown in red) hybridizes specifically to the prematurely terminated strand. The hybridizing site consists of 7 nt of tag sequence plus 14 nt of Septin 9. The helper contains two stretches of 2'-O-methyl nucleotides. The stretch close to the helper's 3'-end (lower case, uuu), in combination with a 3'-terminal mismatch (T/T), is designed to prevent extension of the helper. The centrally located stretch of 2'-O-methyl nucleotides (lower case, cga) is not essential to the HDCR process. It is designed to reduce background that might occur due to inappropriate priming of non-target fragments on the helper. Because the helper shares 5'-sequences with the driver, copying of the helper's 5'-tail regenerates the driver's binding site. (5) A 76°C temperature spike is sufficient to remove the helper used here, allowing the driver to access its regenerated binding site. It is important to note that at this step the driver will be in competition with the helper for binding. Modifications that decrease helper  $T_m$  or that increase driver  $T_m$  (such as the use of 5 MeC as used here) and the use of a 10-fold higher concentration of driver aids binding and thus copying of the Septin 9 strand. Selection for the correct product is maintained throughout the amplification because of the continued need of specific helper binding to the Septin 9 sequence in every cycle.

reaction, we have studied various mixtures of DNAs (Figure 3). The sensitivity of HDCR was determined using samples containing different concentrations of methylated DNA pre-cut with Glal (10 pg–10 ng); the hypermethylation in the Septin 9 gene could be detected in samples containing only 10 pg of Glal-cut genomic DNA (Figure 3A). Because driver hybridization and priming is reduced due to competition from the helper, the efficiency of amplification is significantly reduced compared with standard PCR and detection requires more cycles. Nevertheless, the requirement for specific annealing with sequences internal to the Septin 9 fragment during each cycle leads to a prolonged suppression of background amplification. Specificity was further examined in DNA mixtures containing different proportions of Glal-pre-cut methylated DNA (0 or 0.1% or 100%). HDCR could accurately detect methylated

Septin 9 in samples containing only 0.1% of methylated DNA (Figure 3B).

#### HDCR of the hypermethylated Septin 9 gene in clinical samples

As proof of principle, HDCR was employed to evaluate the extent of hypermethylated Septin 9 gene in CRC and matched normal tissues from 25 cancer patients (four Stage A, nine Stage B, eight Stage C, one Stage D and four unknown stage). The level of methylation was determined relative to that of fully methylated CpGenome DNA. A clear separation of cancer and normal tissue DNA methylation is evident (Figure 4A). In considering individual samples, HDCR could detect hypermethylation in the Septin 9 gene to a level of >5% in 22/25 (88%) patients (Figure 4B). For all but two of the subjects, methylation of the Septin 9 gene was



**Figure 3.** Detection by HDCR of methylated Septin 9 in genomic DNA mixtures pre-cut with Glal. In addition to the drivers and the helpers, Septin 9-specific HEX-labelled Taqman probe was used and amplification was detected by measurement of fluorescence at each cycle. Panel (A): amplification curves 0–10 ng of fully methylated DNA pre-cut with Glal. Panel (B): detection of 0.1% Glal-cut methylated DNA in a background of Glal-cut K562 (unmethylated) DNA (10 ng total DNA).

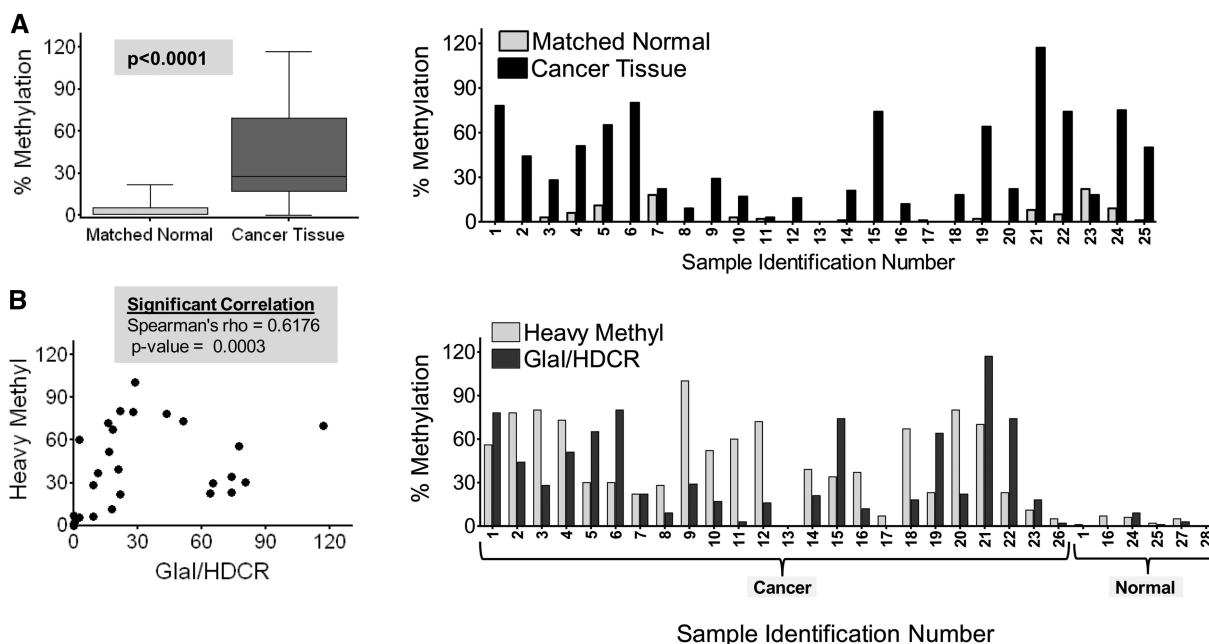
substantially higher in the cancer than matched normal DNA. In two patients, similar levels of methylation were seen in matched normal tissue, indicating methylation variability within normal tissue, methylation in the zone of pre-neoplastic tissue in which the cancers arose, or that the normal tissue was contaminated with neoplastic cells.

The Septin 9 HDCR assay was compared with the heavy methyl assay that is now in clinical use; a significant correlation between the two methods was shown (Figure 4C) ( $P = 0.0003$ , Spearman's  $\rho$ : 0.6176). Despite the generally good correlation, in some patients the methylation level determined by HDCR and heavy methyl assays differed substantially (Figure 4C and D); these differences may reflect the pattern of CpG site

methylation in the individual patients as selection in the two assays is based on different CpG sites in the same region (Supplementary Figure S1).

## DISCUSSION

While several novel and promising methylation-based biomarkers continue to be discovered and characterized, the efficiency of clinical translation of these markers is compromised due to the lack of adequately sensitive, specific and commercially viable assays in simply acquired and feasible clinical specimens. Based on the use of Glal, a methylation-dependent RE and HDCR, a new and sensitive technique for selective amplification,



**Figure 4.** Detection of methylated Septin 9 in clinical samples by HDCR and Glal-based single-tube assay. Genomic DNA from patient samples, cancer biopsies or matched normal tissue was used; HDCR was used to detect methylated Septin 9 after Glal digestion in the PCR mix. For comparison, heavy methyl assay was used to detect methylated Septin 9 using bisulphite-treated DNA. Panel (A): a box plot representing percent methylation in matched normal tissue versus that noted in cancer tissue from 25 CRC patients. Panel (B): the graph shows the same comparison for individual patients [sample identification (ID) numbers, 1–25]. Panel (C): a correlation plot comparing HDCR and heavy methyl-based detection of percent methylation in cancer, matched normal and normal tissue obtained from 30 subjects. Panel (D): graph shows the comparison between the detection by two methods for individual subjects. Cancer tissue is represented by sample ID numbers, 1–23 and 26, matched normal tissue corresponding to sample ID numbers, 1, 16, 24 and 25 and normal healthy colorectal tissue is represented by sample ID numbers, 27 and 28. Identical sample ID numbers in Panels (B) and (D) represent the same patient.

we have been able to detect the methylated Septin 9 gene in a 1000-fold excess of unmethylated DNA (Figures 3 and 4). We have compared the sensitivity of detection on both model DNAs and clinical samples by HDCR to that obtained using the established heavy methyl assay (Figure 4C and D), which is currently being developed for early diagnosis of CRC in blood samples (9,29).

The unique attribute of HDCR is sustained selection of the target sequence throughout the whole reaction, thus yielding high levels of analytical sensitivity and specificity. This selection occurs at two levels; first, only methylated sequences are cut by the methylation-dependent enzyme and second, through use of the helpers, the specific target sequence is tagged and then the same helpers are used to maintain selection for the target sequence in each subsequent cycle of amplification. This results in a very low background; we could detect methylated Septin 9 in as low as 10 pg of methylated genomic DNA when present in a background of 10 ng of unmethylated sample.

### Specific technical features

Once specific DNA ends have been generated, here by RE digestion, a combination of two oligonucleotides, the helper and the driver, at each fragment end is used in an interdependent manner to drive amplification. The helper binds to gene sequences adjacent to the cut end and acts as a template allowing only the cut genomic DNA to be extended (creating a driver-specific Tag to allow driver

binding). The methylation and site-specific cutting of genomic DNA, combined with the hybridization to gene-specific sequence of the helper, provide the specificity at each end. The first important factor is that the helper should be completely end-blocked so that no extension can occur. Here we used a combination of three 2'-O-methyl nucleotides (uuu) followed immediately by a mismatch at the 3'-end to prevent any copying of annealed genomic DNA. Other well-known ways of blocking extension, such as using a terminal dideoxynucleotide or a C3-spacer, could be used (38). A potential source of background is priming on the helper by other fragments of related sequence. Should this happen then the product might have sufficient similarity to the helper that is further amplified (by being 'helped' on all subsequent cycles). In the example shown here we have inserted 2'-O-methyl residues, shown in lower case, 'cga' in the helper sequence (Figures 1 and 2), in order to reduce this. Furthermore, the 2'-O-methyl stretch is positioned such that only fragments with ends expected to result from Glal cleavage will be tagged, thus avoiding amplification of molecules that may have been sheared in this region. Drivers contain a stretch of 2'-O-methyl residues located 6 nt from the 3'-end. Such modifications have been shown to permit priming while blocking the copying of a primer (39).

Another important factor concerns the binding of the driver to its regenerated binding site. At this step, there is competition from the helper, which is a longer



oligonucleotide and binds to a longer, overlapping site in the target sequence. This leads to low reaction efficiency of ~0.3–0.4. Here we have reduced this effect by using 5mC in the drivers to increase  $T_m$  (36,37) and by using low concentrations of the helpers. Modified cycling conditions also help. For example, we used a temperature spike to 76°C to remove the copied helpers so that drivers can bind in the next step (67°C) (Figure 2, Step 4). We have further improved efficiency by using inosines in place of some of the guanosines in the helpers (Supplementary Figures S2 and S3). This modification has been shown to reduce the melting temperatures of oligonucleotides (40). With the inclusion of inosines, the concentration of the helper can be increased and HDCR efficiency is improved to 0.61; with 10 ng of input methylated DNA amplification is detected with a  $C_t$  of ~25. Details of design of helper sequences and use of Inosines are provided in the 'Supplementary Discussion' section.

The use of HDCR for specific amplification of methylated sequences is dependent on the availability of REs that specifically cut methylated target sequences and on the location of restriction sites within target regions of interest. The *GlaI* enzyme cuts different methylated target sites with different efficiencies, dependent on both primary sequence and level of methylation (35), so it is important to evaluate cutting in the context of the specific sequence before designing assays. Cutting at specific sites can be evaluated experimentally using an *in vitro* methylated amplicon covering the target region. While previously few methylation-dependent REs were known, new methylation-dependent enzymes with different sequence specificities have expanded the range of possibilities for assay design. In particular, SibEnzyme, the company that produces *GlaI*, has been adding to its range of methylation-dependent REs. Because these enzymes are relatively new and do not always have straightforward cutting specificities, we advise that the most recent information on cutting specificities is obtained from the company. Methylation-dependent enzymes that belong to the *MspJI* family (*MspJI*, *LpnPI* and *FspE1*) have also recently become commercially available (41). *LpnPI* has a recognition sequence of CMNG, and because 5mC in mammalian DNA is almost always found in the context of a CpG dinucleotide, this specificity effectively becomes that of a methylated *HpaII* site, i.e. CMGG, common in CpG islands. It may be easier to design methylation assays that use enzymes with such straightforward recognition sequences. In the Supplementary Data we demonstrate additional HDCR assays that selectively amplify *LpnPI* fragments of genes shown to be methylated in a high fraction of CRCs, *IRF4* (45) and *GRASP* (unpublished data). If necessary it should be possible to increase selection for methylated fragments by including methylation-sensitive REs such as *HhaI* or *HpaII* if sites occur between the *GlaI* sites.

#### Advantageous features of the *GlaI*-HDCR method

The method described here has several unique features that overcome hurdles faced by currently available

methods and also significantly augment its clinical relevance.

One significant advantage is that it avoids the bisulphite pre-treatment of DNA, which is costly and technically time-consuming, prone to variability due to DNA degradation or inefficient conversion rate (42–44), this is especially undesirable in the context of clinical setting where samples might be in limited supply. While the lower per-cycle efficiency means that HDCR takes additional cycles compared with standard PCR or MSP (up to 150 min for example in Figure 3), this is more than compensated by the removal of the bisulphite modification, normally 4–6 h.

In a standard PCR, specificity can be lost due to mispriming events. Even if the original mispriming only occurred at low efficiency, the full primer binding site is generated in the next round, and priming in subsequent rounds will therefore proceed at high efficiency. This can lead to the amplification of unwanted products or even primer dimers. This might reduce the amplification of the specific target to such a degree that false-negative results are generated. The drivers used in HDCR can also misprime on non-target molecules. However, a driver contains a modification that prevents its binding site from being generated. This prevents amplification of non-target molecules and primer dimers. On every cycle the driver depends upon a helper to regenerate its binding site. But if the driver has misprimed no help will be given if the helper is unable to hybridize to the non-target molecule. Since this selection is maintained throughout the amplification, the overall selection against unwanted products will be very strong.

Within a genomic *GlaI* digest we could detect a small amount of methylated Septin 9 sequence in a mix of cut methylated sequences from other parts of the genome; some other *GlaI*-cut fragments may have sufficiently similar sequences to the target Septin 9 sequence that they would be amplified if not for the continuous selection against them. This sustained specificity would also address any additional complexity that could arise from *GlaI* cutting at sites other than the consensus sites (35).

We have used HDCR both in a format where DNA is pre-cut and added to the amplification reaction and also in a one-tube format where all reaction components are added together and the *GlaI* digestion is done in the same tube prior to initiation of amplification. Using the closed tube approach (involving *GlaI* digestion and subsequent amplification in one reaction), we were able to detect methylated Septin 9 in DNA from 88% of clinical specimens obtained from the cancer patients (Figure 4). Use of HDCR in a one-tube format has clear advantages in a clinical setting. This is dependent on the compatibility of the restriction digestion conditions and those of the PCR buffer. *GlaI*-cutting was only slightly reduced when carried out in the PCR mix. In contrast to methylation detection assays that rely on a lack of cutting of methylated DNA, incomplete cutting by a methylation-dependent enzyme will not reduce the specificity of detection of methylated sequences, but will impact by slightly reducing sensitivity in proportion to the amount of incomplete digestion.

We anticipate that HDCR will be amenable to multiplexing, since a mix of gene-specific helpers sharing a common 'tagging' sequence could be used in combination with a single driver at each end. This would be ideal for use in detection of a panel of biomarkers in clinical samples.

While described here in the context of detection of methylation, HDCR has features that can be adapted for use in a wide range of applications. We envisage that through appropriate target-specific design of the driver and helper oligonucleotides, HDCR could be adapted to detect other types of methylated genes, hypomethylated sequences, gene mutations, microsatellite instability and microRNAs in samples. Because selection for the targeted sequence can be maintained throughout amplification, HDCR should be considered for any applications in which non-specific amplification is a problem.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1–3, Supplementary Figures 1–4.

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