

# Cleavage of mispaired heteroduplex DNA substrates by numerous restriction enzymes

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## Abstract

The utility of restriction endonucleases as a tool in molecular biology is in large part due to the high degree of specificity with which they cleave well-characterized DNA recognition sequences. The specificity of restriction endonucleases is not absolute, yet many commonly used assays of biological phenomena and contemporary molecular biology techniques rely on the premise that restriction enzymes will cleave only perfect cognate recognition sites. In vitro, mispaired heteroduplex DNAs are commonly formed, especially subsequent to polymerase chain reaction amplification. We investigated a panel of restriction endonucleases to determine their ability to cleave mispaired heteroduplex DNA substrates. Two straightforward, non-radioactive assays are used to evaluate mispaired heteroduplex DNA cleavage: a PCR amplification method and an oligonucleotide-based assay. These assays demonstrated that most restriction endonucleases are capable of site-specific double-strand cleavage with heteroduplex mispaired DNA substrates, however, certain mispaired substrates do effectively abrogate cleavage to undetectable levels. These data are consistent with mispaired substrate cleavage previously reported for Eco RI and, importantly, extend our knowledge of mispaired heteroduplex substrate cleavage to 13 additional enzymes.

## Introduction

Restriction endonucleases are the DNA-cleaving components of restriction modification systems employed by bacteria, archaea and certain viruses of unicellular algae to protect against foreign, invading DNA (Pingoud and Jeltsch, 2001; Pingoud et al., 2005). Restriction endonucleases are classified broadly by subunit composition and cofactor requirement into types I, II, III and IV (Bickle and Kruger, 1993; Perona, 2002; Pingoud et al., 2005). The largest class of restriction endonucleases is type II, which is characterized by homodimeric or homotetrameric assemblies that generally require  $Mg^{+2}$  as a cofactor and recognize and cleave close to or within a 4-8 base pair recognition sequence (Bickle and Kruger, 1993; Pingoud and Jeltsch, 2001; Perona, 2002; Pingoud et al., 2005). Most of the enzymes used in modern molecular biology laboratories -- such as in assays of biological phenomena, cloning, mutation detection and site-directed mutagenesis protocols -- are type II endonucleases (Pingoud et al., 1993; Roberts, 2005).

The most valuable attribute of restriction endonucleases is the high degree of specificity with which they recognize and cleave DNA sequences (Roberts, 2005). Understanding the chemical and biophysical basis of this specificity has important biological and biomedical implications: The specificity of protein-DNA interactions underlies a myriad of regulatory and cell biological mechanisms. Crystal structures have been solved for several type II restriction endonucleases, and in some cases, mutant forms. Additionally, some are co-crystals with the enzyme bound to their cognate or noncognate substrates ((Roberts et al., 2007) and sources therein). Biophysical studies using crystallographic data, biochemical and kinetic studies, and molecular dynamics simulations have provided key insights into the protein-DNA interface -- most notably with the EcoRI and EcoRV proteins -- and have provided a foundational understanding of the biophysical basis of site specific interactions (Becker et al., 1988; Lesser et al., 1990; Lesser et al., 1993; Winkler et al., 1993; Engler et al., 1997; Jen-Jacobson, 1997; Horton and Perona, 1998b; Jen-Jacobson, 2000; Watrob et al., 2001; Horton et al., 2002; Parry et al., 2003; Kurpiewski et al., 2004; Hiller et al., 2005).

Many type II restriction endonucleases are known to recognize a symmetrical site within DNA known as a palindrome, however, recently it is becoming increasingly appreciated that this class of enzymes is composed of proteins with a large degree of structural and functional diversity. The two-fold symmetrical relationship of the DNA-protein interface is consistent with the well-supported half-site rotational symmetry model of recognition (Kurpiewski et al., 2004). Functionally, type II restriction enzymes are typically a homodimer where the monomers interface symmetrically with each half-site of the recognition pattern (Alves et al., 1982). Although these enzymes have a high degree of specificity for their cognate recognition sequence, the specificity is not absolute. Extensive, pioneering biophysical studies have demonstrated the molecular basis of non-canonical DNA cleavage: the efficiency of non-canonical substrate cleavage can be predicted once the energetic penalties associated with the changes to the substrate are appreciated (Lesser et al., 1990). Restriction enzymes for which detailed studies of cognate and noncognate substrates have been performed, such as EcoRI, have revealed a dynamic range of dissociation and cleavage rate constants. Cleavage rate constants for noncognate substrates can vary from greater than that of the cognate substrate to a rate so slow that detectable cleavage is not generally observable (Thielking et al., 1990). For the enzymes EcoRV, EcoRI, BamHI the rate of noncognate substrate cleavage has been shown to be exquisitely dependent upon many variables, most notably: in vitro conditions, pH, salt concentrations (Engler et al., 1997), divalent ion concentrations (Thielking et al., 1992; Vermote and Halford, 1992; Vipond and Halford, 1995;

Engler et al., 2001; Sam et al., 2001; Kurpiewski et al., 2004), the presence of glycerol or other water occluding chemicals (Horton and Perona, 1998b; Sapienza et al., 2005; Sapienza et al., 2007), and the context of the site (adjacent nucleotides and the proximity to other recognition sites or DNA termini) (Alves et al., 1984; Engler et al., 1997; Jen-Jacobson, 1997; Horton and Perona, 1998a; Engler et al., 2001). These factors have widely variable thermodynamic contributions directly and indirectly to both the recognition and cleavage steps of restriction endonucleases (Lesser et al., 1990; Martin et al., 1999; Jen-Jacobson et al., 2000). While it is believed that most restriction enzymes share a common conserved functional protein core (Niv et al., 2007), there is very little primary amino acid sequence homology (Kovall and Matthews, 1999). Even among isochizomers, structural requirements for cleavage vary drastically (Wolfes et al., 1985). The lack of structural conservation suggests that mechanistic inferences between these enzymes should be made with extreme caution. Biophysical data pertaining to the mechanism of recognition and cleavage among type II restriction enzymes is currently limited to a few well-studied enzymes, making it exceptionally difficult to predict mechanistic characteristics or the rate of non-cognate substrate cleavage of the many lesser-studied type II restriction enzymes.

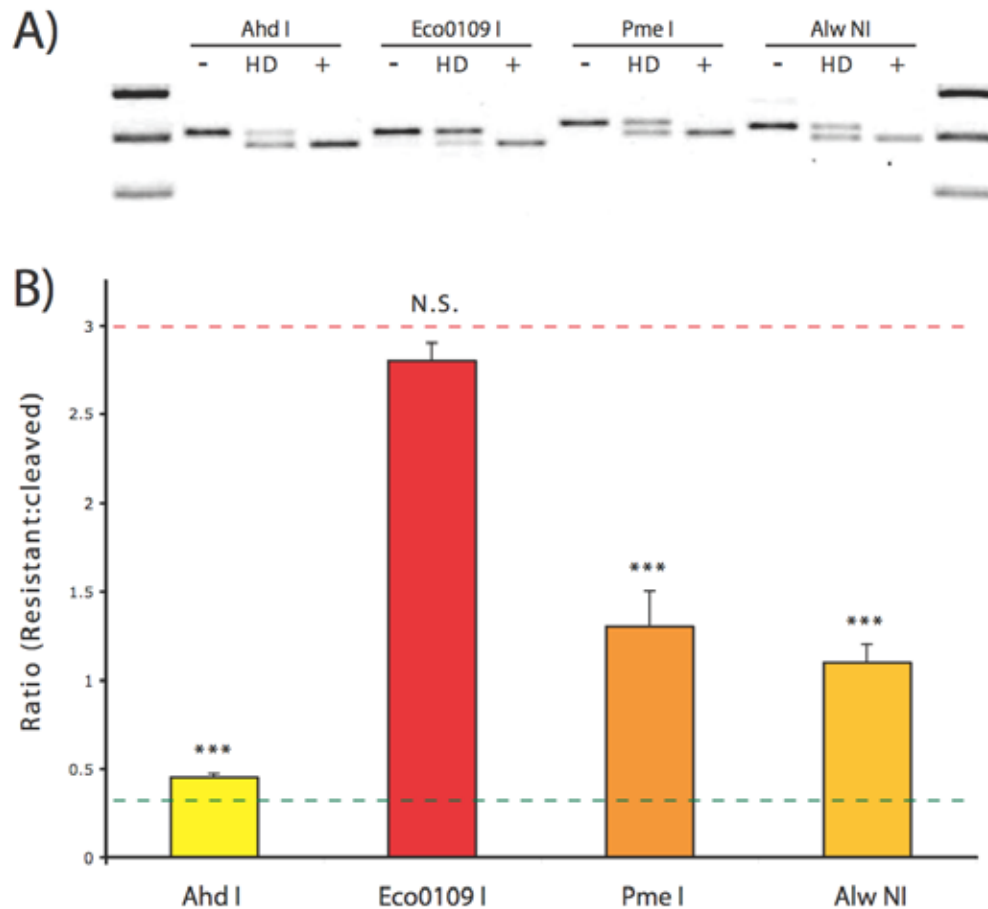
Heteroduplex DNA is not common *in vivo*: DNA mismatches are readily detected and repaired (Modrich, 1995). However, mismatch heteroduplex DNA is commonly found *in vitro*, especially when there is heterogeneity in the DNA and PCR is used to amplify the DNA. This had been elegantly demonstrated by T4 endonuclease VII sensitivity of mixed template PCR amplicons (Jenkins et al., 1999). T4 endonuclease VII has been shown to efficiently cleave mismatched DNA heteroduplexes (Golz et al., 1998a; Golz et al., 1998b). Assays utilized to quantify biological phenomena, such as the frequency of mutation, recombination or RNA editing, or common molecular biology techniques, such as site-directed mutagenesis protocols, must address the issue of heteroduplex DNA when PCR amplification is employed. However, often assays utilize PCR and subsequently quantify the frequency of restriction endonuclease cleavage as a metric of a biological phenomenon, without controlling for heteroduplex DNAs. Numerous reports quantify restriction endonuclease cleavage in assays that ignore heteroduplex DNA formation or assume (sometimes this assumption is explicitly stated) that heteroduplex DNA will not be a viable substrate for double strand DNA cleavage (Iland and Todd, 1992; Paschen and Djuricic, 1994; Belcher and Howe, 1997; Judo et al., 1998; Serth et al., 1998; Jenkins et al., 1999). Protocols used to perform *in vitro* site-directed mutagenesis, namely the unique site elimination (USE) method, also assume heteroduplex DNA will not be cleaved by restriction endonucleases (Deng and Nickoloff, 1992; Ling and Robinson, 1997; Clontech Laboratories, 2001). The assumption that a heteroduplex restriction sequence is not a viable substrate for cleavage is inconsistent with available structural and biophysical data suggesting that many non-cognate substrates may be efficiently cleaved *in vitro*, such as has been shown for the EcoRI endonuclease (Thielking et al., 1990). We investigated whether cleavage of mispaired heteroduplex

DNA substrates was unique to EcoRI or was more widespread among commonly used restriction endonucleases. Both position and type of non-cognate change within the recognition sequence are used to empirically probe the DNA-protein interface for critical alterations that either permit or abrogate efficient cleavage.

## Results

### Heteroduplex DNA cleavage revealed by PCR assay

Site selectivity of restriction endonucleases is a key property that has made these proteins extraordinarily useful in molecular biology. Despite their widespread use experimentally, a detailed understanding of the biophysical basis of this specificity has only been studied for a few enzymes, most notably EcoRI, EcoRV and BamHI (Perona, 2002). Due to the large degree of size and structural diversity of restriction endonucleases (Pingoud et al., 2005), inferences among enzymes should only be made with caution. *In vivo*, restriction enzyme recognition sites in DNA are almost always perfect cognate homoduplexes where the site (often a palindrome) exists on both strands of the DNA. Typically recognition of the two cognate DNA half-sites by the endonuclease (most often a dimer) results in specific double-stranded cleavage. *In vitro*, heteroduplex DNA can be quite common where one half-site may exist but the other half-site has a mismatch (thus the sequence is correct on only one strand). We asked whether such non-cognate sites would be recognized by restriction endonucleases and double-strand cleavage would occur. We developed a PCR-based assay to test whether heteroduplex restriction enzyme recognition sites with a single nucleotide mispair could be cleaved by various endonucleases. The assay utilizes a forward primer encoding the restriction endonuclease recognition site or a non-cognate site bearing a single nucleotide substitution and a reverse primer to produce an ~ 300 based-pair amplicon from a plasmid template. The amplicons were quantified by absorption spectrometry, mixed equimolar, denatured, and annealed to ensure optimal formation of heteroduplex DNA (~50%). The annealed amplicons were utilized as substrate in a restriction endonuclease reaction and the products were resolved using agarose gel electrophoresis (Figure 1A). Control homoduplex samples with (+) and without (-) the cognate restriction recognition site were also tested. Because the heteroduplex substrate is ~ 1/4 homoduplex +, ~1/4 homoduplex -, and ~1/2 heteroduplex DNA, a cleavage pattern of 3/4 cutting and 1/4 resistant is consistent with efficient heteroduplex cleavage. The Eco0109I restriction endonuclease did not significantly cleave the heteroduplex DNA (Figure 1A). However, the other three enzymes examined, AhdI, PmeI and AlwNI, all produced a fragment pattern consistent with cleavage of some or all of the heteroduplex DNA (Figure 1A). Densitometry was performed on reactions performed in triplicate and Chi Square analyses were performed to determine whether the cleavage pattern (ratio of resistant to cleaved) matched that expected if only the homoduplex + DNA were cleaved. Only, Eco0109I produced such a pattern, whereas, AhdI, PmeI and AlwNI deviated significantly from that expected, suggesting the heteroduplex DNA also underwent significant double-strand cleavage (Figure 1B).



**Figure 1: PCR-based assay for heteroduplex DNA cleavage assay.** Oligonucleotide PCR amplicons ~300bp in length were amplified containing the recognition site (+) or with a single nucleotide substitution (-). These amplicons were used to generate heteroduplex DNA (HD) by annealing an equimolar mixture of + and - products. (A) Each enzyme was tested for cleavage of their recognition site (+), a mutated site (-), and heteroduplex substrates. All enzymes efficiently cleaved the positive control (+) and did not cut the mutated site (-). Mutations examined were AhdI #11, Eco0109I #31, PmeI #3V and AlwNI #1V mutations were examined. Nomenclature of mutation is the position number in the enzyme recognition sequence and type of change (transition = I and transversion = V) and is used throughout. (B) Quantification of the ratio of resistant to cleaved DNA is shown. Enzymes unable to cleave heteroduplex DNA are expected to produce a ratio of resistant to cleaved products equal to 3 (red dashed line), whereas, those efficiently cleaving heteroduplex DNA are expected to give a ratio of 0.33 (green dashed line). Only Eco0109I produced a pattern that did not deviate significantly from that expected for not cutting heteroduplex ( $p > 0.95$ ). AhdI, PmeI and AlwNI each deviated significantly from that expected for no heteroduplex cleavage (\*\*\* is  $p < 0.001$ ). Statistical analyses were Chi Square tests,  $n = 3$  for each enzyme.

#### Oligonucleotide assay of heteroduplex DNA cleavage

We aimed to develop a more sensitive and quantitative, yet still non-radioactive, assay of mismatched non-cognate heteroduplex DNA cleavage using annealed oligonucleotides. The oligonucleotide assay utilizes custom synthesized and annealed oligonucleotides as the substrate. Reaction products are resolved using non-denaturing polyacrylamide gel electrophoresis (PAGE) and post stained with ethidium bromide for fluorescence analysis. We hypothesized the oligonucleotide heteroduplex assay would confirm our previous results with the PCR assay and yield more sensitive quantitative results, due to the homogeneity of the substrate. We examined three of the enzymes studied using the PCR assay (AhdI, Eco0109I, and PmeI) and ten additional commonly used restriction enzymes, including: BamHI, EcoRI, EcoRV, HindIII, PstI, SacI, Sall, SpeI, XhoI, XbaI, all for heteroduplex DNA cleavage with a single mismatch. The oligonucleotide assay demonstrated SpeI, AhdI, PmeI and SacI restriction endonucleases cleave the heteroduplex substrate tested bearing a single non-cognate mismatch (Figure 2 and data not shown). XhoI,

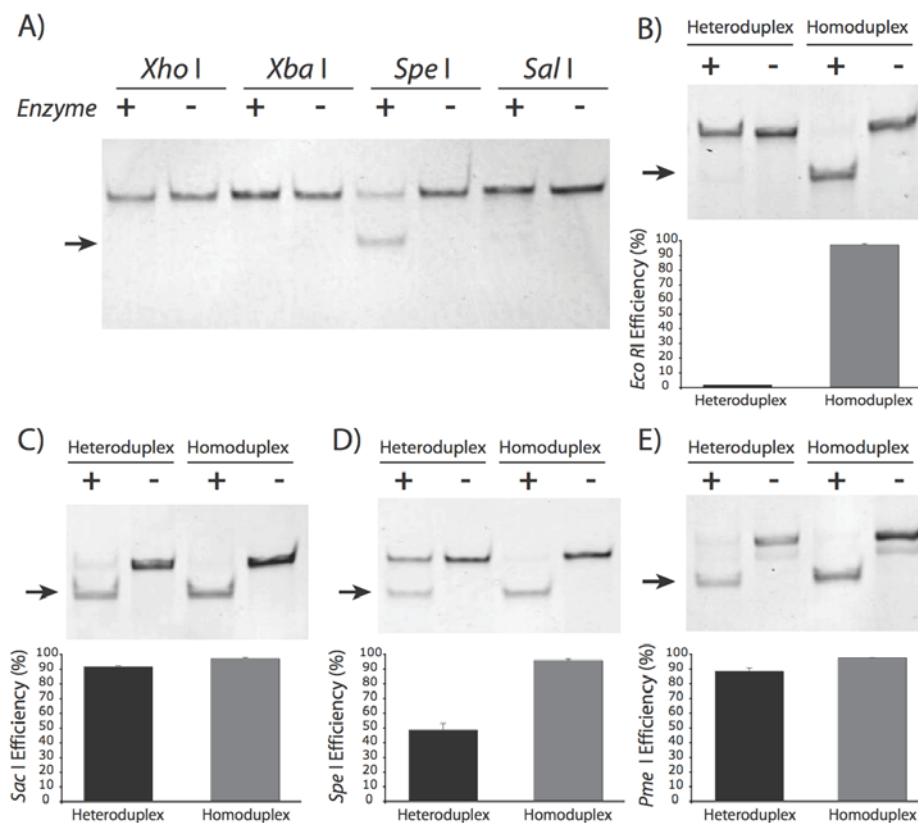
XbaI, EcoRI and Sall failed to efficiently cleave heteroduplex DNA (Figure 2A and 2B). Densitometry was performed to estimate cleavage efficiency with that of the homoduplex DNA bearing the site. In all cases, the minus enzyme lane did not reveal cleavage products and the homoduplex was robustly cleaved. The assays revealed SpeI, PmeI and SacI produced robust heteroduplex cleavage with PmeI and SacI approaching the efficiency of homoduplex cleavage in this assay (Figure 2C-E). Importantly, results obtained using the PCR-based heteroduplex DNA cleavage assay were confirmed with the oligonucleotide assay (Figure 2 E and data not shown).

#### Position and mutation type effect on heteroduplex DNA cleavage

Biophysical studies of certain restriction enzyme recognition sites have established a hierarchy of non-cognate substrate viability from better than the cognate substrate to not a viable substrate at all (Perona, 2002). Mismatched substrates for the EcoRI enzyme are generally considerably better substrates than those

bearing the alteration on both strands of the DNA. However, the specific alteration in mismatched sequences resulted in widely varied cleavage rates spanning numerous orders of magnitude (Thielking et al., 1990). Additionally, the position of the alteration, type of alteration, destabilization of the duplex, perturbation of the DNA backbone and even flanking positions outside the recognition sequence have all been shown to affect substrate structure and or cleavage efficiency (Alves et al., 1984; Wenz et al., 1996; Engler et al., 1997; Jen-Jacobson, 1997; Horton and Perona, 1998a). We examined the effect of position and mutation type on heteroduplex DNA cleavage while maintaining the context of the site constant. There are 18 possibly single mismatch substrates for a restriction endonuclease with a hexanucleotide recognition sequence when restricting the analysis to the common four DNA nucleotides. We reasoned that examining six mismatched substrates for each restriction endonuclease would efficiently estimate the frequency with which mismatched non-cognate substrates are cleaved. To initially probe the requirements of heteroduplex DNA cleavage activity, we generated alterations in each position, testing individually three transitions (I) and three transversions (V) in the recognition site (alternating where practical) and examined cleavage patterns using the oligonucleotide assay (Figure 3). Most of the enzymes examined produced readily detectable double-stranded cleavage fragments and, as predicted, none of the enzymes

cleaved all of the mismatched heteroduplex DNA substrates tested (Figure 3 and Table 1). In all cases, double-strand cleavage of the homoduplex control was observed to be efficient (Figure 3). Importantly, none of the enzymes cleaved all of the mismatched substrates, ruling out contamination with a non-specific mismatch endonuclease. Interestingly, *SpeI* was found to robustly cleave heteroduplex DNA with transitions at positions 1, 3 or 5, but transversions at any position were cleaved less efficiently (Figure 3A). Although there was no strong position effect observed for *SpeI*, a mutation type preference was evident. In contrast, *SacI* revealed a strong position effect but no mutation type preference was observed (Figure 3B). *SacI* robustly cleaved heteroduplex DNA with transitions at positions 3 or 6 and transversions at positions 1 or 2, however, neither the transition at position 4 or the transversion at position 5 were viable substrates (Figure 3B). We extended our heteroduplex substrate position effect experimentation to include six other restriction endonucleases: *XbaI*, *EcoRI*, *EcoRV*, *XhoI*, *BamHI*, and *HindIII*. These experiments identified numerous additional sites where heteroduplex changes either permitted cleavage or abrogated cleavage (Figure 3C-H and Table 1). Some heteroduplex conditions, such as *EcoRV* 4I/+ and 6I/+, *EcoRI* 1V/+ and 2I/+, *XhoI* 1V/+ and 2I/+, and *BamHI* 3I/+ and 6V/+, were efficiently cleaved, whereas, other heteroduplex substrates revealed minimal to no cleavage in these assays.

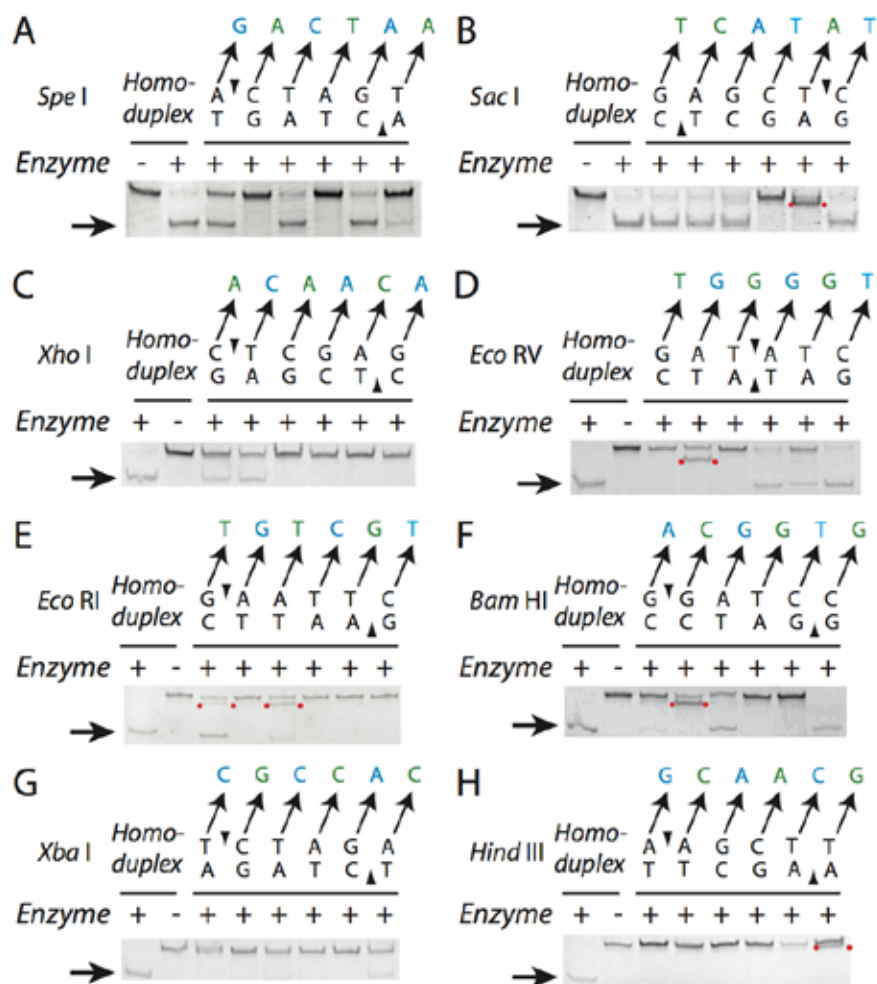


**Figure 2: Oligonucleotide heteroduplex DNA cleavage assay.** Annealed oligonucleotides bearing the restriction recognition site (homoduplex = +/+) or lacking the site on one strand (heteroduplex = HD) were tested for DNA cleavage with various restriction enzymes. (A) *SpeI* #11 / + heteroduplex results in double stranded cleavage, producing a ~25 bp fragment. *XhoI* #6I/+, *XbaI* #2I/+, and *SalI* #2I/+ heteroduplexes show no evidence of cleavage. (B-E) Quantified cleavage efficiency for homoduplex and heteroduplex DNAs from 3 or more independent reactions. (B) *EcoRI* #5V/+ heteroduplex is minimally cleaved. (C) *SacI* #1V/+ heteroduplex is efficiently cleaved. (D) *SpeI* #11/+ heteroduplex resulted in ~ 50% cleavage relative to homoduplex controls. (E) *PmeI* #6I/+ heteroduplex also cleaved efficiently. + and - indicate whether an enzyme was included in the restriction endonuclease reaction. +/+ substrate resulted in efficient cleavage for all enzymes examined. Arrows indicate cleavage fragment.

**Table 1: Summary of heteroduplex DNA cleavage activity for each restriction endonuclease.** The positions examined for each type of non-cognate change are shown in parentheses and whether these were cleaved is indicated. G.C.T. (Glycerol concentration test) indicates whether heteroduplex cleavage requires or is stimulated by high glycerol concentrations.

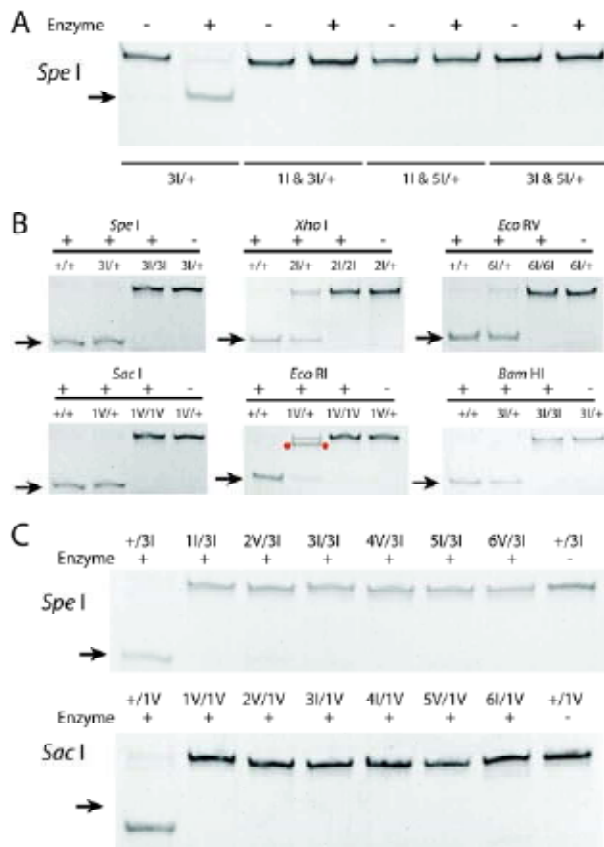
Enzyme	Transition (I)	Transversion (V)	Assay(s)	G.C.T.
AlwNI	N.E.	Yes (1)	PCR	N.E.
AhdI	Yes (1)	Yes (1)	PCR & Oligonucleotide	N.E.
BamHI	No (1,5) Yes (3)	No (2,4,5) Yes (6)	Oligonucleotide	No
Eco0109I	No (3)	No (3)	PCR & Oligonucleotide	N/A
EcoRI	Yes (5 <sup>‡</sup> ) No (2,4,6)	Yes (1 <sup>*</sup> ) No (3,5)	Oligonucleotide	Yes
EcoRV	Yes (3,4,6) No (2)	Yes (5) No (1,3)	Oligonucleotide	No
HindIII	No (1,2,3,5)	No (2,4,6)	Oligonucleotide	N/A
PmeI	Yes (5,6)	Yes (5)	PCR & Oligonucleotide	N.E.
PstI	No (3)	No (3)	Oligonucleotide	N/A
SacI	Yes (3,6) No (4)	Yes (1,2) No (5)	Oligonucleotide	No
Sall	No (2)	No (2)	Oligonucleotide	N/A
SpeI	Yes (1,3,5)	No (2,4,6 <sup>‡</sup> )	Oligonucleotide	No
XbaI	No (1,2,3,5)	No (2,4) Yes (6)	Oligonucleotide	Yes
XhoI	Yes (2) No (4,6)	Yes (1,6) No (3,5)	Oligonucleotide	No

Note: The assay indicates the PCR-based heteroduplex cleavage (PCR) and / or the oligonucleotide heteroduplex cleavage (Oligo.) assays. ‡ = modest cleavage detected is 5-15%. \* Heteroduplex cleavage at this position is glycerol dependent. No cleavage indicates less <5% of the appropriate fragment size was observed. N/A = not applicable. N.E. = not examined.



**Figure 3: Position effect of heteroduplex DNA cleavage.** Position and mutation type affect heteroduplex cleavage differently for each enzyme examined. Three transitions (blue) and three transversions (green) were examined for each enzyme. Heteroduplex substrates were examined at all six positions for: (A) SpeI, (B) SacI, (C) XhoI, (D) EcoRV, (E) EcoRI, (F) BamHI, (G) XbaI, and (H) HindIII. Each profile was tested two or more times for each enzyme and representative data are shown. Arrows indicate cleavage fragment. Red bullets highlight intermediate DNA fragment.





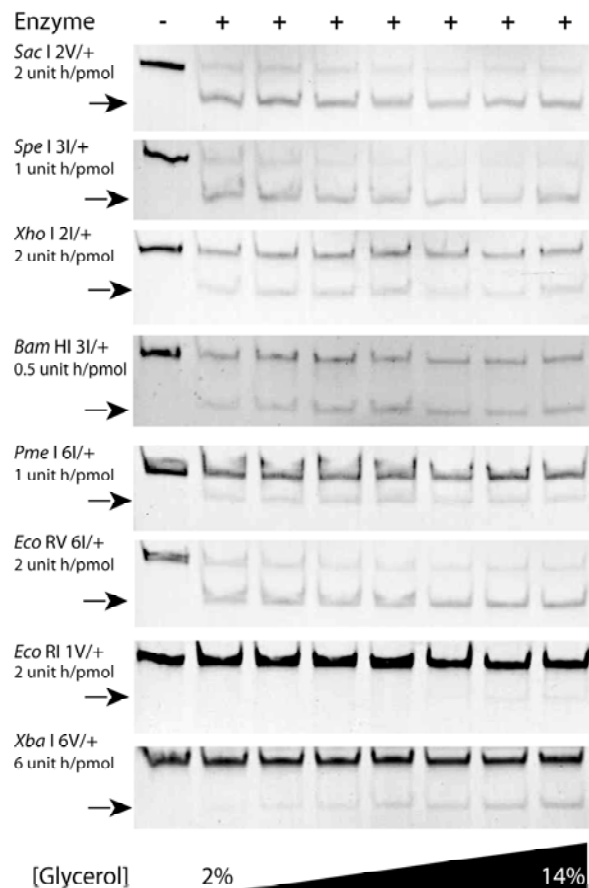
**Figure 4: Double mutant non-cognate cleavage analysis.** (A) SpeI cleaves several single non-cognate 1I/+, 3I/+, or 5I/+ heteroduplex DNAs (Figure 3). Heteroduplex substrates bearing all combinations of two of these non-cognate alterations in a single strand were examined as substrates for the SpeI endonuclease and failed to produce any detectable cleavage fragments. (B) Substrates bearing the homoduplex non-cognate sequence were examined for double-strand DNA cleavage with several enzymes using the oligonucleotide assay. +/+ and heteroduplex substrates produced double-stranded cleavage products (Arrow). Red bullets indicate intermediate sized fragment. (C) Substrates with two non-cognate changes, one alteration on each strand of DNA, were examined as substrates for the SpeI and SacI endonuclease. The single mutation heteroduplex substrates were efficiently cleaved, as before, however, substrates lacking the cognate signal on at least a single strand failed to produce significant DNA cleavage fragments.

Several of the mispaired substrates produced an intermediate sized product in an enzyme dependent manner (Figure 3, red bullets). Control experiments with annealed oligonucleotide standards demonstrated these are consistent in size with single strand DNA cleavage. Kinetic studies with mismatched EcoRI substrates have shown a 400,000 fold range of cleavage rates between the canonical and certain non-canonical half-sites (Thielking et al., 1990), which is consistent with the apparent single strand cleavage observed for certain substrates using this oligonucleotide assay.

#### Double mismatch substrate analysis in cis and trans orientations

The finding that many single nucleotide mismatch substrates are susceptible to double strand DNA cleavage suggests the energetic penalties associated with the mismatch are minimal or at a minimum are often overcome under commonly used, multiple turnover, *in vitro* conditions. Heteroduplex DNAs with individual transition mismatches at positions 1, 3, or 5 in the SpeI recognition sequence resulted substrates susceptible to

cleavage using the oligonucleotide assay (Figure 3). Since each mismatch mutation resulted in cleavage, we asked whether any combination of the two transitions in cis would produce detectable cleavage products. As before, the 3I/+ control reaction produced robust cleavage, however, none of the double missense substrates in cis produced significant cleavage fragments (Figure 4A). For several enzymes that produced robust cleavage of specific mismatch heteroduplex substrates, we examined substrates bearing this mismatch alteration in trans to determine their viability as substrates. In all cases the positive control reactions produced the predicted cleavage fragments, however, the double mismatch alteration in trans abrogated cleavage (Figure 4B). We examined the panel of six mutations all in trans with the SpeI 3I alteration and in trans with the SacI 1V alteration to determine whether any of these combinations resulted in a viable substrate using the oligonucleotide assay. Although the single mismatch positive controls produced robust cleavage products, none of the double mutations in trans resulted in significant cleavage (Figure 4C).



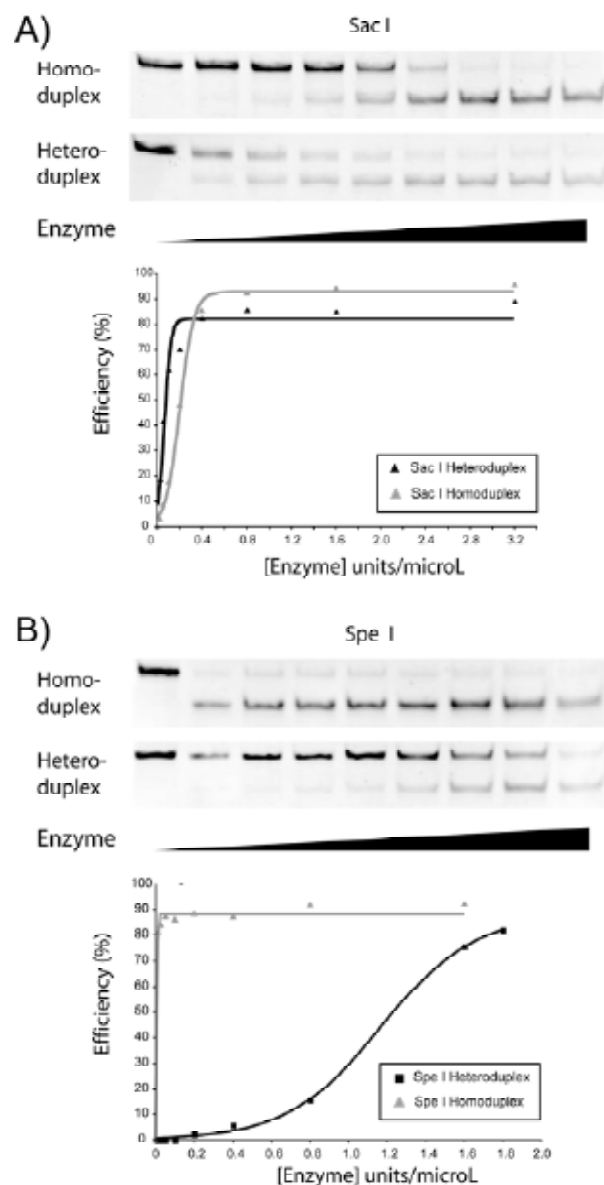
**Figure 5: Effect of glycerol on heteroduplex substrate cleavage.** Glycerol concentrations 2-14% were examined for the SacI 2V/+, SpeI 3I/+, XhoI 2I/+, BamHI 3I/+, PmeI 6I/+, EcoRV 6I/+, EcoRI 1V/+, and XbaI 6V/+ heteroduplex DNA substrates. SacI, SpeI, XhoI, BamHI, PmeI, and EcoRV efficiently produced double-stranded DNA fragments independent of glycerol concentration. Arrows indicate cleavage fragment. In contrast, EcoRI cleavage of the 1V/+ heteroduplex DNA was strictly glycerol dependent and XbaI cleavage of the 6V/+ substrate did not require high glycerol but was stimulated by glycerol. The total unit hours used per picomole of substrate is provided.

### Glycerol effect on mismatch heteroduplex cleavage.

We examined the effect of glycerol on cleavage of several single nucleotide mismatch heteroduplex DNAs. Glycerol and other water occluding chemicals can enhance cleavage of non-cognate substrate cleavage by some restriction endonucleases. Reduced specificity of some restriction enzymes under conditions of high glycerol may result from the dramatic reduction in the effective reaction volume and or by the removal of water molecules from the enzyme-substrate interface that are required for specificity. We performed our oligonucleotide assay with varying concentrations of glycerol (2-14%) to determine whether heteroduplex DNA cleavage required or was stimulated by high glycerol concentrations. The reaction volume was increased 3 fold for these experiments and samples were taken at multiple time points, reducing the total reaction time 25-85%. In all cases, the most informative partial digestion is shown (Figure 5). Only the EcoRI 5I/+ and 1V/+ sites were found to require high glycerol concentration to produce detectable cleavage. The XbaI 6V/+ DNA did not require high glycerol concentration but glycerol appeared to stimulate heteroduplex DNA cleavage of this substrate. The remaining enzymes tested, including SacI, SpeI, XhoI, BamHI, PmeI and EcoRV, neither required high glycerol for heteroduplex DNA cleavage nor were detectably stimulated by glycerol for the substrate examined (Figure 5).

### Heteroduplex cleavage is especially abundant under multiple-turnover conditions.

The prevalence of commonly used restriction enzymes that cleave heteroduplex DNA has broad implications due to their extensive use in research laboratories. This is especially true of restriction enzymes used in assays that quantify the cleavage of PCR or RT-PCR amplicons as a metric of biological phenomena. We examined in more detail the conditions required to observe efficient cleavage of mismatched heteroduplex DNA substrates. The SacI 1V/+ and SpeI 1I/+ substrates were utilized and compared to cognate control substrates using oligonucleotide cleavage assays with increasing amounts of enzyme (Figure 6A). We discovered that the SacI restriction enzyme cleaved heteroduplex mismatched DNA substrate at a lower enzyme concentrations than the cognate homoduplex substrates (Figure 6A and 6B). The enzyme concentration required to achieve 50 % of maximal cleavage was used to compare the substrates. The amount of SacI required to cleave heteroduplex DNA ( $0.07 \pm 0.06$  units enzyme/ $\mu$ L) was significantly less than that required to cleave the control DNA substrate ( $0.20 \pm 0.02$  units enzyme/ $\mu$ L) under multiple turnover conditions (Student's t-test,  $p < 0.0001$ ,  $n=3$ ). In contrast, the SpeI restriction enzyme cleavage of heteroduplex DNA substrate was significantly right shifted relative to the cleavage of the cognate homoduplex control (Figure 6B). The enzyme concentration required to achieve 50% of maximal cleavage was dramatically and significantly increased for the SpeI 1I/+ heteroduplex ( $1.05 \pm 0.02$  units enzyme/ $\mu$ L) over the homoduplex cognate control ( $4.0 \times 10^{-5} \pm 7.4 \times 10^{-5}$  units enzyme/ $\mu$ L) substrate under multiple turnover conditions (Student's t-test,  $p < 1.0 \times 10^{-6}$ ,  $n=3$ ).



**Figure 6: Enzyme concentration analysis of heteroduplex cleavage activity.** Oligonucleotide DNA cleavage assays were performed with increasing units of enzyme and cognate homoduplex or heteroduplex substrate. (A) SacI reveals robust cleavage of both heteroduplex and cognate substrates. (B) SpeI reveals reduced cleavage of heteroduplex substrate, whereas, robust cleavage of the cognate substrate was observed. Cleavage efficiencies from three independent trials with each substrate were obtained. Typical results were plotted.

### Discussion

Restriction endonucleases have revolutionized modern biology. The extraordinarily high degree of specificity with which they cleave specific, well-characterized DNA sequences is paramount to their utility. In addition to their use as a tool to the molecular biologist, these enzymes have proven a valuable model system to study the nature of the protein-DNA interaction that underlies their specificity. Although detailed biophysical studies have only been performed with a few endonucleases, these and other studies have provided a foundational understanding of the energetic basis for site specificity that is key to elucidating a myriad of other essential cellular process regulated by specific protein-DNA

interactions. Although the restriction enzyme - DNA interaction is highly specific, biophysical studies have demonstrated the basis of this specificity and its limitations. Elucidating the conditions of non-cognate cleavage has proven a valuable way to better understand specificity of protein-DNA interactions. *In vitro* conditions, types of non-cognate changes to the recognition sequence and flanking sequence, and site-directed mutagenesis of the enzymes that alter their specificity have been used to provide insight into this process. An interesting class of non-cognate DNA is the mispaired substrate formed from heteroduplex DNA with the cognate sequence on one strand and a single non-cognate change on the other. Although mispaired heteroduplex has not been extensively studied, pioneering work with one enzyme, EcoRI, has demonstrated efficient cleavage of certain mispaired heteroduplex EcoRI sites (Thielking et al., 1990).

Mispaired heteroduplex DNA has several relevant attributes: (1) due to avid repair pathways, mispaired DNA is not common *in vivo*; (2) mismatched DNA is extremely prevalent *in vitro*, especially in procedures following PCR amplification; (3) mispaired DNA alters the normal helical structure of the molecule; (4) mispaired DNA substrates have not been the focus of study with many endonucleases; and (5) the misconception that heteroduplex DNA will not cleave or that such cleavage is a unique feature of EcoRI seems prevalent. The finding that many single nucleotide mismatch substrates remain viable for double strand DNA cleavage with many commonly-used restriction enzymes is in accord with previously published EcoRI mismatch substrate data. These data suggest that the energetic penalties associated with the non-cognate change are minimal or at least are readily overcome under commonly used multiple turnover conditions *in vitro*. Importantly, in cases where restriction endonuclease cleavage is used as a biological assay, reaction conditions equivalent to a several fold over-digestion, such as we have reported here, are often used to avoid the confound of incomplete digestion. These data suggest that under such conditions heteroduplex cleavage DNA is an important consideration that should not be overlooked.

Our data demonstrate that the cleavage of mispaired heteroduplex DNA is a common property observable with many commercially available restriction enzymes and is not unique to EcoRI. The fact that EcoRI is known to exhibit "star" or noncognate cleavage activity under certain *in vitro* conditions, notably elevated glycerol concentrations, may have contributed to the misconception that mispaired cleavage was a unique property of this enzyme. Extending our knowledge of mispaired heteroduplex cleavage to 13 additional restriction enzymes is especially relevant given the extensive use of restriction enzymes in assays and techniques following PCR. Examples of protocols where heteroduplex DNA is of concern include, but are not limited to, assays of RNA editing, recombination and mutation frequency and techniques such as site-directed mutagenesis (unique site elimination), and methods to detect spontaneous or rare mutagenic events (Deng and Nickoloff, 1992; Iland and Todd, 1992; Paschen and Djuricic, 1994; Belcher and Howe, 1997; Ling and

Robinson, 1997; Judo et al., 1998; Serth et al., 1998; Jenkins et al., 1999; Clontech Laboratories, 2001). In many cases, heteroduplex DNA is not addressed or controlled for, however, in others it is specifically stated that the heteroduplex DNA will be resistant to cleavage without data to support this assertion (Iland and Todd, 1992; Judo et al., 1998; Serth et al., 1998; Jenkins et al., 1999). When restriction endonuclease cleavage data are quantified and the data are attributed to a biological phenomenon without controlling for the generation of heteroduplex or utilizing an appropriate standard curve to correct for this assumption, the data are potentially erroneous. The extent of the error in the data depends upon the amount of heteroduplex present and the efficiency of heteroduplex cleavage, which can vary wildly dependent upon the precise substrate and enzyme used. Our goal is not to dispute previously published findings and thus we did not specifically examine any of the enzymes with the precise substrates utilized by other researchers we have cited. We do believe our data adequately demonstrate that most commonly used restriction enzymes are capable of cleaving certain mispaired heteroduplex DNAs and that empirical data is required to determine whether the efficiency of substrate cleavage will interfere with a specific assay or protocol. Thus, assumptions about whether heteroduplex DNA substrates will cleave without appropriate controls to prevent their formation or inclusion in the analysis, or to appropriately standardize the data, are generally quite problematic.

## Materials and Methods

**PCR Assay.** Forward oligonucleotide primers 24 to 36 nucleotides in length were designed to a plasmid clone (DES TOPO, Invitrogen) containing the specific enzyme recognition site or the specific enzyme recognition site with a single nucleotide substitution each with 15 5' flanking nucleotides. A reverse oligonucleotide primer was designed starting ~300 bp downstream on the plasmid. Oligonucleotides used with this assay are provided (Table 2). Oligonucleotides were synthesized by Integrated DNA technologies for this and all other experiments. PCR amplification was performed using standard methods and the resulting ~315 bp amplicons were verified on an agarose gel. After quantification with absorbance spectrometry, the PCR fragment containing the single nucleotide mutation (-) was mixed equimolar with the PCR fragment containing the restriction enzyme recognition site (+). The mixture was denatured for 5 minutes at 95 °C and annealed on ice to form heteroduplex (HD) DNA. Typically a 20X restriction endonuclease treatment of 1 microgram DNA (+, -, or HD) was performed per the manufacturer's instructions for each enzyme examined. For this and all other experiments, commercially purified restriction enzymes were used exclusively (New England Biolabs and Promega) in their recommended buffer at the optimal temperature. BSA was included when recommended. The reactions were separated by agarose gel electrophoresis (2.5%), stained with ethidium bromide and densitometry was performed on sub-saturating images (NIH ImageJ). Raw intensity was corrected using base pair weighting. The assay was performed in triplicate and Chi Square tests were performed to determine if the fragment



patterns deviated from that expected for no heteroduplex cleavage.

**Oligo Assay.** Forward oligonucleotides 36 nucleotides in length were designed containing the specific enzyme recognition site and the specific enzyme recognition site with a single nucleotide substitution with ~10 nucleotides flanking the site on the 5' end and ~20 nucleotides flanking on the 3' end. The complementary reverse oligonucleotide was designed containing the enzyme recognition site. Oligonucleotides used in this assay are provided (Table 3). Paired oligonucleotides were mixed equimolar, denatured for 5 minutes at 95 °C and annealed with cooling to 4°C at a rate of 0.1 °C / second. Restriction endonuclease treatment of ~10 pmoles of duplex DNA (+ or HD) was performed with 25-50 units of enzyme in a 50 microliter total volume reaction per the manufacturers' instructions. The reactions were covered with ~20 microliters mineral oil to prevent evaporation and incubated at the optimal temperature for 4 hours. 20% non-denaturing polyacrylamide gel electrophoresis (PAGE) was used to resolve ~ 1/3 of the reaction volume. Xylene cyanol and bromophenol blue were used as approximate size markers and to preserve orientation. Control experiments were performed to ensure adequate separation of unannealed oligos and double strand cleavage products. Typically, electrophoresis was performed at 150V for 75 to 180 minutes. The gel was

stained for 45 minutes with 0.05% ethidium bromide solution and sub-saturation images were obtained under UV light. The percentage of double-strand DNA cleavage was calculated using densitometry and NIH ImageJ. Raw intensity was corrected using base pair weighting. Greater than 5% heteroduplex DNA cleavage was deemed significant. The glycerol concentration assay used the standard oligo assay with the following exceptions: a 150 microliter reaction volume was used, varying glycerol concentration of 2-14 % was used, the reaction was incubated for 3 hours, the glycerol concentration of 1/3 of the reaction was adjusted to 6% with cold water or 33% glycerol, and samples were concentrated to reduce their volume, as needed.

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**Table 2: Oligonucleotides used in the PCR heteroduplex assay.** PCR primers are designated forward (end in F) or reverse (end in R). Restriction recognition sites are underlined and correspond to the known site (+) or a mutated site (-). The mutation position in the recognition site (#) and the type of mutation, transversion (V) or transition (I) are shown.

Enzyme	Name	Oligonucleotide Sequence
<i>AhdI</i>	AhdI-PCR	ATG AAG CCA TAC CAA ACG ACG AGC
<i>AhdI</i>	AhdI+PCR	CGT TCA TCC ATA GTT GCC <u>TGA CTC CCC GTC</u> GTG TAG
<i>AhdI</i>	AhdI-PCR(#11)F	CGT TCA TCC ATA GTT GCC <u>TAA CTC CCC GTC</u> GTG TAG
<i>AlwNI</i>	AlwNI-PCR	AAA CCC GAC AGG ACT ATA AAG ATA
<i>AlwNI</i>	AlwNI+PCR	GCT CTG CTA ATC CTG TTA <u>CCA GTG GCT GCT</u> G
<i>AlwNI</i>	AlwNI-PCR(#1V)F	GCT CTG CTA ATC CTG TTA <u>CAA GTG GCT GCT</u> G
<i>Eco0109 I</i>	Eco0109-PCR	TTA CTT TCA CCA GCG TTT CTG GGT
<i>Eco0109 I</i>	Eco0109+PCR	ATC ACC GAA ACG CGC GAA CGA <u>AAG GGC CTC</u> GTG ATA CGC CT
<i>Eco0109 I</i>	Eco0109-PCR(#3I)F	ATC ACC GAA ACG CGC GAA CGA <u>AAG AGC CTC</u> GTG ATA CGC CT
<i>PmeI</i>	PmeI-PCR	TAC GCC AAG CTT GCA TGC CTG CAG GTC GAC TGA TCA
<i>PmeI</i>	PmeI+PCR	TAT GAG CTC CTC ATT GAT TAA <u>GTT TAA ACC</u> CGC T
<i>PmeI</i>	PmeI-PCR(#5V)F	TAT GAG CTC CTC ATT GAT TAA <u>GTT TCA ACC</u> CGC T

**Table 3: Oligonucleotides used in the Oligonucleotide heteroduplex assay.** Oligonucleotides are designated top (end in T) or bottom (end in B). Restriction recognition sites are underlined and correspond to the known site (+) or a mutated site (-). The mutation position in the recognition site (#) and the type of mutation, transversion (V) or transition (I) are shown.

Enzyme	Name	Oligonucleotide Sequence
<i>AhdI</i>	AhdI+T	TTG CAG CAC TGG GGC CAG ATG GTA AGC CCT CCC GTA TCG TAG TTA-TCT ACA <u>CGA CCG GGA GTC</u> AGG CAA CTA TGG ATG AAC GAA ATA GAC
<i>AhdI</i>	AhdI+B	GTC TAT TTC GTT CAT CCA TAG TTG CCT <u>GAC TCC CCG TCG</u> TGT AGA-TAA CTA CGA TAC GGG AGG GCT TAC CAT CTG GCC CCA GTG CTG CAA
<i>AhdI</i>	AhdI-(#11)B	GTC TAT TTC GTT CAT CCA TAG TTG CCT <u>AAC TCC CCG TCG</u> TGT AGA-TAA CTA CGA TAC GGG AGG GCT TAC CAT CTG GCC CCA GTG CTG CAA
<i>AhdI</i>	AhdI-(#1V)B	GTC TAT TTC GTT CAT CCA TAG TTG CCT <u>TAC TCC CCG TCG</u> TGT AGA-TAA CTA CGA TAC GGG AGG GCT TAC CAT CTG GCC CCA GTG CTG CAA
<i>BamHI</i>	BamHI+T	GCG CGA AAT GGT CAC CGG GTG <u>GAT CCC</u> TTT CCG GGA
<i>BamHI</i>	BamHI-(#6V)T	GCG CGA AAT GGT CAC CGG GTG <u>GAT CGC</u> TTT CCG GGA
<i>BamHI</i>	BamHI+B	TCC CGG AAA <u>GGG ATC CAC</u> CCG GTG ACC ATT TCG CGC
<i>BamHI</i>	BamHI-(#5V)B	TCC CGG AAA <u>GGG ATA CAC</u> CCG GTG ACC ATT TCG CGC
<i>BamHI</i>	BamHI-(#5I)B	TCC CGG AAA <u>GGG ATT CAC</u> CCG GTG ACC ATT TCG CGC
<i>BamHI</i>	BamHI-(#11)B	TCC CGG AAA <u>GAG ATC CAC</u> CCG GTG ACC ATT TCG CGC
<i>BamHI</i>	BamHI-(#2V)B	TCC CGG AAA <u>GGC ATC CAC</u> CCG GTG ACC ATT TCG CGC

Table 3 (continued): Oligonucleotides used in the Oligonucleotide heteroduplex assay.

Enzyme	Name	Oligonucleotide Sequence
<i>Bam</i> HI	BamHI-(#3I)B	TCC CGG AAA <u>GGG GTC CAC</u> CCG GTG ACC ATT TCG CGC
<i>Bam</i> HI	BamHI-(#4V)B	TCC CGG AAA <u>GGG AGC CAC</u> CCG GTG ACC ATT TCG CGC
<i>Bam</i> HI	BamHI-(#6V)B	TCC CGG AAA <u>GGG ATC GAC</u> CCG GTG ACC ATT TCG CGC
<i>Eco</i> 0109I	Eco0+T	AGG CGT ATC ACG <u>AGG CCC TTT</u> CGT TCG CGC GTT TCG GTG AT
<i>Eco</i> 0109I	Eco0+B	ATC ACC GAA ACG CGC GAA CGA AAG <u>GGC CTC</u> GTG ATA CGC CT
<i>Eco</i> 0109I	Eco0-(#3I)B	ATC ACC GAA ACG CGC GAA CGA AAG <u>AGC CTC</u> GTG ATA CGC CT
<i>Eco</i> 0109I	Eco0-(#3V)B	ATC ACC GAA ACG CGC GAA CGA AAG <u>CGC CTC</u> GTG ATA CGC CT
<i>Eco</i> RI	EcoRI+T	GCG CGA AAT GGT CAC CGG GTG AAT <u>TCC TTT</u> CCG GGA
<i>Eco</i> RI	EcoRI-(#1V)T	GCG CGA AAT GGT CAC CGG GTT <u>AAT TCC</u> TTT CCG GGA
<i>Eco</i> RI	EcoRI+B	TCC CGG AAA <u>GGA ATT CAC</u> CCG GTG ACC ATT TCG CGC
<i>Eco</i> RI	EcoRI-(#1V)B	TCC CGG AAA <u>GTA ATT CAC</u> CCG GTG ACC ATT TCG CGC
<i>Eco</i> RI	EcoRI-(#2I)B	TCC CGG AAA <u>GGG ATT CAC</u> CCG GTG ACC ATT TCG CGC
<i>Eco</i> RI	EcoRI-(#3V)B	TCC CGG AAA <u>GGA TTT CAC</u> CCG GTG ACC ATT TCG CGC
<i>Eco</i> RI	EcoRI-(#4I)B	TCC CGG AAA <u>GGA ACT CAC</u> CCG GTG ACC ATT TCG CGC
<i>Eco</i> RI	EcoRI-(#5V)B	TCC CGG AAA <u>GGA ATG CAC</u> CCG GTG ACC ATT TCG CGC
<i>Eco</i> RI	EcoRI-(#5I)B	TCC CGG AAA <u>GGA ATC CAC</u> CCG GTG ACC ATT TCG CGC
<i>Eco</i> RI	EcoRI-(#6I)B	TCC CGG AAA <u>GGA ATT TAC</u> CCG GTG ACC ATT TCG CGC
<i>Eco</i> RV	EcoRV+T	GCG CGA AAT GGT CAC CGG GTG ATA <u>TCC TTT</u> CCG GGA
<i>Eco</i> RV	EcoRV-(#6I)T	GCG CGA AAT GGT CAC CGG GTG <u>ATA TTC</u> TTT CCG GGA
<i>Eco</i> RV	EcoRV+B	TCC CGG AAA <u>GGA TAT CAC</u> CCG GTG ACC ATT TCG CGC
<i>Eco</i> RV	EcoRV-(#1V)B	TCC CGG AAA <u>GTA TAT CAC</u> CCG GTG ACC ATT TCG CGC
<i>Eco</i> RV	EcoRV-(#2I)B	TCC CGG AAA <u>GGG TAT CAC</u> CCG GTG ACC ATT TCG CGC
<i>Eco</i> RV	EcoRV-(#3I)B	TCC CGG AAA <u>GGA CAT CAC</u> CCG GTG ACC ATT TCG CGC
<i>Eco</i> RV	EcoRV-(#3V)B	TCC CGG AAA <u>GGA GAT CAC</u> CCG GTG ACC ATT TCG CGC
<i>Eco</i> RV	EcoRV-(#4I)B	TCC CGG AAA <u>GGA TGT CAC</u> CCG GTG ACC ATT TCG CGC
<i>Eco</i> RV	EcoRV-(#5V)B	TCC CGG AAA <u>GGA TAG CAC</u> CCG GTG ACC ATT TCG CGC
<i>Eco</i> RV	EcoRV-(#6I)B	TCC CGG AAA <u>GGA TAT TAC</u> CCG GTG ACC ATT TCG CGC
<i>Hind</i> III	Hind3+T	GCG CGA AAT GGT CAC CGG GTA <u>AGC TTC</u> TTT CCG GGA
<i>Hind</i> III	Hind3+B	TCC CGG AAA <u>GAA GCT TAC</u> CCG GTG ACC ATT TCG CGC
<i>Hind</i> III	Hind3-(#2V)B	TCC CGG AAA <u>GAC GCT TAC</u> CCG GTG ACC ATT TCG CGC
<i>Hind</i> III	Hind3-(#2I)B	TCC CGG AAA <u>GAG GCT TAC</u> CCG GTG ACC ATT TCG CGC
<i>Hind</i> III	Hind3-(#1I)B	TCC CGG AAA <u>GGA GCT TAC</u> CCG GTG ACC ATT TCG CGC
<i>Hind</i> III	Hind3-(#3I)B	TCC CGG AAA <u>GAA ACT TAC</u> CCG GTG ACC ATT TCG CGC
<i>Hind</i> III	Hind3-(#4V)B	TCC CGG AAA <u>GAA GAT TAC</u> CCG GTG ACC ATT TCG CGC
<i>Hind</i> III	Hind3-(#5I)B	TCC CGG AAA <u>GAA GCC TAC</u> CCG GTG ACC ATT TCG CGC
<i>Hind</i> III	Hind3-(#6V)B	TCC CGG AAA <u>GAA GCT GAC</u> CCG GTG ACC ATT TCG CGC
<i>Pme</i> I	PmeI+T	GCT GAT CAG CGG <u>GTT TAA ACT</u> TAA TCA ATG AGG AGC TCA TA
<i>Pme</i> I	PmeI-(#6I)T	AGC GGG <u>TTT AGA CTT</u> AAT CAA TGA GGA GCT CAT A
<i>Pme</i> I	PmeI+B	TAT GAG CTC CTC ATT GAT TAA <u>GTT TAA ACC</u> CGC T
<i>Pme</i> I	PmeI-(#6I)B	TAT GAG CTC CTC ATT GAT TAA <u>GTT TAG ACC</u> CGC T
<i>Pme</i> I	PmeI-(#5V)B	TAT GAG CTC CTC ATT GAT TAA <u>GTT TCA ACC</u> CGC T
<i>Pme</i> I	PmeI-(#5I)B	TAT GAG CTC CTC ATT GAT TAA <u>GTT TGA ACC</u> CGC T
<i>Pst</i> I	PstI+T	GCG CGA AAT GGT CAC CGG GTC <u>TGC AGC</u> TTT CCG GGA
<i>Pst</i> I	PstI+B	TCC CGG AAA <u>GCT GCA GAC</u> CCG GTG ACC ATT TCG CGC
<i>Pst</i> I	PstI-(#3V)	TCC CGG AAA <u>GCT TCA GAC</u> CCG GTG ACC ATT TCG CGC
<i>Pst</i> I	PstI-(#3I)	TCC CGG AAA <u>GCT ACA GAC</u> CCG GTG ACC ATT TCG CGC
<i>Sac</i> I	SacI+T	GCG CGA AAT GGT CAC CGG GTG <u>AGC TCC</u> TTT CCG GGA
<i>Sac</i> I	SacI-(#1V)T	GCG CGA AAT GGT CAC CGG GTT <u>AGC TCC</u> TTT CCG GGA
<i>Sac</i> I	SacI+B	TCC CGG AAA <u>GGA GCT CAC</u> CCG GTG ACC ATT TCG CGC
<i>Sac</i> I	SacI-(#1V)B	TCC CGG AAA <u>GTA GCT CAC</u> CCG GTG ACC ATT TCG CGC
<i>Sac</i> I	SacI-(#2V)B	TCC CGG AAA <u>GGC GCT CAC</u> CCG GTG ACC ATT TCG CGC
<i>Sac</i> I	SacI-(#3I)B	TCC CGG AAA <u>GGA ACT CAC</u> CCG GTG ACC ATT TCG CGC
<i>Sac</i> I	SacI-(#4I)B	TCC CGG AAA <u>GGA GTT CAC</u> CCG GTG ACC ATT TCG CGC
<i>Sac</i> I	SacI-(#5V)B	TCC CGG AAA <u>GGA GCA CAC</u> CCG GTG ACC ATT TCG CGC
<i>Sac</i> I	SacI-(#6I)B	TCC CGG AAA <u>GGA GCT TAC</u> CCG GTG ACC ATT TCG CGC
<i>Sal</i> I	SalI+T	GCG CGA AAT GGT CAC CGG GTG <u>TGC ACC</u> TTT CCG GGA
<i>Sal</i> I	SalI+B	TCC CGG AAA <u>GGT CGA CAC</u> CCG GTG ACC ATT TCG CGC
<i>Sal</i> I	SalI-(#2I)B	TCC CGG AAA <u>GGC CGA CAC</u> CCG GTG ACC ATT TCG CGC
<i>Sal</i> I	SalI-(#2V)B	TCC CGG AAA <u>GGG CGA CAC</u> CCG GTG ACC ATT TCG CGC
<i>Spe</i> I	SpeI+T	GCG CGA AAT GGT CAC CGG GTA <u>CTA GTC</u> TTT CCG GGA
<i>Spe</i> I	SpeI-(#3I)T	GCG CGA AAT GGT CAC CGG GTT <u>CCA GTC</u> TTT CCG GGA
<i>Spe</i> I	SpeI+B	TCC CGG AAA <u>GAC TAG TAC</u> CCG GTG ACC ATT TCG CGC
<i>Spe</i> I	SpeI-(#1I)B	TCC CGG AAA <u>GGC TAG TAC</u> CCG GTG ACC ATT TCG CGC
<i>Spe</i> I	SpeI-(#2V)B	TCC CGG AAA <u>GAA TAG TAC</u> CCG GTG ACC ATT TCG CGC
<i>Spe</i> I	SpeI-(#3I)B	TCC CGG AAA <u>GAC CAG TAC</u> CCG GTG ACC ATT TCG CGC
<i>Spe</i> I	SpeI-(#4V)B	TCC CGG AAA <u>GAC TTG TAC</u> CCG GTG ACC ATT TCG CGC
<i>Spe</i> I	SpeI-(#5I)B	TCC CGG AAA <u>GAC TAA TAC</u> CCG GTG ACC ATT TCG CGC
<i>Spe</i> I	SpeI-(#6V)B	TCC CGG AAA <u>GAC TAG AAC</u> CCG GTG ACC ATT TCG CGC
<i>Spe</i> I	SpeI-(#13I)B	TCC CGG AAA <u>GGC CAG TAC</u> CCG GTG ACC ATT TCG CGC
<i>Spe</i> I	SpeI-(#15I)B	TCC CGG AAA <u>GGC TAA TAC</u> CCG GTG ACC ATT TCG CGC
<i>Spe</i> I	SpeI-(#315I)B	TCC CGG AAA <u>GAC CAA TAC</u> CCG GTG ACC ATT TCG CGC

Table 3 (continued): Oligonucleotides used in the Oligonucleotide heteroduplex assay.

Enzyme	Name	Oligonucleotide Sequence
XbaI	XbaI+T	GCG CGA AAT GGT CAC CGG GTT CTA GAC TTT CCG GGA
XbaI	XbaI-(6V)T	GCG CGA AAT GGT CAC CGG GTT CTA GCC TTT CCG GGA
XbaI	XbaI+B	TCC CGG AAA GTC TAG AAC CCG GTG ACC ATT TCG CGC
XbaI	XbaI-(#21)B	TCC CGG AAA GTT TAG AAC CCG GTG ACC ATT TCG CGC
XbaI	XbaI-(#2V)B	TCC CGG AAA GTG TAG AAC CCG GTG ACC ATT TCG CGC
XbaI	XbaI-(#11)B	TCC CGG AAA GCC TAG AAC CCG GTG ACC ATT TCG CGC
XbaI	XbaI-(#31)B	TCC CGG AAA GTC CAG AAC CCG GTG ACC ATT TCG CGC
XbaI	XbaI-(#4V)B	TCC CGG AAA GTC TCG AAC CCG GTG ACC ATT TCG CGC
XbaI	XbaI-(#51)B	TCC CGG AAA GTC TAA AAC CCG GTG ACC ATT TCG CGC
XbaI	XbaI-(#6V)B	TCC CGG AAA GTC TAG CAC CCG GTG ACC ATT TCG CGC
XhoI	XhoI+T	GCG CGA AAT GGT CAC CGG GTC TCG AGC TTT CCG GGA
XhoI	XhoI-(#21)T	GCG CGA AAT GGT CAC CGG GTC CCG AGC TTT CCG GGA
XhoI	XhoI+B	TCC CGG AAA GCT CGA GAC CCG GTG ACC ATT TCG CGC
XhoI	XhoI-(#1V)B	TCC CGG AAA GAT CGA GAC CCG GTG ACC ATT TCG CGC
XhoI	XhoI-(#11)B	TCC CGG AAA GCC CGA GAC CCG GTG ACC ATT TCG CGC
XhoI	XhoI-(#3V)B	TCC CGG AAA GCT AGA GAC CCG GTG ACC ATT TCG CGC
XhoI	XhoI-(#41)B	TCC CGG AAA GCT CAA GAC CCG GTG ACC ATT TCG CGC
XhoI	XhoI-(#5V)B	TCC CGG AAA GCT CGC GAC CCG GTG ACC ATT TCG CGC
XhoI	XhoI-(#61)B	TCC CGG AAA GCT CGA AAC CCG GTG ACC ATT TCG CGC
XhoI	XhoI-(#6V)B	TCC CGG AAA GCT CGA CAC CCG GTG ACC ATT TCG CGC

Notes: *XbaI* has an 11 nucleotide recognition site and required longer oligomers to assay cleavage. *PmeI*+T without the 7 nucleotide overhang gave similar results.

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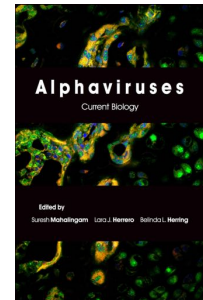
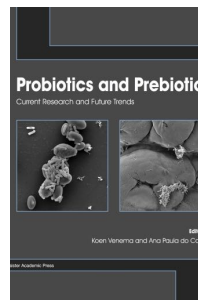
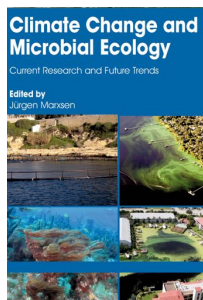
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