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Enhanced DNA sequencing by hybridization

(streptavidin/biotin/stacking interaction/T4 DNA ligase/DNA polymerase)

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ABSTRACT **An enhanced version of DNA sequencing by hybridization (SBH), termed positional SBH (PSBH), has been developed. PSBH uses duplex probes containing single-stranded 3′ overhangs, instead of simple single-stranded probes. Stacking interactions between the duplex probe and a single-stranded target should provide enhanced stringency in distinguishing perfectly matched 3′ sequences. A second enhancement is the use of enzyme-catalyzed steps, instead of pure physical hybridization. The feasibility of this scheme has been investigated using biotinylated duplex probes containing single-stranded 5-base 3′ overhangs, immobilized on streptavidin-coated magnetic beads. Ligation of a single-stranded target, hybridized to the single-stranded region of the duplex probes, provided enhanced discrimination of perfectly matched targets from those containing mismatches. In distinction to the serious complications caused by base composition effects in ordinary SBH, there was little effect of base composition in PSBH. The hardest mismatch to discriminate was the one furthest from the phosphodiester bond formed by ligation. However, mismatches in this position were efficiently discriminated by 3′ extension of the duplex probe using a template-dependent DNA polymerase. These results demonstrate that PSBH offers considerable promise to facilitate actual implementations of SBH.**

Two general approaches of DNA sequencing by hybridization (SBH) have been proposed, and their potential practicality has been demonstrated in pilot studies. In one format, a complete set of 4^n oligonucleotides of length n is immobilized as an ordered array on a solid surface, and an unknown DNA sequence is hybridized to this array (1–4). If the data can be interpreted perfectly, the resulting hybridization pattern provides all n-tuple words in the sequence. This is sufficient to determine short sequences, except for simple tandem repeats. In the second format, an array of immobilized samples is hybridized with one short oligonucleotide at a time (5). If this is repeated 4^n times, once for each oligonucleotide of length n, much of the sequence of all of the immobilized samples would be determined. In both approaches, the intrinsic power of SBH is that many sequences are determined in parallel. Another powerful aspect is that sequence information obtained is quite redundant, particularly as the size of the oligonucleotide probes grows. Thus, the method should be quite resistant to experimental errors, and far fewer than all 4^n probes are required to obtain reliable sequence information (6, 7).

In spite of the overall optimistic outlook, there are still a number of potentially severe drawbacks to actual implementations of SBH. One drawback of current SBH technology is the level of discrimination between a perfectly matched duplex and an end-mismatched duplex. Internal mismatches are presumably ignorable, because their thermodynamic stability is much lower than that of perfect matches. A related problem is distinguishing between weak but correct duplex formation and nonspecific binding of target or probe DNA to underlying solid supports. A second potential drawback is the effect of secondary structure in the target DNA. This could lead to blocks of sequences that are unreadable if this secondary structure is more stable than the oligonucleotide complex with the corresponding target region. A third drawback is the possibility that certain oligonucleotide sequences will have anomalous behavior and, for one reason or another, will be recalcitrant to hybridization under standard conditions used. A fourth drawback is ambiguities in reading sequences longer than a few hundred base pairs because of recurrences. A final drawback is that 4^n rapidly becomes quite a large number if the chemical synthesis of all of the nucleotide probes is actually contemplated. These inherent drawbacks currently prevent SBH from actual implementation in large-scale DNA sequencing efforts.

Here, we propose an enhanced version of SBH, termed positional SBH (PSBH). PSBH uses duplex probes containing single-stranded 3′ overhangs instead of simple single-stranded probes. Two enzyme-catalyzed steps, instead of pure physical hybridization, are used to enhance the accuracy of sequence information obtained.

MATERIALS AND METHODS

Oligonucleotides. Oligonucleotides used (Table 1) were purchased from Operon Technologies (Alameda, CA). Their 5′ termini were labeled (8) using T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP (Amersham) (8).

Ligation of Target DNA to Immobilized Duplex Probes. A biotinylated duplex probe containing a 5-base 3′ overhang was prepared by annealing equimolar amounts of two complementary single-stranded oligonucleotides in TE (10 mM Tris·HCl, pH 8/1 mM EDTA) by heating to 70°C and slowly cooling to room temperature. The duplex has a 5′-biotinylated 23-mer, made of an 18-base 5′ constant region and a 5-base 3′ variable region, and an 18-mer complementary to the constant region of the 23-mer. The biotinylated duplex probe (4 pmol) was immobilized on streptavidin-coated magnetic beads (0.1 mg; Dynal, Oslo). The beads were washed to remove unbound duplex probes and suspended in 10 μl of a ligation mixture containing 1 pmol of a 5′-^{32}P-labeled single-stranded target oligonucleotide in 10% polyethylene glycol, M_r 6000 (PEG)/66 mM Tris·HCl, pH 7.5/6.6 mM $MgCl_2$/10 mM dithiothreitol/1 mM ATP and salt as indicated (see text). Then, T4 DNA ligase (400 cohesive end units; New England Biolabs) was added, and the mixture was incubated at 37°C for 30 min. The reaction was terminated by adding 1 μl of 0.5 M EDTA. The magnetic beads were cooled on ice for 5 min and washed twice at 4°C with 100 μl of TE to remove unligated targets. Ligated targets were released from the beads by washing at 90°C with 100 μl of TE. The radioactivity of each fraction was determined by Cerenkov counting.

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Abbreviations: SBH, sequencing by hybridization; PSBH, positional SBH.

Table 1. Oligonucleotides used

No(s).	Sequence (5′ → 3′)
1, 2, 3	(C,T,G)TGATGCGTCGGATCATC
4, 5	TCGGTTCCAAGGGC(C, T)
6, 7	TCGGTTCCAAGA(G, T)CT
8, 9	TCGGTTCCAA(G, A)ATAT
10	b-GATGATCCGACGCATCAAGGCCC
11	b-GATGATCCGACGCATCAGGGCCC
12	b-GATGATCCGACGCATCAGGGCCT
13	b-GATGATCCGACGCATCACAGCTC
14–35	b-GATGATCCGACGCATCAG(NNNNN)
36	GATCTAGCTGGGCCGAACTAGGCCACATT

Bases indicated in parentheses such as (A, G) are alternates contained at a single position in the indicated alternative oligonucleotides. Oligonucleotides 10–35 have a biotinylated (b) 5′ terminus. Oligonucleotides 10 and 13 have one altered base adjacent to the 3′ overhang to avoid self-priming in the DNA polymerase extension reaction (see *Results*). Overhang sequences for oligonucleotides 14–35 are shown in Tables 2 and 5.

Extension of Free 3′ Termini of Duplex Probes. A target oligonucleotide was hybridized and ligated to a biotinylated duplex probe as described above. After ligation, the magnetic beads were washed twice at 4°C with 100 μl of TE to remove unligated targets, and suspended in 10 μl of 40 mM Tris·HCl, pH 7.5/20 mM $MgCl_2$/50 mM NaCl/40 μM dATP/40 μM dGTP/40 μM dCTP/2 μM [α-^{32}P]dTTP (2–4 Ci/mmol, 1 Ci = 37 GBq; Amersham). Extension of the free 3′ terminus of a duplex probe containing a ligated target was initiated by the addition of 3 units of Sequenase version 2.0 (United States Biochemical) and incubated for 10 min at room temperature. The reaction was terminated by adding 1 μl of 0.5 M EDTA. The magnetic beads were washed twice at room temperature with 100 μl of TE to remove unincorporated [α-^{32}P]dTTP, and the radioactivity incorporated into the duplex probes was determined by Cerenkov counting. Similar extension reactions were carried out with annealed but nonligated targets. *Taq* DNA polymerase (Boehringer Mannheim) was also tested for extension reactions at 37°C for 30 min.

Preparation of Nested Target. Nested target A is an equimolar mixture of 29-, 28-, 26-, 24-, 22-, 20-, 19-, 18-, 16-, 14-, 12-, 10-, 9-, 8-, 7-, 6-, and 5-mers, with a common 5′ sequence. The sequence of the 29-mer of target A is shown in Table 1 as oligonucleotide 36.

RESULTS

Principles of PSBH. PSBH was developed to enhance current SBH technology. PSBH uses duplex probes, instead of the simple single-stranded probes used in ordinary SBH. These probes consist of a double-stranded constant region and a variable single-stranded 3′ overhang. A single-stranded target DNA is added to a duplex probe immobilized on a solid support (Fig. 1). The 3′-terminal sequence of the target should anneal to the single-stranded probe region. The duplex probe should provide enhanced sequence stringency in detecting the 3′-terminal sequence of target DNA, because of base stacking between the preformed DNA duplex and the newly formed duplex (9). The probe–target configuration used in PSBH allows DNA ligase to join the adjacent DNA strands. This ligation step should increase the discrimination of perfectly matched target DNAs from those containing mismatches, because DNA ligase is sensitive to single-base mismatches near the ligation site (10). An additional enzyme-catalyzed step should distinguish targets that are mismatched at the 5′ end. Here, extension of the free 3′ terminus of the probe using a template-dependent DNA polymerase (lacking a 3′ → 5′ exonuclease and terminal transferase activity) should require correct duplex formation between the free 3′ terminus and the ligated target. Here, PSBH has been tested in model systems to determine whether these two enzyme-catalyzed steps, together with stacking hybridization of a target to the preformed duplex probe, are effective in reducing ambiguities in SBH.

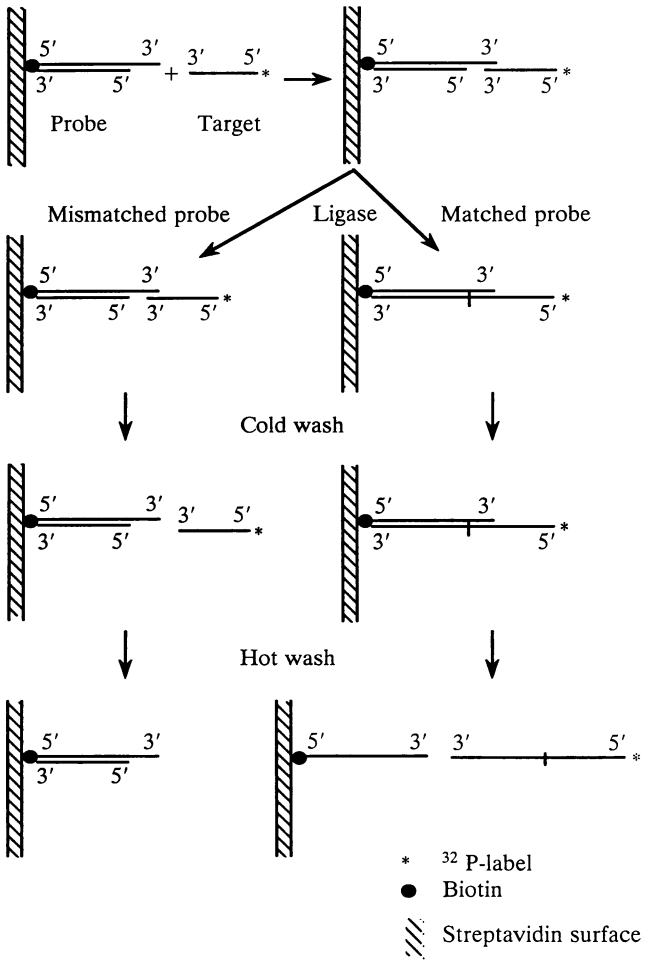

FIG. 1. Outline of experiments to test ligation discrimination for matched and mismatched targets bound to duplex probes, immobilized on streptavidin-coated magnetic beads.

Ligation as a Step in SBH. Experiments to investigate the discrimination power of ligation are outlined in Fig. 1. Streptavidin-coated magnetic beads were used as the solid support, because the beads can be easily manipulated and retain biotinylated duplex probes even under harsh conditions. An end-biotinylated duplex probe containing a 5-base 3′ overhang was immobilized on the streptavidin-coated magnetic beads, and target oligonucleotides, 5′-labeled with ^{32}P, were allowed to hybridize and ligate to the duplex probes. Unligated and ligated targets were removed by washing the beads at 4°C and 90°C, respectively. The 90°C wash melts the nonbiotinylated strand from the immobilized (biotinylated) strand. The ligation efficiency, defined as the ratio of ligated target to the total target used, was taken as the ratio of the ^{32}P released by the 90°C wash to the total ^{32}P used. The relative ligation efficiency of perfectly matched and mismatched targets is defined as the discrimination factor.

The ligation conditions that produce the highest discrimination factor were determined by varying the kind and concentration of salt, the reaction temperature, and the position of single mismatches. The ideal ligation step should proceed for all DNA sequences with similar efficiency. Thus, the influence of the target base composition was also investigated. All ligation reactions were performed in the presence

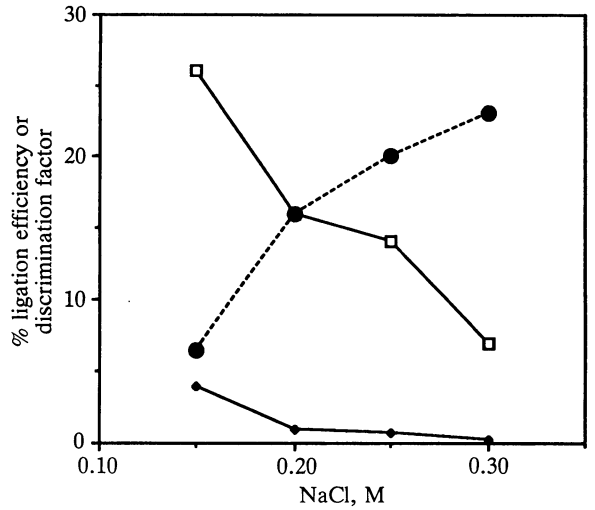

FIG. 2. Effect of NaCl concentration on ligation of matched (□) and mismatched (◆) targets to duplex probes (ligation efficiency). Probes used oligonucleotides 11 and 12 (Table 1), with a constant complementary strand, oligonucleotide 1, tethered to streptavidin-coated magnetic beads. Thus, the single-stranded 3′ overhang of the duplex probes was either 5′-GGCCT-3′ or 5′-GGCCC-3′. A 5′-^{32}P-labeled oligonucleotide 4 (3′-terminal sequence, 5′-GGGCC-3′) was added to each of the two duplex probes. The discrimination factor (●) is also shown.

of 10% (wt/vol) PEG, which stimulates T4 DNA ligase activity and overcomes the inhibitory effects of high concentrations of salts (11–13).

Typical ligation results at 37°C at various NaCl concentrations are shown in Fig. 2. The ligation efficiency and the discrimination factors are plotted as a function of NaCl concentration for two probes, one perfectly matched and the other with a 3′-end mismatch. The discrimination factor increased as the NaCl concentration was raised from 0.15 M to 0.30 M while the ligation efficiency was substantially reduced. Similar results were obtained when ligation was performed in KCl or potassium glutamate (data not shown). As a compromise between yield and discrimination, all subsequent ligation reactions were performed in 0.2 M NaCl or 0.25 M KCl. Lower ligation temperatures did not improve the discrimination against mismatches. For example, ligation at room temperature (≈22°C) yielded higher ligation efficiencies than at 37°C, but produced lower discrimination factors. An example of the effect of mismatch position on ligation in 0.25 M KCl is shown in Table 2. In general, the closer the mismatches are to the ligation point, the higher is the discrimination factor.

The effect of the base composition of the single-stranded region of the probe on ligation discrimination is summarized in Table 3. For the sequences tested, the discrimination factor in favor of the matched sequence varied from 10 to 24,

Table 3. Ligation efficiency of a single-stranded target to duplex probes with 5-base overhangs with different A+T contents

Probe (5′ → 3′)		A+T content	Ligation efficiency, %	Discrimination factor
Match	GGCCC	0	30	
Mismatch	GGCCT		3	10
Match	AGCCC	1	36	
Mismatch	AGCTC		2	18
Match	AGCTC	2	17	
Mismatch	AGCTT		1	17
Match	AGATC	3	24	
Mismatch	AGATT		1	24
Match	ATATC	4	17	
Mismatch	ATATT		1	17
Match	ATATT	5	31	
Mismatch	ATATC		2	16

Only the variable overhanging portion of the probe sequence is shown. The entire sequences of the probe and the target can be found in Table 1 (probes 14–35 and targets 4–9). Mismatched bases are indicated by boldface type.

but there was no systematic effect of base composition. This indicates that PSBH should avoid the serious base composition effects seen in ordinary SBH. Duplex probes with 6-base 3′ overhangs were also tested but showed significantly lower discrimination factors than those with 5-base overhangs (data not shown). Thus, the latter appear to be the most practical and efficient for PSBH.

DNA Polymerase Extension as a Step in SBH. Discrimination by ligation was worst, as expected, for mismatches most distal to the ligation point. Discrimination of such end mismatches was tested using DNA polymerase extension of the free 3′ terminus of the duplex probe containing a ligated target (Fig. 3). Biotinylated duplex probes containing 5-base 3′ overhangs were tethered to streptavidin-coated magnetic beads, and single-stranded oligonucleotide targets were hybridized and ligated to the duplex probes. After ligation, the magnetic beads were washed to remove unligated targets, and then the free 3′ termini of the duplex probes were extended by a template-dependent DNA polymerase in the presence of [^{32}P]dTTP and three other unlabeled dNTPs. A 3′-end mismatch should inhibit extension by a DNA polymerase (14). No extension should occur on duplex probes without ligated targets. The replacement of mismatches and template-independent extension reactions were avoided by using a genetically engineered T7 DNA polymerase, Sequenase version 2.0, which has neither 3′ → 5′ exonuclease nor terminal transferase activity.

The discrimination factor after the extension step was almost the same as that after ligation alone (Table 4). However, when extension reactions were done with hybridized but unligated targets, the discrimination factor was significantly higher. This can be explained by the relatively higher

Table 2. Ligation efficiency of a single-stranded ^{32}P-labeled (5′ end) target (3′-TCGAGAACCTTGGCT-5′) to duplexes with 5-base overhangs with different mismatches

Probe	Ligation efficiency, %	Discrimination factor
3′-CTACTAGGCTGCGTAGTC-5′		
5′-b-GATGATCCGACGCATCAGAGCTC-3′	17	
5′-b-GATGATCCGACGCATCAGAGCTT-3′	1	17
5′-b-GATGATCCGACGCATCAGAGCTA-3′	0.5	34
5′-b-GATGATCCGACGCATCAGAGCCC-3′	0.2	85
5′-b-GATGATCCGACGCATCAGAGTTC-3′	0.4	42
5′-b-GATGATCCGACGCATCAGAACTC-3′	0.1	170

Complete duplex probe is shown only for the perfect match. The 18-mer of the duplex was constant (oligonucleotide 1 in Table 1). Mismatches are shown in boldface type. b, Biotin. The target was the same in each case (oligonucleotide 6 in Table 1).

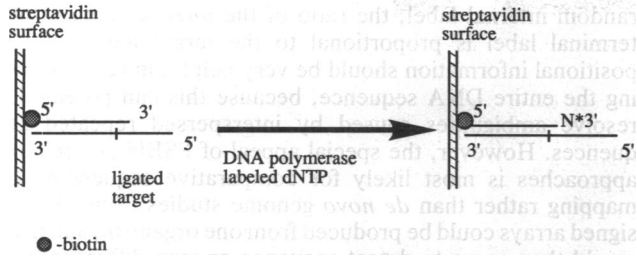

FIG. 3. Use of DNA polymerase extension to enhance PSBH.

concentrations of matched duplexes present without ligation, because the average ligation efficiency was only 15–20%. The very high 3′ mismatch discrimination provided by polymerase extension provides a very useful enhancement for PSBH.

Several duplex probes with palindromic overhangs (e.g., probes 10 and 13 in Table 1) displayed extensive self-priming extensions in the absence of targets. Such intramolecular secondary-structure formation was easily eliminated by changing one base in the duplex portion (otherwise constant) of the probe. Such considerations of possible secondary-structure formation will have to be taken into account in the design of future probe arrays.

A Pilot Test of PSBH. The effectiveness of the ligation and extension steps in a format closer to actual DNA sequencing was examined using nested target and a set of 25 biotinylated duplex probes. Nested target A was prepared from a 29-mer, oligonucleotide 36 (Table 1). It was an equimolar mixture of synthetic oligonucleotides of various lengths, sharing a common 5′ terminus. Ligation or extension reactions (without ligation) were carried out with duplex probes immobilized on streptavidin-coated magnetic beads. The average signal from >20 experiments for mismatched samples for ligation and extension reactions was 600 ± 100 cpm and 150 ± 50 cpm, respectively. Based on these results, ligation and extension signals >1000 cpm and >500 cpm, respectively, were considered positive. If the ligation and extension results were consistent, the analysis was scored either positive or negative. If ligation and extension results were inconsistent, an additional step, extension after ligation, was performed to resolve ambiguities. Results of a typical experiment are presented in Table 5. The average discrimination factor seen by extension alone was higher than that by ligation. In most cases, the results obtained by ligation and extension were consistent, indicating that each of these reactions has sufficiently high discrimination power. Two target sequences (probes AGCTA and AATGT, Table 5) were detected by ligation only. However, these sequences were efficiently detected and discriminated by performing extension reactions after ligation (data not shown). Probe AGCTT (Table 5) was positive in ligation and negative in extension. It was also negative in extension performed after ligation. Therefore, it was considered as negative. From the results obtained, the 25-base sequence of the target A was easily reconstructed.

Table 5. PSBH analysis of oligonucleotide A (target A)

Probe (5′ → 3′)	Ligation, cpm	Extension, cpm	Result observed	Result expected
AGCTC	855	65	−	−
AGCTT	1,101	101	−	−
AGCCC	255	212	−	−
AGTTC	3,533	838	+	+
AACTC	707	59	−	−
AGATC	556	386	−	±*
AGATT	228	212	−	−
ATATT	165	168	−	−
GGCCC	4,082	2212	+	+
GGCCT	1,977	3096	+	+
AGCTA	50,947	243	+†	+
AATGT	1,245	232	+†	+
AATTT	623	118	−	−
GCGGA	155	387	−	−
ATGTG	2,266	2974	+	+
GTTCG	2,516	710	+	+
TGTGC	416	192	−	−
CCTAG	2,537	7846	+	+
CCCAG	3,079	6304	+	+
GTGGC	3,674	5433	+	+
TGGCC	2,254	4840	+	+
TAGTT	3,516	4360	+	+
CAGCT	1,257	2811	+	+
GCTCG	450	131	−	−

Only the variable overhanging portion of the probe sequences is shown. The sequences of the probe can be found in Table 1 (oligonucleotides 10–35). For the definition of positive (+) and negative (−) results, see text. The expected result is based on the sequence of the target.

*This probe corresponds to the very 5′ portion of the target. It should be positive for ligation and negative for extension.

†Signal was positive when the extension reaction was done after the ligation step.

DISCUSSION

Calculations based on the simple all-or-none model for oligonucleotide hybridization (15) indicate that hybridization of an oligonucleotide next to a preformed duplex can allow shorter probe sequences to be used because of the extra stacking energy. This in turn provides greater mismatch discrimination than in ordinary hybridization (C.R.C., unpublished data). The results we have obtained nicely confirm these predictions. They indicate that, by using stacking hybridization, probe size can be reduced to 5-base single-stranded overhangs. This means that only 1024 (4^5) different probes are needed to cover all possible sequences, which should greatly facilitate the construction of ordered probe

Table 4. Enhanced discrimination of matched duplexes using a DNA polymerase extension reaction

Probe	Ligation, cpm	Extension, cpm	
		+ ligation	− ligation
3′-CTACTAGGCTGCGTAGTC-5′			
5′-b-GATGATCCGACGCATCAGAGATC-3′	7870	21,550	29,500
5′-b-GATGATCCGACGCATCAGAGATT-3′	330	825	250
Discrimination	24	26	118

Duplex probe is shown only for the perfect match. The 18-base complementary strand and the ^{32}P-labeled (5′ end) target (3′-TCTAGAACCTTGGCT-5′) were constant. The mismatched base is indicated in boldface type. b, Biotin. For extension plus ligation, extension with Sequenase version 2.0 with ^{32}P-labeled dTTP and three other unlabeled dNTPs was done on a ligated target–probe complex after the removal of unligated target. For extension minus ligation, extension was carried out with annealed but unligated target.

arrays needed for full sequencing. Ordinary SBH approaches use 8- or 9-base probes.

Enzymatic steps can further improve the discrimination against mismatches. Ligation at a high salt concentration and an elevated temperature proceeds efficiently with perfectly matched duplexes but not mismatches. Similar ligation conditions have been used for the detection of single base mutations (10, 16). Another important advantage of using a ligation step in SBH is that there is comparatively little effect of the base composition on match–mismatch discrimination (Table 3), which is a serious problem seen in ordinary SBH. Thus, the use of ligation in SBH allows the analysis of both A+T-rich and G+C-rich targets at the same temperature. Note that the ligation discrimination with worst cases, G·T and G·A, end mismatches, is ≈20. This is comparable to the average discrimination seen with internal mismatches in ordinary SBH (4). In addition to the reasons already cited, the high discrimination PSBH version may also be due to the suppression of any interference from the surface of the bead because the double-stranded portion of the probe acts as a 50-Å stiff spacer.

Discrimination between matched and mismatched target sequences was considerably enhanced by polymerase extension of the free 3′ terminus of a duplex probe. This is particularly helpful for discriminating against end mismatches. The extension step can also be used to enhance the power of ordered probe arrays. For instance, the use of labeled dNTPs one at a time can determine the identity of one additional nucleotide of a target DNA. Thus, an array of only 1024 probes containing 5-base variable sequences would actually have the sequencing power of an array of 4096 hexamers.

The PSBH scheme can read only the 3′-terminal sequence of a target. Thus, the determination of the entire sequence of a target requires a set of nested 3′ deletions. If the distribution of the 3′ termini of nested targets is sufficiently broad, the hybridization pattern will reveal the entire sequence of the target (excepting tandemly repeated sequences). The set of nested deletions need not have a common 5′ terminus. Thus, digestion of a target with both exo- and endonucleases could be used to produce a set of molecules with a broad distribution of 3′ termini. In preliminary experiments, partial digestion of a single-stranded target with both venom phosphodiesterase and DNase I produced a sufficiently broad distribution of 3′ ends to reveal the entire sequence of the target by PSBH (unpublished data).

Because PSBH detects only the 3′-terminal sequence of a target, positional information about the distance between the 3′ sequence and a known reference point could be obtained by using nested targets with a common 5′ end and variable 3′ ends. For example, if a target has one 5′ label and a second random internal label, the ratio of the internal label to the terminal label is proportional to the target length. Such positional information should be very helpful in reconstructing the entire DNA sequence, because this can potentially resolve ambiguities caused by interspersed repeated sequences. However, the special appeal of PSBH and related approaches is most likely for comparative sequencing or mapping rather than *de novo* genome studies. Suitably designed arrays could be produced from one organism, and they would then serve to detect sequence or map differences in closely related organisms.

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