

Rapid SNP diagnostics using asymmetric isothermal amplification and a new mismatch-suppression technology

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We developed a rapid single nucleotide polymorphism (SNP) detection system named smart amplification process version 2 (SMAP 2). Because DNA amplification only occurred with a perfect primer match, amplification alone was sufficient to identify the target allele. To achieve the requisite fidelity to support this claim, we used two new and complementary approaches to suppress exponential background DNA amplification that resulted from mispriming events. SMAP 2 is isothermal and achieved SNP detection from whole human blood in 30 min when performed with a new DNA polymerase that was cloned and isolated from *Alicyclobacillus acidocaldarius* (*Aac pol*). Furthermore, to assist the scientific community in configuring SMAP 2 assays, we developed software specific for SMAP 2 primer design. With these new tools, a high-precision and rapid DNA amplification technology becomes available to aid in pharmacogenomic research and molecular-diagnostics applications.

The availability of the human genome sequence^{1,2} and genome diversity databases^{3–5} at the beginning of the 21st century are causing a paradigm shift away from the standard protocol of medical care toward genotyped medicine. This new type of medicine is based on the accumulating knowledge of gene polymorphisms (SNPs) and their relationship to specific phenotypes, such as disease predisposition, drug metabolism and disease development. A key step for the development of individualized medicine is the ability to rapidly test patients for these SNPs and/or

other mutations correlated to diseases and disease predisposition. Supporting this point, the US Food and Drug Administration has required the drug industry to publicly provide SNP data examined in the process of procuring a drug license. Today SNP genotyping technologies^{6–9} are still a bottleneck in drug discovery research and clinical applications. But high-throughput gene analysis and SNP detection technologies will inevitably become both cheaper and faster in the future. Besides SNP genotyping, these improved sequence-detection technologies would also allow and advance studies in other disciplines such as population genetics, the global surveillance of infectious disease and the study of somatic mutations in human cancer.

Almost all previously developed SNP-detection systems consist of two steps: amplification (usually by PCR) and detection of SNP (using DNA fragments amplified in the first step). This approach is reasonably fast, but to shorten the time required and simplify the detection, it is ideal to develop a one-step method, in which the amplification itself can be the SNP detection signal. The difficulty in developing such a technology is in the suppression of the background amplification. For example, primers for allele-specific primer PCR are designed with the nucleotide mismatch at the 3' end of the PCR primers, but the misamplified PCR products primed from mismatched primers are still exponentially amplified, producing background signals that must be addressed.

Here we report SMAP 2, the first rapid one-step SNP detection technology in which the amplification of the targeted DNA is the signal of the target SNP itself.

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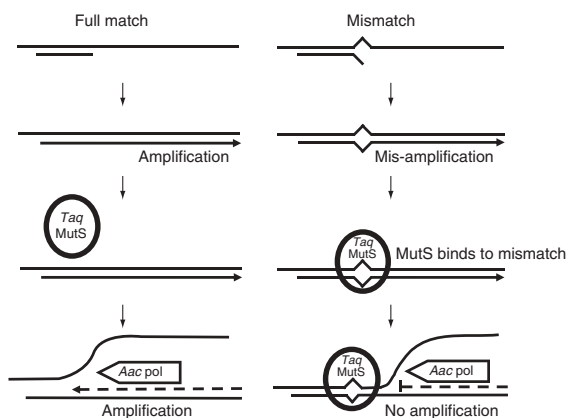


Figure 1 | The mechanism of allele discrimination as mediated by *Taq* MutS. SNP typing with a wild-type allele-specific primer, using the wild-type allele (left) and the mutant allele (right) as templates. The wild-type allele discrimination primer is designed to encompass the SNP nucleotide site at a position close to the 3' end. Hybridization of the wild-type discrimination primer to the wild-type allele promotes amplification. On occasion, however, misamplification occurs using the wild-type discrimination primer on a mutant allele. This mismatch amplification event generates a dsDNA with a single mismatched base pair. In SMAP 2, *Taq* MutS tightly binds to mismatched nucleotides in dsDNA and *Aac* DNA polymerase cannot strand-displace or extend through the nucleoprotein complex. Hence mismatch amplification is inhibited.

RESULTS

Suppression of background amplification

To avoid exponential background amplification, we developed two fundamental technologies. Together these technologies ensure that primer sets designed to amplify highly related sequences, such as a SNP variant and a wild-type allele, do not misamplify the incorrect sequence. The first technology uses *Thermus aquaticus* MutS (*Taq* MutS)¹⁰ in combination with an isothermal amplification procedure (Fig. 1). In a SMAP 2 assay for SNP genotyping, one primer is designed with homology to the SNP sequence and flanking DNA. We refer to this primer as the discrimination primer. As with any DNA polymerase *in vitro*, occasionally mispriming occurs. In SMAP 2, however, the *Taq* MutS protein binds to the nucleotides consisting of a mismatched duplex between the target DNA and the extended discrimination primer. This protein binding blocks the disassociation of the mismatched DNA duplex by the strand-displacing DNA polymerase and thus prevents exponential background amplification. The effectiveness of the *Taq* MutS is a consequence of using it in an isothermal amplification protocol rather than in a thermal cycling system like PCR, in which the *Taq* MutS dissociates at high temperatures.

The second strategy to suppress the background signals in SMAP 2 is the asymmetric design of the primers. Two asymmetric primers with unique sequences flanking the target sequence, a folding primer (FP) and a turn-back primer (TP), are used to amplify a specific genomic region.

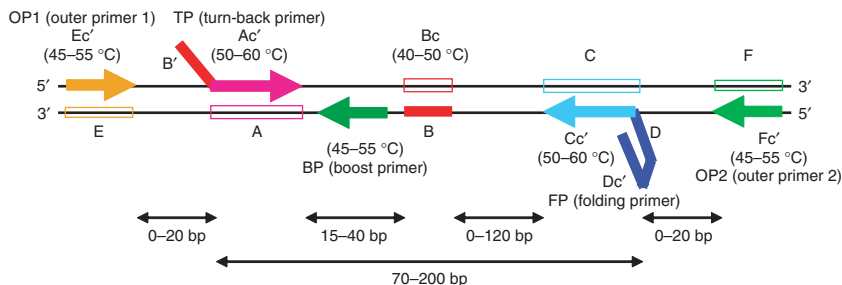
Primer design and amplification principle

The entire process of amplification by SMAP 2 requires five primers: TP, FP, boost primer (BP) and two outer primers (OP1

and OP2; Figs. 2 and 3). The TP 3'-end sequence is complementary to the target sequence and the 5'-end sequence is complementary to a sequence approximately 15–40 base pairs downstream on the same strand of DNA. The FP 3'-end sequence is complementary to a target genome sequence, and the 5'-end sequence is self-annealing and creates a hairpin structure. OP1 and OP2 strand-displace the DNA synthesized in FP and TP primer extension. The discrimination primer can be FP, TP or BP. For assay design any choice may suffice, but occasionally one specific primer design strategy may be more effective. At present this can only be determined empirically. The variable-nucleotide position in the discrimination primer can be at the 3'-end n , $n-1$ or $n-2$ position of FP, TP or BP, and the 5'-end n , $n-1$ or $n-2$ position of TP. The role of BP is to boost the speed of amplification, but it can also be used as a discrimination primer. Coordinated melting temperature (T_m) values and proper distancing between primers annealing sites are important in SMAP 2 assay design (Fig. 2). When designing primers, one should avoid sequences that have the potential to form secondary structures and follow the same rules that apply to the design of PCR primers. We have been successful in designing primers with 40–60% G+C content. Furthermore, an amplicon length of less than 200 bp is required to achieve the highest-efficiency (fastest) amplification.

The genomic sequence between and including the TP and FP primers is the target region to be amplified by the SMAP 2 reaction (Figs. 2 and 3). In the first step, FP and TP hybridize to the template genomic DNA. Both products primed from the FP and TP are then dissociated from the template genomic DNA by the strand-displacement activity of the DNA polymerase, whose extension is primed by OP1 and OP2. These displaced single-stranded products then become templates in the second step for the opposing FP and TP. Single-stranded displaced DNA products, which we refer to as intermediate products (IM1 and IM2), are then generated by the strand-displacement activity of the DNA

Figure 2 | SMAP 2 primer design. Primers for SMAP 2 amplification with the recommended distance between primer regions and a T_m range for each primer type. Genomic DNA regions of unique sequence are designated A, B, C, F and E. Primers are indicated by solid arrows and given the corresponding capital letter designation followed by c', indicating it is a complementary sequence. The D and Dc' region of FP are self-complementary and have no homology to genomic DNA. The region B' of the TP is complementary to the Bc region on the same strand and is responsible for 'loop-back' self-priming that occurs later on intermediate species. In SMAP 2, the target SNP (or mutation) must be present between the 5'-end of the Ac' region of the TP and the 5'-end of the Cc' region of the FP, as these define the outer regions of the genomic DNA to be amplified in the SMAP 2 amplicon.



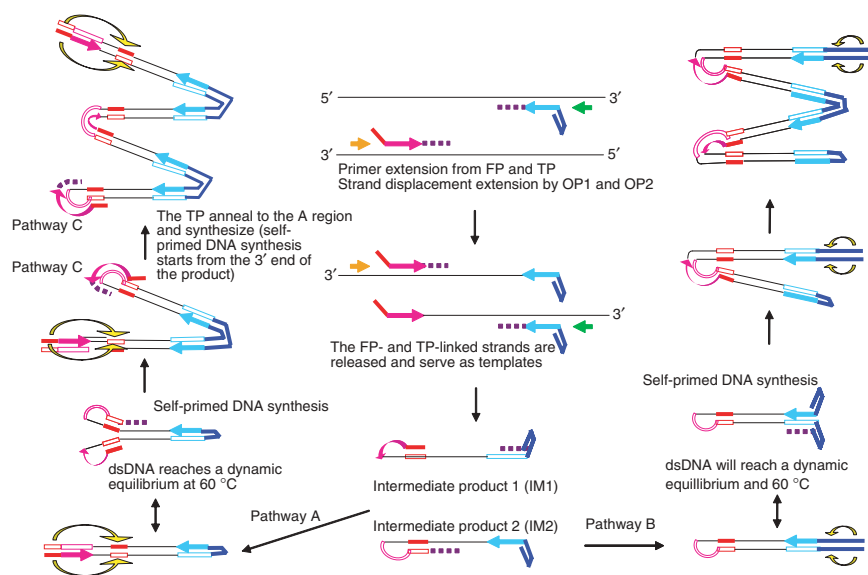


Figure 3 | SMAP amplification process. The initial priming events of the SMAP amplification process generate two intermediates, IM1 and IM2. Next self-priming DNA synthesis from each of these intermediates create hairpin molecules via pathway A or B. These structures lead to further self-primed DNA synthesis to create a dimeric amplicon and then subsequent larger species. Pathway C is a third possibility, in which a free TP anneals to the amplicon at an internal complementary site (A region; see Fig. 2) and primes strand-displacement DNA synthesis. Amplification proceeds until reaction components are exhausted. Typical yields of SMAP 2 exceed those of PCR by 100-fold.

polymerase primed from the flanking OP regions adjacent to the target sequence. IM1 and IM2 are milestone products for the subsequent amplification steps. IM1 has the TP sequence at the 5' end and the FP complementary sequence at the 3' end, and IM2 is complementary to IM1. The initial self-priming elongation site on IM1 is the 3'-end of the FP sequence of IM1, and this priming also occurs later on other multiple-unit-length (more mature) products. Concatenated products of IM1 are synthesized by an elongation process termed pathway A. The characteristic feature of the products of pathway A is that the free 5' and 3' ends carry TP and its complementary sequence, forming long double-stranded hairpin DNA. The initial self-priming elongation site on IM2 is located at the 3' end of the TP sequence of IM2, and this priming also occurs later on other multiple-unit-length (more mature) products. Long concatenated DNA products are synthesized as in pathway A, but denoted as pathway B because the end products are different. These long-hairpin DNA products carry FP and its complementary sequence at the free 5' and 3' ends, respectively. There is one other elongation event that is defined as pathway C. The elongation via pathway C starts from the 3' end of a free TP-primer that hybridizes to the looping structure of the TP complementary sequence, which is located at the intermediate region of the long products of pathway A.

One key feature responsible for the suppression of the background is the asymmetric design of FP and TP. On comparison with other isothermal amplification technologies, SMAP 2 is most similar to LAMP¹¹. LAMP technology, however, uses a symmetrical primer design with two TP primers, such that a dumbbell form is the milestone amplification product. A consequence of this LAMP primer design strategy is that additional opportunities exist for free-primer hybridization and priming events that otherwise do not occur in SMAP because self-priming events are thermodynamically

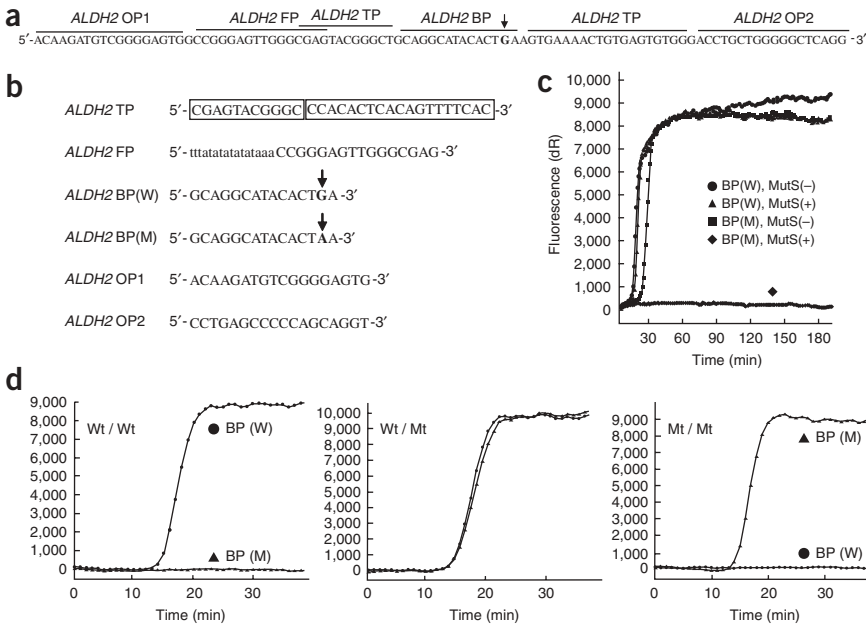
favoured by the FP when incorporated in the amplicon. Hence the primer design of SMAP 2 minimizes the alternative misamplification pathways and consequential background products (Supplementary Fig. 1 online).

To test and observe the effect of these primer design and technique differences, we performed SMAP 2 and LAMP with closely matched primer sets on an identical target. We performed the assays on whole blood from a homozygous wild-type individual, with wild-type and mutant primer sets designed to amplify the human type 2 iodothyronine deiodinase¹² gene (*DIO2*). We performed the amplification reaction directly from lysed blood samples without purifying the DNA. We achieved real-time monitoring of the amplification by using intercalating SYBR Green I to detect the generation of double-stranded DNA at 60 °C. Both SMAP 2 and LAMP used the same BP, OP1, OP2 and TP. The TP was designed as the discrimination primer with the SNP detection nucleotide on the 5' end. The other 'reversing' primer, which is TP for LAMP and FP for SMAP 2, had 3'-end

sequence homologous to the target, but differed on the 5' end consistent with design requirements for the respective technique (Supplementary Fig. 2 online). Furthermore, the reaction condition and enzymes for each of the techniques were identical, and experiments were performed with both full-match and mismatched primer sets, with and without *Taq* MutS. The data showed that LAMP amplification proceeds slightly faster than SMAP 2, and that *Taq* MutS slightly delayed full-match amplification in both techniques. On mismatched targets, however, the effect of *Taq* MutS on LAMP was substantially less than compared to the total suppression of mismatch amplification that was evident with SMAP 2 under the same conditions (Supplementary Fig. 3 online). This is due to the greater abundance of mismatch product in the LAMP reaction that was generated early in the process via other priming pathways. Analysis of the assay products on an agarose gel revealed a dissimilar banding pattern for SMAP 2 and LAMP, indicating different self-priming mechanisms of amplification (Supplementary Fig. 4 online).

The effect of *Taq* MutS on SNP typing of *ALDH2*

To demonstrate the amplification efficiency and specificity of SMAP 2, we used the SNP (G1543A) of aldehyde dehydrogenase 2 (*ALDH2*), which is the gene involved in alcohol sensitivity¹³, as the test model system. On a blood sample from an individual known to be homozygous wild-type (as determined by PCR sequencing; data not shown), amplification occurred when using a wild-type primer, BP(W), both in the presence of *Taq* MutS and in its absence (Fig. 4). When using the SNP-variant primer, BP(M), on the wild-type blood sample, we observed misamplification of the wild-type target after 25 min in the absence of *Taq* MutS. But the amplification was completely suppressed by inclusion of *Taq* MutS in the reaction, with no signal observed even after 255 min of

**Figure 4** | SNP typing of *ALDH2* allele.

(a) Sequence of *ALDH2* gene. The arrowhead indicates the position of the SNP nucleotides of the *ALDH2* allele (G1543A). DNA sequences used for primer design are marked. (b) Primer set for SNP typing of *ALDH2*. BP(W) and BP(M) were designed to be wild-type allele-specific and mutant allele-specific, respectively. The arrowheads indicate the nucleotides corresponding to SNP site. (c) The effect of *Taq* MutS on SNP detection of *ALDH2* gene. The intensity of fluorescence of SYBR Green I dye monitored during SMAP 2 reaction with or without *Taq* MutS. The blood sample of a homozygous wild-type DNA was used as a template for all reactions. dR, baseline-subtracted fluorescence reading. (d) Amplification time course of SMAP 2 reaction with *ALDH2* allele-specific primers using human blood specimens. Three possible diploid genotypes of *ALDH2* alleles are shown. The left, center and right graphs are time courses for a homozygous wild type, heterozygote and a homozygous mutant, respectively.

incubation. These data indicated that *Taq* MutS was capable of completely suppressing the mismatch background amplification.

To demonstrate the accuracy and clinical utility of this assay for handling multiple samples, we next tested 63 blood samples of healthy candidates. The fluorescence of both wild-type and mutant alleles could be detected at 17 min after incubation at 60 °C. Absolutely no background signal was evident, indicating that mismatch amplification was completely suppressed. To validate these results, we also detected the same SNP by using PCR-restriction fragment length polymorphism (PCR-RFLP)¹⁴. Of the SMAP 2 assays performed on 63 individuals, 41 tested homozygous for wild-type, 20 were heterozygous and 2 were homozygous mutant for the *ALDH2* alleles. All the data for the 63 samples obtained by PCR-RFLP and SMAP 2 showed perfect concordance (data not shown).

Accurately genotyping gene-family members with SMAP 2

The cytochrome *P450* proteins comprise a large gene family and are challenging to distinguish with hybridization or amplification techniques. SMAP 2 was capable of discrimination of not only these gene family members, but also of unique SNP variants of a specific subtype. We designed primer sets for amplification of the wild-type cytochrome *P450*, family 2, subfamily C, polypeptide 19 gene (*CYP2C19*1*) and an allelic variant known as *CYP2C19*2*(G681A)¹⁵. We chose this example to illustrate the amplification specificity and discrimination power of SMAP 2. In the case of *CYP2C19*, it is necessary to discriminate the signals from highly related subtypes *CYP2C8*, *CYP2C9* and *CYP2C18*, as all four of these genes have a high degree of sequence similarity (Fig. 5). We selected SMAP 2 primer sequences (BP, FP and OP) that are unique to the *CYP2C19* subtype to allow the amplification of it alone, by

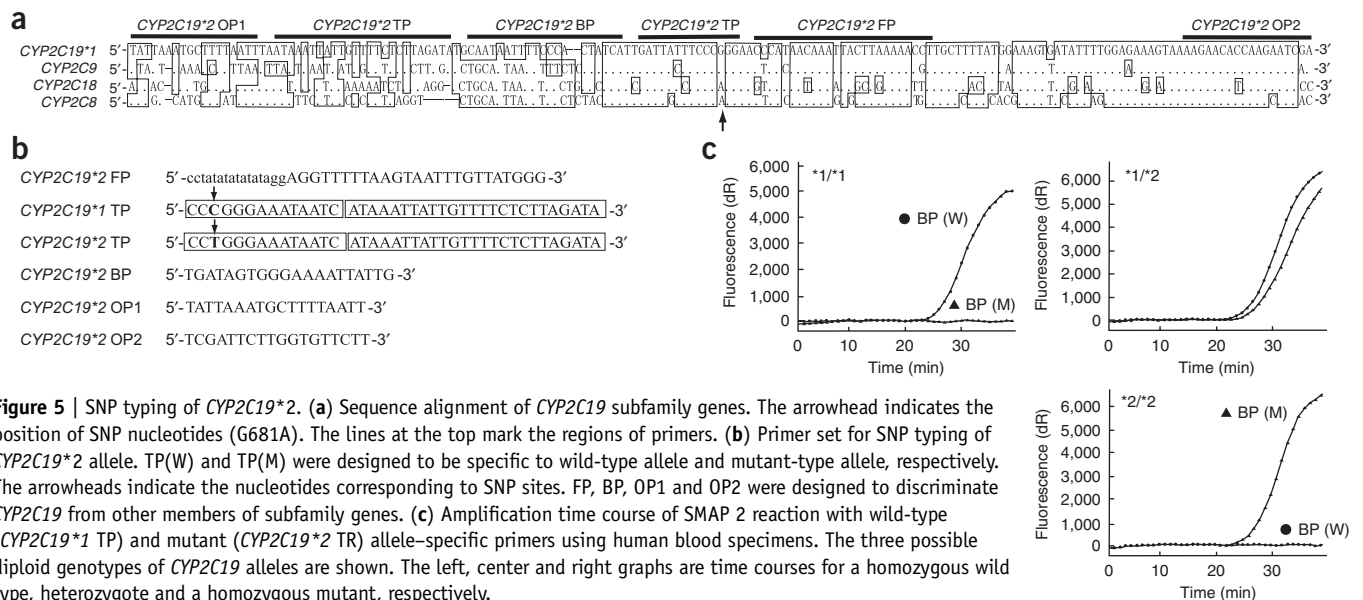


Figure 5 | SNP typing of *CYP2C19*2*. (a) Sequence alignment of *CYP2C19* subfamily genes. The arrowhead indicates the position of SNP nucleotides (G681A). The lines at the top mark the regions of primers. (b) Primer set for SNP typing of *CYP2C19*2* allele. TP(W) and TP(M) were designed to be specific to wild-type allele and mutant-type allele, respectively. The arrowheads indicate the nucleotides corresponding to SNP sites. FP, BP, OP1 and OP2 were designed to discriminate *CYP2C19* from other members of subfamily genes. (c) Amplification time course of SMAP 2 reaction with wild-type (*CYP2C19*1* TP) and mutant (*CYP2C19*2* TR) allele-specific primers using human blood specimens. The three possible diploid genotypes of *CYP2C19* alleles are shown. The left, center and right graphs are time courses for a homozygous wild type, heterozygote and a homozygous mutant, respectively.

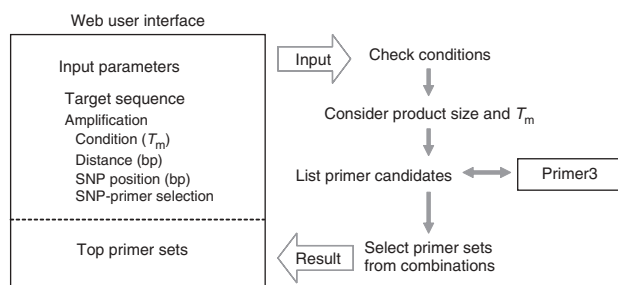


Figure 6 | SMAP primer design software version 1. Web user input variables and design process are outlined for the SMAP primer design software. The software generates primers designed in both forward and reverse directions with respect to the position of BP, TP and FP on the template.

being an imperfect match to all other family members (*CYP2C8*, *CYP2C9* and *CYP2C18*). We used TP as a discrimination primer to base pair with the target guanine in the wild type (*CYP219C*1* TP) and the adenine in the variant (*CYP2C19*2* TP). We verified all the data for the human blood samples by PCR-sequencing, and found perfect concordance with the SMAP 2 results, indicating that the SMAP 2 method is versatile and that the use of specific primers for differentiation of highly related targets was achievable.

SMAP Primer design software version 1

To facilitate the design of primer sets for SMAP applications we developed the SMAP server and algorithms specifically for SMAP 2 primer design (Fig. 6). The program, developed in Java and Struts uses Primer3 [Primer3]¹⁶ to generate a set of potential sequences for each of the primers required. Primer selection is based on the Primer3 scoring algorithms using optimal T_m and product-size range. Also, the requirements for TP and FP as well as all other primers are generated and combined to form all possible sets of primers where the SNP is in a valid location on either the FP, BP or TP. Primer sets are then ranked based on the Primer3 scoring for each of the primers. The SMAP 2 primer design software is freeware, available to assist researchers in generating unique primer sets for amplification of target sequences of interest. Presently there is not enough information to fully understand what constitutes the best SMAP 2 primers or primer combinations. Typical PCR rules applied by the Primer3 software are relevant, but other poorly understood SMAP-specific requirements clearly exist as not all primer sets that are theoretically designed for SNP detection, can faithfully discriminate the SNP from wild-type sequence. Hence the SMAP 2 primer design software cannot rank-order the primer sets based on theoretical performance, nor purposefully generate the best primer combinations for the target sequence. We found, however, that the software is an effective tool for initial experiments. It is recommended that the primer sets be refined through empirical experimentation (modification of primer length, position and other parameters), if an increase in speed and specificity of the SMAP reaction is desired.

We tested the primer-design software on two different gene targets, iodothyronine deiodinase type 2 (*DIO2*) and $\beta 3$ adrenergic receptor (*ADRB3*)¹⁷. We used the software to design several primer sets for each gene and SNP variant, which we then synthesized and ran SMAP 2 trial experiments. We selected the best primer sets

based on specificity and speed of the reaction. Then we optimized these primer sets by further rational design until a set providing optimal speed and specificity was configured. These optimized primers performed well and yielded accurate SNP typing data that was evident in 30 min (Supplementary Figs. 2 and 5 online). Information on how to use the software and how to optimize the primers by incorporating minor changes (length changes, repositioning), is available on the RIKEN SMAP server.

DISCUSSION

SMAP 2 is based on the concept that DNA amplification is itself the signal for detection of a specific target sequence. It is conceivable that SMAP 2 background suppression technologies can also benefit other types of isothermal amplification, although we observed minimal impact when MutS was used in LAMP assays (Supplementary Fig. 3). The technique is simple, requires no DNA purification and is performed in a closed tube, which reduces the risk of contamination. We included data generated on real-time PCR detection systems in this report; however, end-point detection, including colorimetric or spectrophotometric analysis may be possible because of the background suppression and high yields ($>100\times$ PCR yields in equivalent assay volumes). End-point determination is essentially digital and may enable simple and cost-effective detection methodologies that could be used in field applications or in countries with limited financial resources for health-care diagnostics. Furthermore, owing to the high yields and isothermal nature, very small-scale microfluidic designs for high-throughput SNP screening applications may be feasible.

In SMAP 2 flexibility of primer design to optimize performance is greater than in many other traditional isothermal amplification methods^{18–20}. We demonstrated that each of the primers (FP, TP and BP) can be engineered as a discrimination primer, facilitating the ability to detect any type of nucleotide change. In addition to single-base alterations, insertions and deletions of 1–18 bases have also been detected in clinical samples from human cancer and other tissues (unpublished data). The SMAP 2 assay is sensitive enough to detect a few molecules (Supplementary Fig. 6 online) making it potentially useful for mutation-screening of tumor samples that frequently display defined mutations in specific genes.

SMAP 2 likely will not replace PCR as a basic research tool for amplification because of the complexity of its primer design and optimization. Furthermore, the concatenated form of the amplified material is not useful for most research applications such as cloning, sequencing and expression. We have demonstrated, however, that SMAP 2 has strong potential for SNP genotyping with high accuracy, and unlike any other method, gives a reliable diagnostic result based exclusively on amplification alone.

METHODS

Preparation of blood sample. We collected blood samples from volunteers after obtaining their written informed consent for each collection. All donors were researchers and employees of the institutions represented by the authors of this paper. Institutional approval for conducting research using human material was obtained from the RIKEN Ethical Advisory Committee before initiating the study. We collected $\sim 30\ \mu\text{l}$ of blood by pricking the finger of volunteers. We divided the collected samples and used one aliquot directly for SMAP 2 reactions; we diluted the

remaining blood samples approximately threefold with 50 mM NaOH and heated them at 98 °C for 3 min. For each 25- μ l SMAP reaction, we added 1 μ l of the diluted and heated blood sample directly to the assay. We purified genomic DNA from a second aliquot of each sample with the QuickGene-mini80 (FUJIFILM) and used this for PCR-RFLP of *ALDH2*. To examine the accuracy of the other SMAP 2 data reported in this paper, we PCR-amplified the SNP targets and directly sequenced the amplified DNA to verify the SNP genotype results.

Reaction mix for SMAP (asymmetrical primer amplification).

We typically performed SMAP reactions in a 25- μ l volume. Enzymatic components (*Aac* DNA polymerase and *Taq* MutS) for the SMAP 2 assays were supplied by DNAFORM K.K. in a prototype SMAP 2 core kit. Each reaction contained 3.2 μ M each of FP and TP, 0.4 μ M each of OP1 and OP2, 1.6 μ M BP, 1.4 mM dNTPs, 5% DMSO, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM (NH₄)₂SO₄, 8 mM MgSO₄, 0.1% Tween 20, 1/100,000 diluted original SYBR Green I, 40 units of *Aac* DNA polymerase, 1.5–2.4 μ g of *Taq* MutS and 1 μ l of prepared blood sample. We performed SMAP 2 reactions at 60 °C.

Reaction mix for LAMP (symmetrical primer amplification). We performed LAMP reactions in a 25- μ l volume using *Aac* DNA polymerase (DNAFORM K.K.). The assay composition was identical to the reaction mix for SMAP 2 reactions with the exception of the FP. For LAMP we designed a second TP primer (TP2; **Supplementary Figs. 2 and 3**) and used at the same concentration (3.2 μ M) as the other TP (TP1). We prepared the LAMP and SMAP 2 comparative samples identically and carried out the reactions at 60 °C.

Data analysis and diagnostic judgment. We evaluated the application of SMAP 2 in practical diagnostics according to the principle of amplification versus non-amplification compared to threshold values. The presence of intercalating SYBR Green I dye during the reaction was monitored with Mx3000P system (Stratagene) and SNP typing was determined according to the fluorescence intensity. Generally, we considered amplification to be positive if the fluorescence (dR: baseline-subtracted fluorescence reading) strength was higher than a value of 1,000, and that no amplification occurred if the signal was less than 1,000.

Additional methods. Descriptions of the SMAP 2 assay design strategy, sample preparation, amplification-mix setup and additional information are available in **Supplementary Methods** online.

URLs. Information about availability of reagents and licenses for conducting SMAP technology assay development and commercial use is available from the DNAFORM K.K. website (http://www.dnaform.jp/index_e.html). The RIKEN SMAP server web site for SMAP 2 primer design software and suggestions for further optimization can be found at (<http://www.smappedna.com>).

Note: Supplementary information is available on the Nature Methods website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Methods* website for details).

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