Defined Oligonucleotide Tag Pools and PCR Screening in Signature-Tagged Mutagenesis of Essential Genes from Bacteria

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ABSTRACT

We describe a fast and simple method for signature-tagged mutagenesis (STM) using defined oligonucleotides for tag construction into mini-Tn5 and PCR instead of hybridization for rapid screening of bacterial mutants in vivo. A collection of 12 unique 21-mers were synthesized as complementary DNA strands to tag bacterial mutants constructed by insertional mutagenesis using pUTmini-Tn5Km2 plasmids. Tags were tested in a combination of assays by PCR and compared to hybridization for specificity and for large-scale screening. Each defined tag has the same melting temperature, an invariable region to optimize PCRs and a variable region for specific amplification by PCR. A series of "suicide" plasmids carrying mini-Tn5s, each with a specific tag, were transferred into Pseudomonas aeruginosa, *giving 12 libraries of mutants; groups of 12 mutants were pooled and arrayed into 96-well microplates, representing approximately one-sixth of the* P. aeruginosa *5.9-Mb genome. This simple STM method can be adapted to any bacterial system and used for genome scanning in various growth conditions.*

INTRODUCTION

Bacterial pathogens produce a plethora of different virulence factors; some of which bypass the immune system and cause disease. The expression of most known virulence factors is regulated by specialized signal-transduction systems during bacterial growth and infection (4). The identification of many virulence factors has been limited by the ability to reproduce host environmental conditions in the laboratory and by the few methods available for selecting bacterial mutants having defects in genes that express these factors.

Signature-tagged mutagenesis (STM) allows identification of genes coding for proteins that are essential for in vivo survival and/or responsible for the infection. STM is based upon a negative selection in which individual mutants are specifically tagged and identified within complex pools of bacteria. By using this method, a large number of mutants can be analyzed in parallel for negative phenotypes, including attenuation of virulence (8). To identify specific mutants within a pool, individual tagging of bacteria is necessary (7). In the original STM (8), tagging was accomplished by ligating oligonucleotides as 40-bp "signatures" containing semi-random sequences (7). Tags contained a variable region ($[NK]_{20}$) having 10^{17} different sequences and an invariable region for amplification by radioactive polymerase chain reaction (PCR) to generate specific hybridization probes. Tags were double-stranded by PCR and cloned into a mini-transposon to generate a library of mutants.

It became rapidly apparent that some tags gave better hybridization signals than others. Other limitations included cross-hybridization of tags obtained from the low specificity of degenerated tags that have homologous sequences. In addition, tags representing bacteria grown in vitro and in vivo had to be screened by hybridization and compared carefully to identify nonambiguous positive signals. The mutants arrayed in 96-well microplates were grown in vitro and represented a library of mutagenized bacteria (the in vitro pool) each with a different tag and unique signature. Mutants were pooled in a single in vitro culture from which an aliquot is used for in vivo passage. Based upon negative selection, this in vivo step eliminates mutants incapable of survival and/or maintenance. Bacterial genomic DNA is extracted from bacteria grown in vitro and from those recovered after in vivo passage and used as a template for preparation of probes. These DNA probes were hybridized with DNA from colony blots corresponding to arrayed mutants for identification of clones that are unable to survive in vivo. Since the complexity of tags have a variable region containing 10^{17} possibilities, we noted weak hybridization signals with several clones of bacteria recovered in vitro and in vivo (not shown). These limitations can lead to ambiguity and false interpretation of STM data.

PCR is a simple, rapid and useful technique used in most laboratories in almost all applications of molecular biology (6). PCRs can also be done directly with minimal treatment of cells as DNA template, including bacterial colonies (9). An important step in PCR is the design of sequence-specific primers between 18-mers to 24-mers in length (6). Moreover, higher free energy for duplex formation (ΔG) (1) caused by insertion of certain nucleotides at the 5′ end rather than at the 3′ end of a PCR primer stabilized primer-template duplex and optimized amplification reactions (12); whereas, the 3′-terminal position in primers was found essential for controlling mispriming (10). The insertion of a nucleotide mismatch at the 3′ terminus of a primer-template duplex is more detrimental to PCR amplification than internal mismatches (10). Instead of using degenerated oligonucleotides, which are difficult to design for optimizing PCR, we decided to design specific oligonucleotides in the objectives to optimize and to have high specificity during the PCR screening step.

To eliminate the screening steps for identification of colonies containing unique tags and to facilitate interpretation of results in STM, we decided to synthesize a simpler mixture of oligonucleotide tags based upon these rules. Also, we decided to use PCR instead of hybridization because it is a more rapid and easier method than hybridization for tag analysis and for selection of bacterial clones. These important modifications eliminate the use of probe markers, the labeling procedures and DNA purification and simplify the time-consuming screening steps. Our experimental scheme involves designing 12 pairs of 21-mers synthesized as complementary DNA (cDNA) strands for cloning into mini-Tn*5*. The sets of 12 tags are repeatedly used (11) to construct 12 libraries and for specific PCR amplification as signature tags that are easily detectable by PCR. Tagged products from arrayed bacterial clones can be compared as DNA products of specific length sepa-

rated by agarose gel electrophoresis. STM was tested with *Pseudomonas aeruginosa*, an opportunistic pathogen whose genome is being sequenced (**http://www.pathogenesis.com**).

MATERIALS AND METHODS

DNA Synthesis of Signature Tags

Table 1 lists the tags used; they were synthesized following the supplier's recommendations for oligonucleotides (PE Biosystems, Foster City, CA, USA). Twelve pairs of 21-mers were designed following three basic rules: *(i)* similar melting temperature (T_m) of 64°C to simplify tag comparisons by using one step of PCRs; *(ii)* invariable 5′ ends with higher ΔG than at the 3′ end to optimize PCR amplification reactions (10); and *(iii)* a variable 3′ end for an optimized yield of specific amplification product from each tag (12).

Table 1. DNA Sequences of Oligonucleotides Synthesized for STM

Tag Number	Nucleotide Sequence
1	5′-GTACCGCGCTTAA**ACGTTCA**G-3′
2	5′-GTACCGCGCTTAA**ATAGCCT**G-3′
3	5′-GTACCGCGCTTAA**AAGTCTC**G-3′
4	5′-GTACCGCGCTTAA**TAACGTG**G-3′
5	5′-GTACCGCGCTTAA**ACTGGTA**G-3′
6	5′-GTACCGCGCTTAA**GCATGTT**G-3′
7	5′-GTACCGCGCTTAA**TGTAACC**G-3′
8	5′-GTACCGCGCTTAA**AATCTCG**G-3′
9	5′-GTACCGCGCTTAA**TAGGCAA**G-3′
10	5′-GTACCGCGCTTAA**CAATCGT**G-3′
11	5′-GTACCGCGCTTAA**TCAAGAC**G-3′
12	5′-GTACCGCGCTTAA**CTAGTAG**G-3′

Each 21-mers has a T_m of 64°C and permits PCRs in one step when primer combinations are used for screening. The consensus 5′ ends comprising the first 13 nucleotides have higher ΔGs for optimizing PCRs. The variable 3′ ends indicated in bold define tag specificity and allow amplification of specific DNA fragments. Each tag is used as a primer in PCR with a primer synthesized within the Km-resistant gene of mini-Tn*5Km2*. The set of 12 21-mers representing the cDNA strand in each tag is not represented and can be deduced from the sequences presented.

Construction of Tagged Plasmids

Tags were synthesized as 24 oligonucleotides capable of annealing as 12 21-mers of cDNA strands. Annealing reactions contained 50 pmol of both complementary oligonucleotides in 50 µL of TE buffer (10 mM Tris-HCl, pH 7.4, 0.5 mM EDTA). The mixture was heated for 5 min at 95°C, left to cool slowly at room temperature and kept on ice. Double-stranded (ds)DNA tags were prepared in 12 separate reactions with pUTmini-Tn*5Km2* plasmid DNA (5) digested with *Kpn*I (New England Biolabs, Mississauga, ONT, Canada), and recombinant molecules were constructed in vitro by blunt-end, fill-in with T4 DNA Polymerase (Life Technologies, Gaithersburg, MD, USA) and T4 DNA ligase, following the manufacturer's recommendations. Plasmids were transformed into *Escherichia coli* S17-1λ*pir* (15) by electroporation, and transformants were selected on LB agar (Difco Laboratories, Detroit, MI, USA) supplemented with 50 µg/mL of ampicillin (Amp) and kanamycin (Km) (both from Sigma, St. Louis, MO, USA). Single colonies were selected, purified and screened by colony PCR in 50-µL reaction volumes containing the following: 10 µL of boiled bacterial colony in 100 µL of TE PCR (10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA), 5 µL of 10× *Taq* DNA Polymerase reaction buffer (Life Technologies); 1.5 mM $MgCl_2$, 200 µM of each dNTPs, 10 pmol of each 21-mers. The 21-mers, numbered 1–12 in Table 1, were used in combination with the pUTKanaR1 (5′-GCGGCCTCGAGCAAGACGTTT-3′) primer from the Km-resistant gene. Five units *Taq* DNA polymerase were used in each PCR. Amplification conditions were the following: hot start for 7 min at 95°C, 2 cycles at 95°C for 1 min, 65° to 55°C for 1 min and at 72°C for 1 min followed by 10 cycles at 95°C for 1 min, 55°C for 1 min and 72°C for 1 min (touchdown PCR) in a DNA Thermal Cycler (Perkin-Elmer, Norwalk, CT, USA). Amplified products were analyzed in a 1% agarose gel, 1× Tris-borate EDTA buffer and stained in 0.5 µg/mL ethidium bromide solution.

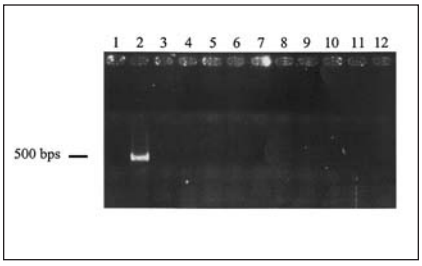

Figure 1. Agarose gel electrophoresis of specific tag2 amplification product obtained when using total DNA from cells of *E. coli* S17-1λ*pir* transformed with the pUTminiTn*5*Tag2. Lanes 1–12 represent PCR products obtained using the 1–12 21-mers in Table 1 and the Km primer.

Construction of *P. aeruginosa* Mini-Tn*5Km2* Mutant Libraries

E. coli S17-1λ*pir* with pUTmini-Tn*5Km2* tagged plasmids was used as donor for conjugal transfer into the recipient host strain *P. aeruginosa* PAO-1293, a PAO1 derivative containing

phage Etv79 and a chloramphenicol (Cm) mutation (kindly provided by Dr. Bruce Holloway, Monash University, Queensland, Australia). Matings were done as previously described (14) with the following modifications: a ratio of 1 donor cell for 10 recipient cells was mixed and spotted as a 50-µL drop on a nylon membrane and placed on a non-selective Brain Heart Infusion Agar (BHIA) plate (Difco Laboratories). Plates were incubated at 30°C for 8 h, and filters were washed with 10 mL of phosphate-buffered saline (PBS) to recover bacteria. Exconjugants were selected on BHIA supplemented with Cm (5 µg/mL) (Sigma) and Km (500 µg/mL) and incubated overnight at 37°C. Colonies resistant to Km (mini-Tn*5* marker inserted into the chromosome of *P. aeruginosa*) and Cm (*P. aeruginosa* marker) were transferred by "toothpicking" on BHIA supplemented with piperacillin (10 µg/mL) (Sigma) (to exclude bacteria having the "suicide" donor plasmid pGP704 inserted into the chromosome). As shown in Figure 2, colonies of exconjugants from the same tag pool were grown overnight at 37°C in a 96-well microplate with 1.5 mL of TSB (Difco Laboratories) supplemented with Km (300 µg/mL). Dimethyl sulfoxide (DMSO) (7%) was added, and plates were kept frozen at -80°C. For STM, one mutant from each library was picked to form pools of 12 unique tagged mutants.

Screening of Tagged Mutants

The 12 *P. aeruginosa* mutants from the same pool were grown separately overnight at 37°C. Aliquots of these cultures were pooled, and a sample was removed for PCR analysis (the in vitro pool). A second sample from the same pool containing 10^5 bacterial cells per 100 µL was analyzed by bacterial counts on agar plates (colony-forming units) and introduced into agar beads (2). Young adult, Sprague-Dawley rats of 140–160 g in weight (The Jackson Laboratories, Bar Harbor, ME, USA) were anesthesized with isofluorane, and 100 µL of the agar bead mixture were injected intratracheally into the left lobe of lungs. Bacteria were recovered 8 days after inoculation from the lungs of animals by plating homogenized tissues on BHIA supplemented with Cm and Km. An aliquot of 10^4 colonies recovered after in vivo selection were pooled and used for colony PCR (the in vivo pool). PCR amplification products of the tags present in the in vivo pool were compared with amplified products of tags present in the in vitro pool, as depicted in Figure 3. *P. aeruginosa* PAO909, used as a negative control for PCR screening, is a transduction mutant (E79tv-2) purine auxotroph (*pur-67*) of the parent strain PAO1 obtained from the *Pseudomonas* Genetic Stock Center (East Carolina University, Greenville, NC; **http://www.pseudomonas.med.ecu.edu**).

RESULTS AND DISCUSSION

Elaboration of Twelve Specific STM Tags

Table 1 lists the 12 21-mers that were synthesized. All STM tags showed specificity as a unique DNA amplification product by PCR when using primers 1–12 in combination with the Km primer. The example shown in Figure 1 demonstrates that there was no cross-amplification from different tags, and the expected 500-bp product was amplified. All other tags used as PCR primers gave specific amplifications from their corresponding pUTmini-Tn*5Km2* tagged plasmid (data not shown). These results confirmed that modifications of a few nucleotides at the 3′ end of a primer can lead to a very specific PCR and optimal amplification product. It has been shown that PCR can detect a single mismatch and differentiate specific alleles (10). In addition, a higher ΔG at the 5′ terminus of a primer allowed optimal amplification products (12). Touchdown PCR was preferred to the standard PCR cycle because it facilitated establishment of optimal PCR conditions for two primers and increased specificity of the amplification products obtained (6).

Construction of Libraries

As shown in Figure 2, construction of 12 libraries with plasmids pUTmini-Tn*5*tag1 to mini-Tn*5*tag12 was done for obtaining pools of mutants. By using

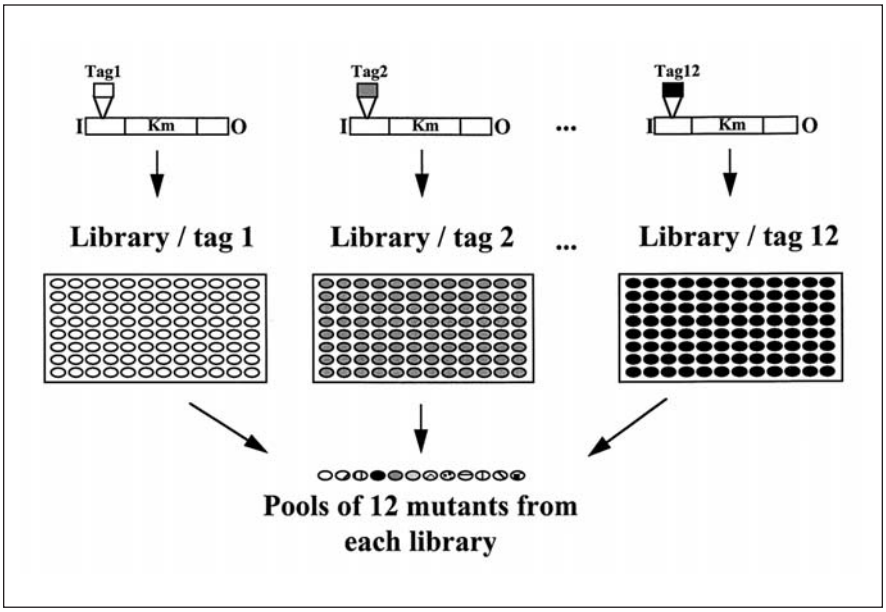

Figure 2. Construction of 12 libraries of *P. aeruginosa* mutants tagged with mini-Tn*5Km2*. dsDNA tags were cloned into the pUTmini-Tn*5Km2* plasmid (see Materials and Methods). Km-resistant exconjugants were arrayed as libraries of 96 clones. In a defined library, each mutant has the same tag but inserted at different locations in the bacterial chromosome. One mutant from each library is picked to form 8 pools of 12 mutants with a unique tag for each. Pools are arrayed into 96-well microplates, and pools are injected into 12 individual rats for in vivo selection. The differences between tags are represented by gray-scale colors and motifs. O and I represent the 19-bp inverted repeats at each extremity of the mini-

this method, there was no *a priori* screening necessary to confirm that mutants in a given pool are tagged more than once, which is caused by two or more conjugation events in the same bacterial cell. Assuming a random nonspecific insertion of the mini-Tn*5Km2* transposon, statistical analysis suggests that for the 5.9-Mb chromosome of *P. aeruginosa*, the possibility of obtaining the same tag twice by insertion of the mini-Tn*5* into the same gene is 1/5900. Following this scheme, we obtained 8 pools of 12 mutants per microplate, and each mutant in the same pool has a different and unique tag. To identify *P. aeruginosa* mutants that are unable to survive in vivo, these pools were then incorporated into agar beads and injected into rat lungs (2).

STM Selection

In the first STM step shown in Figure 3, in vitro cultures were prepared separately for each tagged bacterial clone. In a single culture of pooled bacteria, we anticipated that certain mutants can have defects, resulting in slow growth; whereas, others grow as the wild-type. In this case, slow-growth mutants will not necessarily be recovered or could be underrepresented. Furthermore, to meet the objective that all mutants of a pool must be equally represented, clones were grown individually and then pooled. In Figure 3, the comparisons made between amplified products obtained from in vitro and in vivo pools showed that the mutant with tag1 was not recovered after the in vivo passage in rat lungs. This negative control in STM was a tag1 mutant constructed with *P. aeruginosa* strain PAO909, a purine auxotroph of PAO1. This strain is highly attenuated and used as an artificial STM negative control (to test the efficiency of the in vivo selection step) confirmed by the presence of specific PCR product in vitro but not after in vivo passage in rat lungs. These results showed that PCR can be used to compare tagged mutants present before and after the in vivo selection step. In Figure 3, the amount of PCR product obtained with the in vitro pool seems to be greater than with the in vivo pool. These variations are expected and typical of PCR amplification products obtained from bacterial cells. Differences in intensity between these two pools were expected because the quantity of template is not the same, and PCR products have been analyzed in two distinct agarose gels. However, one can compare the intensity of PCR products obtained from each tag in the same pool. One might compare the ratio of intensity of PCR product of tags from a pool with the ratio of intensity of PCR product from the other pool. Another advantage of PCR is that a very low concentration of product indicated that the mutant is present. This overcomes the possibility to identify negative mutants underrepresented in the in vivo pool.

By using this new approach, we screened 1012 mutants, each with a unique tag in the rat lung model for chronic lung infections (2) and identified 45 virulence attenuated mutants (not published) representing approximately 4% of mutants screened. This percentage corresponded to the ratio of mutants obtained by STM with *Salmonella typhimurium* (8), with *Staphylococcus aureus* (11,13) and with *Vibrio cholerae* (3). Thus, PCR can be used to rapidly and efficiently detect STM mutants, saving time-consuming efforts in

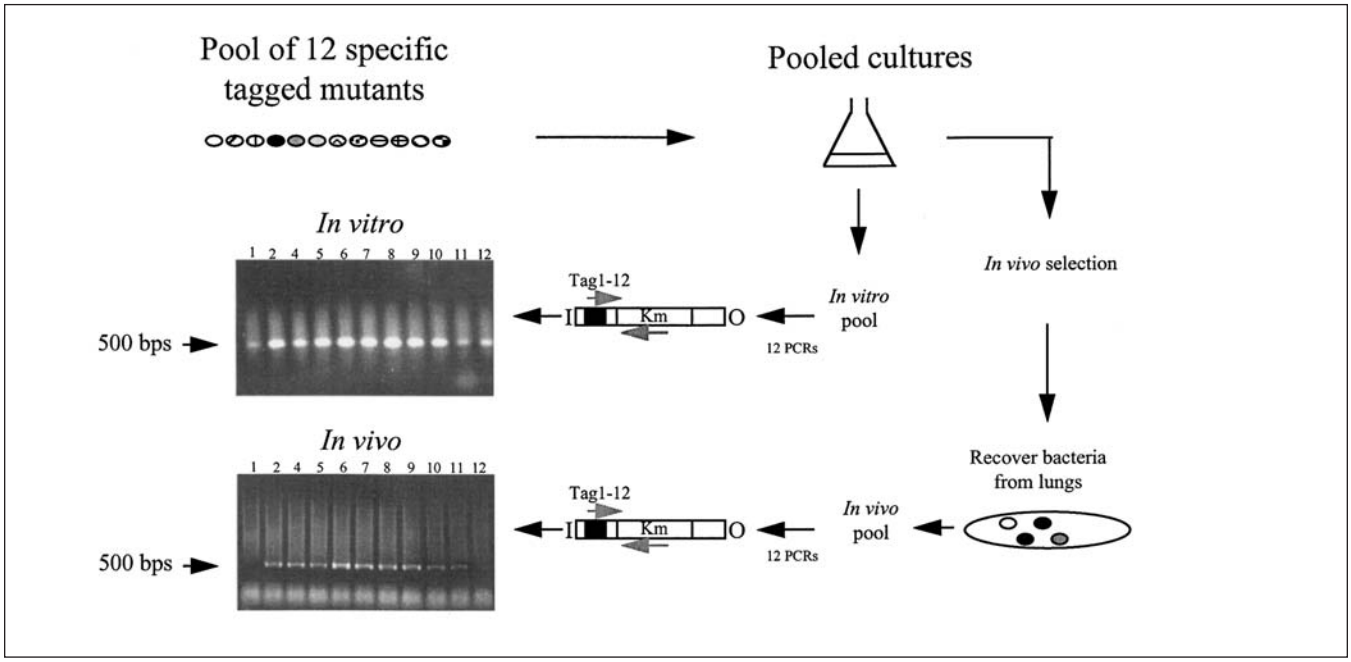

Figure 3. STM scheme for comparisons between the in vitro and in vivo negative selection step. Mutants from the same pool were grown as separate cultures. An aliquot was kept as the in vitro pool, and a second aliquot was used for chronic lung infection in rats. After this passage, bacteria were recovered from lungs and constitute the in vivo pool. The in vitro and in vivo pools of bacteria were used to prepare DNA templates in 12 PCRs using the 21-mers 1–12 in Table 1 and the Km primer. PCR products were analyzed by agarose gel electrophoresis. Lanes 1–12: PCR products obtained with the primers 1–12. In this example, the mutant with tag3 was not used, and the mutant with tag1 was a negative control (strain PAO909 mutagenized with mini-Tn*5*/Tag1).

testing oligonucleotide specificity and screening of mutants for the construction of pools of tagged mutants. A limitation of the original STM method was the complexity (number of differently tagged mutants) of pools that gave weaker hybridization signals (9). In fact, there are two factors that restrict the complexity of pools of different mutants for use as inocula in infection studies. First, as demonstrated by Chiang and Mekalanos (3), the animal model might limit the complexity of the pool. This is true because when the pool is more complex, there is a higher probability that some mutants will not be in sufficient numbers in the organs of an infected animal to produce enough labeled probe for analysis by hybridization (3,8). This first factor is really animal-model-dependent. Second, the amount of labeled tag for each transposon is inversely proportional to the complexity of the tag pool (8). The size of the aforementioned pool is limited, as hybridization signals can become too weak for autoradiography detection. However, in the STM we describe here, there was no limitation in the complexity of pools after in vivo selection. In fact, the method presented here as a PCR screening has only one target sequence and was not affected by the latter pool-complexity factor. One has to take into consideration signals from attenuated mutants, which are sufficient to generate a PCR product after the in vivo selection. As demonstrated in the basic method, and depending on the animal model selected and mutant types, the attenuation of mutants needs additional testing by other methods such as LD_{50} (8) and/or competitive infectivity index test (3). A collection of 24, 48 or 96 tagged oligonucleotides could be designed with the modifications of STM presented; however, these oligonucleotides should be analyzed to eliminate PCR amplification cross-reactions, and tags should be carefully designed. This would increase the number of mutants in a pool, but would reduce the number of animals used for screening mutants in vivo. The number of tags used is only limited by the ability to produce the expected DNA product as amplified by PCR. Compared to hybridization, PCRs gave a clear distinction between the presence or absence of a specific amplification product and had more sensitivity. Using PCR for identification of mutants makes it possible to use robot-based screening and facilitates large-scale screening of mutants. Also, false negatives and the ramifications of hybridization are eliminated. To simplify the PCR data obtained, we used a limited number of tags; however, additional tags can be used. Finally, these modifications give a rapid and easy method for STM screening.

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