Improved Protocol for SAGE Tag-to-Gene Allocation

BioTechniques 34:1212-1219 (June 2003)

ABSTRACT

*Serial analysis of gene expression (SAGE) is a powerful method for large-scale analysis of gene expression patterns. SAGE yields digital information on transcript abundance by the use of short sequence fragments (tags). Because SAGE does not require a priori knowledge of the expressed genes in the starting material, SAGE can be used for gene discovery. Unfortunately, correct tag-to-gene allocation after SAGE remains problematic when the short sequence of the tag corresponds to more than one gene in the reference database or when novel yet uncloned genes were detected. To overcome this problem, we developed an improved protocol for the proper identification of tag-corresponding genes. It relies on the isolation of 3'-terminal cDNA restriction fragments by the use of paramagnetic streptavidin beads and the ligation of linkers before the amplification step. Our protocol benefits from additional information encoded in each SAGE tag: its location 3'-terminal to the last *Nla*III restriction site in the cDNA. In contrast to previously described protocols, stringent PCR conditions can be applied because of the length of the specific primers, which are composed of linker- and tag-specific sequences. Additionally, we demonstrate that our protocol yields quantitative information that can be used for further expression analysis of specific SAGE tags.*

INTRODUCTION

Serial analysis of gene expression (SAGE) (1), a method for large-scale expression analysis, has been applied to many different organisms, tissues, and pathological conditions. The principle is based on the reduction of each expressed transcript sequence to short (9–11 bp) yet representative sequences (tags). The number of times a particular tag is detected in a SAGE library pro-

vides a quantitative and digital measure of gene expression (1–3).

Unfortunately, a major problem exists when analyzing SAGE data: although each specific tag theoretically contains enough information for correct assignment of the corresponding gene (1), an identification of the underlying gene by database comparison sometimes remains difficult, if not impossible (4,5). Homology searches in expressed sequence tag (EST) databases may fail to detect homologous sequences (gene discovery aspect of SAGE) or may result in multiple hits. These situations require the direct isolation of the corresponding transcript sequence by molecular biological means. However, the isolation of the tag-specific transcripts has its hurdles. Because of the short length of the tag sequences (9–11 bp), standard techniques based on PCR amplification or colony screening are difficult to perform. Nevertheless, correct clones have been identified successfully by screening with short oligonucleotide probes (1) or modified PCR protocols (4,5). Screening with short oligonucleotide probes, however, is time-consuming and laborious, whereas the published PCR protocols sometimes fail in proper tag-to-gene annotation or had to be performed under sophisticated PCR conditions, which have to be optimized for each tag (4).

With regard to the considerable expenditure necessary for generating a SAGE expression pattern, as well as to take full advantage of the gene discovery potential of SAGE, a reliable and fast method for tag-to-gene allocation is urgently required. Ideally, such a method should be robust and applicable to all tag sequences with similar efficiency, suited for automation, and, when possible, would furthermore be able to confirm or disprove the expression changes predicted by SAGE data.

The following protocol presents such an optimized tag-to-gene approach. Because the principle is related to rapid amplification of cDNA ends (RACE)-PCR (6), we termed our approach SARA-PCR, where SA stands for SAGE and RA for RACE-PCR. In contrast to a similar procedure published more recently (7), which also uses 3'-terminal restriction fragments,

SARA-PCR is more protected against nonspecific amplification because it circumvents a re-amplification step and because it benefits from the additional linker sequence instead of using only short (14 bp) tag-specific primers with a 5'-terminal 6-bp mismatch sequence (*Bam*HI site), which restricts stringent PCR conditions (7). Moreover, we demonstrate that our protocol yields quantitative information, so it can be employed both to identify and to explore the expression of specific candidate genes.

MATERIALS AND METHODS

SAGE Tags

SAGE tags were generated as described previously (3) and were analyzed using SAGE software V3.01 (kindly provided by K.W. Kinzler and colleagues) and reference database (file, *SAGEmap tag ug-rel-Nla3-Mm.txt* derived from *Mus musculus* UniGene Build #96) according to Lal et al. (8).

Preparation of cDNA

Five micrograms of poly(A)⁺-RNA were freshly prepared from adult male C57b6 mouse brain (BGVV, Berlin, Germany) using TRIzol[®] reagent and MessageMaker[™] kit (both from Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using cDNA synthesis system (Invitrogen) in combination with PAGE-purified and 250 pmol biotin-labeled 20-nucleotide M13-specific oligo(dT) primers (biotin-5'-CTAG-TTGTAACGACGCGCCAGTTTTT-TTTTTTTTTTTTTTTTTT-3'; Eurogentec, Seraing, Belgium) (Figure 1A).

Modification of cDNA

Five micrograms of biotinylated double-stranded cDNA were digested with 50 U *Nla*III (New England Biolabs, Beverly, MA, USA) for 1 h at 37°C in a volume of 200 µL (Figure 1B). cDNA was purified with phenol:chloroform and washed twice. The most 3'-terminal restriction fragments were recovered by binding to streptavidin-coated paramagnetic beads (Dyna, Oslo, Norway). cDNA restriction prod-

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ucts were added to 200 μ g beads and mixed intermittently for 1 h at room temperature (Figure 1C). To remove the unbound non-biotinylated 5' cDNA fragments, beads were washed three times with 2 volumes of 1 $\times$ B+W Buffer (2 $\times$: 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 2 M NaCl). One microgram of PAGE-purified linker A (5'-TTTGGAT-TTGCTGGTGCAGTACAACTAGGC-TTAATAGGGACATG-3' hybridized to 5'-[phosphorylated]TCCCTATTAAGC-CTAGTTGTACTGCACCAGCAAAT-CC[amino modified C7]-3'; Amersham Biosciences, Buckinghamshire, UK) was added to bound cDNA, incubated for 2 min at 50°C, and cooled to room temperature for 15 min. Ligation of linker A was performed in a final volume of 40 μ L for 2 h at 16°C using 2 U T4 Ligase (Invitrogen, Eggenstein, Germany) (Figure 1D). cDNA prepared this

way directly served as template for PCR (Figure 1E), either using multiplex PCR in combination with gel electrophoresis (see Quantification I below) or real-time PCR (see Quantification II below).

Primer Design

Forward primers: primers 5'-terminal contained 15 bp corresponding to the linker A, the *Nla*III restriction site (CATG), and a 3'-terminal 10-bp sequence specific for each SAGE tag [5'-TAGGCTTAATAGGGACATG (tag)_{10bp}-3']. Reverse primer—universal for all tags—contained a 20-nucleotide M13-specific sequence (5'-AGTTGTAAAACGACGGCCAG-3') corresponding to the M13-specific sequence of the oligonucleotide used for cDNA synthesis (Figure 1E).

Tag sequences were derived from a

previous SAGE project (3). Tags with an ambiguous (tags 6–16) or unknown (tags 17 and 18) gene identity, tags with a low expression level as calculated by SAGE data (tags 8, 9, and 14–16), or tags of known origin (tags 1–5)—some of them with a differential expression that has been proven by Northern blotting (tags 3–5)—were chosen for the evaluation of our protocol (tag 1: gggttaaatgt; tag 2: tgtgaagtag; tag 3: tctacactgc; tag 4: taactgacaa; tag 5: gggcctgtgg; tag 6: gaaataataa; tag 7: ttgtgcaaac; tag 8: aagccacaga; tag 9: ttgctgtgt; tag 10: aggcattgtc; tag 11: gaagttaaat; tag 12: tcgctgcttt; tag 13: tgctgagatc; tag 14: ggtagcgtaa; tag 15: atttagaggt; tag 16: tcttaacgtc; tag 17: tcattggtcg; tag 18: aaaattattg).

Standard PCR

PCR was performed on a TRIO Thermoblock (Biometra, Göttingen, Germany) in a volume of 25 μ L using 1 $\times$ MgCl₂-free DyNAzyme™ buffer (Biometra), 2 mM MgCl₂, 100 μ M dNTP, 12.5 pmol each primer, 1 U DyNAzyme DNA polymerase (Biometra), and 0.5 μ L modified cDNA (1:100). The following amplification protocol was used: 5 min at 95°C, followed by 35 cycles of 45 s at 95°C, 45 s at 60°C, 45 s at 72°C, and a final extension for 7 min at 72°C. All reactions were performed with a hot start. Optimization of PCR conditions was carried out by varying the MgCl₂ concentration and annealing temperature. Nevertheless, for most tags, the protocol described above proved to be optimal.

To test the effects of different DNA polymerases and buffers, alternatively, 1 U Tag-Polymerase (Perkin Elmer) in combination with SAGE-PCR buffer [10 $\times$: 166 mM (NH₄)₂SO₄, 670 mM Tris, pH 8.8, 67 mM MgCl₂, 100 mM β -mercaptoethanol (1)] or PCR-Buffer II (Perkin Elmer), 2 mM MgCl₂ was used.

In cases with a high background signal, a pre-amplification was performed by an additional 10 cycles of the standard amplification program containing only 50 pmol tag-specific (forward) primer before the M13 (reverse) primer was added to the reaction mixture in a minor amount (5 pmol). With both primers, “exponential” amplification

Figure 1. Principle of SARA-PCR. cDNA synthesis with biotinylated oligo(dT) primers including a M13-specific sequence (A); *Nla*III restriction (B); binding of the 3'-terminal restriction fragments with a biotin label to streptavidin-coated paramagnetic beads (C); followed by a washing step to eliminate the 5'-terminal restriction fragments; ligation of the linkers at the *Nla*III restriction site (D); and amplification with the tag-specific forward primer (composed of the linker sequence and a tag-specific sequence) and the reverse primer (with a M13-specific sequence) (E).

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was continued for a further 35 cycles. Because of the long half-life of Dy-nazyme DNA polymerase at 95°C, no further enzyme was added.

Northern Blotting

Tag-specific products from SARA-PCR (as gene-specific probes) were gel-purified using the QIAquick™ Gel Extraction Kit (Qiagen, Hilden, Germany) and labeled with [α -³²P]dCTP (6000 Ci/mmol) using the Rediprime™ DNA Labeling System (Amersham Biosciences, Braunschweig, Germany). Total RNA was separated on a 1% sodium phosphate-buffered agarose gel and capillary-blotted onto a positively charged nylon membrane (Roche Applied Science, Mannheim, Germany) according to a standard protocol (9). The blot was hybridized to radioactive probes at 64°C using QuickHyb® hybridization solution (Stratagene, La Jolla, CA, USA), stringently washed for 30 min in 0.1% SDS, 0.1× SSC at 68°C, and exposed to a phosphor screen (Typhoon® phosphorimager; Molecular Dynamics, Sunnyvale, CA, USA).

Quantification I: Multiplex RT-PCR with Internal Standard

SAGE tag-specific fragments were co-amplified with an internal standard in the same reaction tube. The product of tag 2 was chosen as an internal standard because it has been proven by Northern blotting not to be regulated in our model (data not shown). For each tag, PCR was performed using three different dilutions (1:200, 1:500, and 1:1000) of ischemia and control cDNA. Each 25-μL reaction volume was derived from a master mixture and contained 1× MgCl₂-free DyNAzyme buffer, 2 mM MgCl₂, 100 μM dNTP, 25 pmol reverse primer (M13), 12.5 pmol forward primer for the internal standard (tag 2), 12.5 pmol forward primer for each tag, and 1 U DyNAzyme DNA polymerase. Amplification was performed as described above.

For quantification, 8 μL each PCR product was separated on a 2% TBE agarose gel stained with ethidium bromide. After electrophoresis, the gel was scanned with a Typhoon phosphorimager. The intensity of amplified frag-

ments was quantified using Image-Quant™ software (Molecular Dynamics). For each cDNA dilution, a ratio was calculated by dividing the intensity value of the candidate gene product by the intensity value of the co-amplified internal standard product. Mean values of those ratios (derived from three different template concentrations for each condition) were compared between ischemic and control conditions (see Figure 3B). Differences between the mean values of the conditions compared greater than two standard deviations (calculated from the three different dilutions for each condition) were regarded as significant. Calculated standard deviations in almost all cases were less than 10%.

Quantification II: Real-Time PCR

Relative quantification of target gene expression levels was also performed by real-time RT-PCR using the Light-Cycler® in combination with FastStart DNA Master SYBR® Green I (both from Roche Applied Science) according to the manufacturer's instructions. Each 20-μL reaction contained 10 pmol tag-specific forward primer, 10 pmol M13-specific reverse primer, 2.4 μL 4 mM MgCl₂, 2 μL FastStart mixture (enzyme reaction mixture with SYBR Green I dye), 2 μL modified cDNA (1:100 dilution), and water. All reactions with ischemia and control cDNA were performed twice. To determine the relative expression levels, GAPDH was used as a housekeeping gene. The PCR program was as follows: denaturation at 95°C for 10 min, amplification and quantification with 45 cycles at 95°C for 15 s, 55°–65°C for 10 s, 72°C for 12 s, a single fluorescence measurement at target-specific temperature, and a melting curve program with continuous fluorescence measurement from 65°–95°C with a heating rate of 0.1°C/s. Determination of crossing points at which fluorescence rises appreciably above the background fluorescence was performed with the Second Derivative Maximum Method by the LightCycler software. The specificity of amplified PCR products was confirmed by melting curve analysis (see Figure 3E). The relative expression of the specific tag was calculated using the following equation: ratio (AU) = 2^{-(delta CP sample -}

delta CP control). PCR efficiencies of target and reference gene amplification were assumed to be 2. This should be sufficiently accurate to obtain an estimation of the relative expression of the target genes investigated. Melting curve analysis after amplification renders further gel electrophoresis unnecessary (see Figure 3E). Because SAGE data and Northern blot analysis indicated a 16% down-regulation of GAPDH in ischemia, all calculated target gene ratios from ischemic conditions were adjusted by a correction factor of 0.84. Obtained ratios were compared between ischemic and control condition.

All surgical procedures were performed in accordance with the Guidelines for the Use of Animals in Neuroscience Research from the Society for Neuroscience.

RESULTS AND DISCUSSION

Our protocol for correct SAGE tag-to-gene allocation benefits from the additional information contained in each tag: its location 3'-terminal of the most 3' *Nla*III restriction site in the cDNA. This position is claimed for SAGE tags because of the principle of the SAGE procedure. Thus, the use of 3'-terminal cDNA fragments helps to avoid false annealing of the forward primer at homologous sequences, located 5'-terminal of the last *Nla*III-recognition site. In contrast to other protocols (4,5,7), SARA-PCR further benefits from the resulting length of the forward primers, which are not only composed of the tag-specific sequence but also contain the linker-specific sequence. The resulting long primer sequences allow a robust and highly stringent PCR protocol, with no need for further optimization in most cases.

Principle

The 3'-terminal cDNA restriction fragments are generated by the use of an oligo(dT)-M13-biotin-primed (Figure 1A) and *Nla*III-digested (Figure 1B) cDNA prepared from the tissue of interest. Binding of terminal restriction fragments to streptavidin-coated beads (Figure 1C) allows the elimination of 5'-located restriction fragments by a washing step. Linkers known

Table 1. Sensitivity and Specificity of SARA-PCR

Tags Tested (n)	PCR Product (%)	Specificity/Accuracy of Amplification ^a (%)
7 known genes	100	100
27 ESTs	92.5	96
10 no-match tags	80	50

^aObtained PCR products that correctly corresponded to predicted genes/sequences. Nonspecific products were mainly due to false primer annealing.

from the standard SAGE protocol (2) are ligated to the *Nla*III restriction fragments (Figure 1D) before the amplification step (Figure 1E). cDNA modified that way contains exactly defined sequences on both ends, which simplifies the primer design.

SARA-PCR Identifies Tag-Specific Transcripts

Our protocol produced a single and reliable product for almost all forward primers tested (Figure 2 and Table 1). The specificity of the amplification was tested by sequencing. The sequence of those amplimers that corresponded to tags derived from already completely sequenced genes confirmed the correct identity of the PCR products tested. None of the products contained a *Nla*III restriction site in addition to the one located in each forward primer, indicating the correct 3'-terminal position of the fragments (as recommended by the SAGE procedure). Products derived from tags of already known genes matched the existing database entries for those genes. The tags, which are

matching different Unigene clusters could be assigned to single clusters or specific ESTs. The length of all PCR products varied between 87 and 675 bp, with a mean of 219 bp (Figure 2).

Effect of DNA Polymerase Buffer

We observed that PCR buffers and DNA polymerases played an important role in amplification efficiency. Concerning the efficiency and specificity of amplification, the best results were obtained by the use of Dynazyme buffer in combination with Dynazyme DNA polymerase.

Effect of Concentration of Paramagnetic Beads

Recently, an inhibitory effect of the paramagnetic beads in PCR has been described (10,11). Therefore, the effect of various concentrations of beads on PCR efficiency was examined. A reduction of amplification efficiency is only observed at high concentrations (20 μ g beads/ μ L reaction mixture). Thus, we do not expect a relevant inhibitory in-

Figure 2. PCR amplification of tag-specific transcripts. Lanes 2–19, PCR performed with forward primers for selected tags containing the tag-specific sequence and part of the linker sequence; reverse primers were derived from the M13-specific sequence. cDNA derived from ischemic mouse brain, *Nla*III-digested and bound to streptavidin-coated paramagnetic beads, were used as template. Eight microliters of each 50- μ L reaction were loaded per lane in a 2% agarose gel. Lane M, 1-kb DNA standard.

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fluence of the paramagnetic beads at those concentrations (0.04 $\mu\text{g}/\mu\text{L}$) that are used in our SARA-PCR protocol.

Effect of "Linear" Pre-Amplification

Sometimes, after amplification, only slight specific bands and a relevant background signal appeared. Because

of the relatively high amount of template used in each reaction—each carrying an M13-specific sequence at the 3'-terminal end—we suspected that the nonexponential amplification of all M13 sequence-bearing cDNA fragments (in addition to the specific and exponential amplification of the specific cDNA containing both correct primer

binding sites) would be the cause for the background signal, which appears as a smear after gel electrophoresis.

Thus, we enhanced the proportion of specific cDNA fragments (with the M13-specific sequence at one end and the additional tag-specific forward primer binding site at the other end) by adding a pre-amplification step to the protocol in which only the tag-specific forward primer is used. The second (reverse) primer was added after the pre-amplification step, and the standard SARA-PCR protocol (now including both primers) was performed. As expected, a further increase of the amount of specific PCR product was achieved (data not shown).

After establishing the amplification protocol, we tested whether it could be used for quantifying purposes, especially whether it could be used to confirm expression differences postulated by the SAGE data. Therefore, we compared two alternative amplification protocols and Northern blotting as a reference standard for expression analysis. In the first semi-quantitative PCR procedure, an internal standard is co-amplified, and the PCR product is analyzed by standard gel electrophoresis (Figure 3A), whereas the second protocol is a real-time PCR procedure with no need for gel electrophoresis (Figure 3, D and E).

Quantification by Multiplex RT-PCR with Internal Standard

Highly reproducible results were obtained by semi-quantitative PCR with an internal standard (Figure 3, A and B), despite several modification steps of cDNA template. However, this is not really surprising, since SAGE itself relies on even more manipulations and nonetheless has been shown to produce highly quantitative data (1–3).

We observed that our protocol with co-amplification of an internal standard reliably detects differences of expression levels for those genes that were differentially expressed at least 2-fold in Northern analysis.

Differences of expression levels calculated by the mean values high-

Figure 3. Comparison of SAGE tag-specific PCR methods and Northern blotting. Shown as an example are the results of the expression analysis of three differentially expressed (tags 4, 5, and 12) and three unregulated (tags 10, 11, and 14) candidate genes. (A) Quantification by multiplex RT-PCR with internal standard. SAGE tag-specific fragments (target gene, TG) were co-amplified with an internal standard (IS) in the same reaction tube. Eight microliters of each reaction generated with a 1:200 cDNA dilution as template—either derived from ischemic (I) or control (C) mouse brain tissue—were loaded on a 2% agarose gel. First lane, DNA size standard. (B) Comparison of mean values of intensity ratios (IR_m), calculated by the division of the intensity value of the tag-specific amplicon by the intensity values of the internal standard amplicon. Mean intensity ratios were determined using three independent reactions with different template dilutions for each condition. Intensities were determined by fluorescence scanning using a Typhoon phosphorimager. (C) Quantification by Northern blotting. Expression analysis by Northern blotting. Ten micrograms of total RNA loaded each lane, derived either from ischemic mouse brain or control mice. Blot was hybridized with [α - ^{32}P]-labeled and transcript-specific probes. (D) Quantification by real time RT-PCR. Comparison of estimated relative expression levels of the target gene (target gene ratios, AU) in comparison to a housekeeping gene (GAPDH) in ischemia and control. After real-time PCR, crossing points (CP) were determined and relative expression ratios (AU) were calculated using the following equation [$AU = 2^{-(\text{delta CP sample} - \text{delta CP control})}$]. All reactions were performed twice. Absolute SAGE tag counts (control/ischemia) of differentially expressed tags were as follows: tag 4: 6/48; tag 5: 0/8; tag 12: 0/11; tag 10: 2/11; tag 11: 1/8; tag 14: 1/4 (compare Reference 3). (E) Confirmation of specificity of amplified PCR products by melting curve analysis after real-time RT-PCR. Each peak represents one single tag-specific product. Melting curves were converted to melting peaks by blotting the negative derivative of the fluorescence with respect to temperature ($-dF/dT$) against temperature.

ly correlate with differential expression as measured by Northern blotting (Figure 3C), albeit comparison of both methods showed that the absolute expression difference observed by PCR always underestimates the differential expression as measured by Northern analysis.

Quantification by Real-Time RT-PCR

We further checked whether the quantification could be performed by real-time PCR to speed up the process time. The specificity of amplification was proved by melting curve analysis (Figure 3E) and gel electrophoresis: the presence of a single product was demonstrated (data not shown). Results generated by real-time PCR of all genes tested corresponded very well with the data obtained by Northern analysis (Figure 3C) and SAGE, when tags with highly significant ($P < 0.001$) tag-ratios were considered.

CONCLUSION

We developed a fast and highly specific PCR-based procedure for the proper identification of corresponding transcripts for specific SAGE tags. We demonstrate that SARA-PCR can be used for the confirmation of differential expression of candidate tags because of its semi-quantitative abilities. This method should be of great value for all those who want to extract complete information of their own or publicly available SAGE libraries. The properties of our protocol enable use in large-scale applications (high-throughput transcript profiling) when many candidate genes have to be confirmed or expression kinetics have to be exploited. The protocol is timesaving and not very labor-intensive, thereby allowing one to take the full advantage of SAGE data previously generated.

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Supported by the Deutsche Forschungsgemeinschaft (ME1562/1-1) and the Hermann und Lilly Schilling Stiftung. SAGE software (V 3.01) and Detailed Protocol (V 1.0c) were kindly provided by Dr. K. Kinzler. Address correspondence to Dr. George Trendelenburg, Department of Neurology, Charité, Humboldt Universität Berlin, Schumannstraße 20/21, D-10098 Berlin, Germany. e-mail: george.trendelenburg@charite.de

Received 3 February 2003; accepted 20 March 2003.

Ute Kannbley, Krisztian Kapinya, Ulrich Dirnagl, and George Trendelenburg
*Humboldt University
Berlin, Germany*