

Increasing the efficiency of SAGE adaptor ligation by directed ligation chemistry

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ABSTRACT

The ability of Serial Analysis of Gene Expression (SAGE) to provide a quantitative picture of global gene expression relies not only on the depth and accuracy of sequencing into the SAGE library, but also on the efficiency of each step required to generate the SAGE library from the starting mRNA material. The first critical step is the ligation of adaptors containing a Type IIS recognition sequence to the anchored 3' end cDNA population that permits the release of short sequence tags (SSTs) from defined sites within the 3' end of each transcript. Using an *in vitro* transcript as a template, we observed that only a small fraction of anchored 3' end cDNA are successfully ligated with added SAGE adaptors under typical reaction conditions currently used in the SAGE protocol. Although the introduction of ~500-fold molar excess of adaptor or the inclusion of 15% (w/v) PEG-8000 increased the yield of the adaptor-modified product, complete conversion to the desired adaptor:cDNA hetero-ligation product is not achieved. An alternative method of ligation, termed as directed ligation, is described which exploits a favourable mass-action condition created by the presence of NlaIII during ligation in combination with a novel SAGE adaptor containing a methylated base within the ligation site. Using this strategy, we were able to achieve near complete conversion of the anchored 3' end cDNA into the desired adaptor-modified product. This new protocol therefore greatly increases the probability that a SST will be generated from every transcript, greatly enhancing the fidelity of SAGE. Directed ligation also provides a powerful means to achieve near-complete ligation of any appropriately designed adaptor to its respective target.

INTRODUCTION

The development of technologies aimed towards monitoring gene expression on a global scale has revolutionized the study of biology from a systems perspective (1). This perspective embraces the idea that the functional significance of

gene products is not only related to their quantity in the cell, but also to how they interact and are strung together to form genetic and biochemical networks. Numerous technologies have been developed over the past decade, with the greatest attention being given to approaches based on either high-throughput sequencing or massively parallel analysis of the transcriptome (i.e. the set of all expressed genes weighted by transcript abundance) using array hybridization technology. The sequencing approach to monitoring gene expression on a global scale typically involves the creation of short representations of each transcript, such as expressed sequence tags (ESTs) or short sequence tags (SSTs) generated using Serial Analysis of Gene Expression (SAGE) technology (2,3). DNA microarray technology attempts to resolve the transcriptome by selectively binding and quantifying each transcript at one or more complementary registers of a high-density array (4–6). These technologies are now routinely used to identify families of genes—in many cases incompletely characterized or with previously unidentified functionality—which act in concert to define a given cell fate or outcome (7), and have been used to identify upstream sequence elements involved in directing the expression of these gene families. Although microarray technology offers an increasingly reliable and sensitive analysis of gene expression, its use is dependent on an a priori knowledge of genes, which are expressed under a given cell state, currently restricting application of the technology to the identification and quantification of these subsets of genes.

SAGE technology (2), in contrast, directly samples the entire transcriptome of an organism under a given cellular state through the generation of SSTs of 9–22 bp in length. Because a 9–10mer oligonucleotide can theoretically identify 4^9 (262 144) or 4^{10} (1 048 576) unique sequences, the entire transcript population of any organism can potentially be represented (2,3). First, a cDNA copy of the mRNA population is digested with a restriction endonuclease (RE; e.g. NlaIII) and the most 3' end restriction fragments of the digested population are purified. A short oligonucleotide adaptor that contains a unique primer sequence and a recognition sequence for a Type IIS RE is then ligated to the anchored cDNA. Because Type IIS REs are capable of cleaving DNA outside their recognition sequence (8), subsequent cleavage with a Type IIS RE (e.g. BsmFI) releases SSTs of equal length (2). A library of these SSTs is created through subsequent dimerization, amplification via the PCR, concatemerization and insertion into an appropriate vector. Finally, a representative

Table 1. Outline of the enzymatic, purification and isolation steps involved in the SAGE and microSAGE protocols (<http://www.sagenet.org/protocol/index.htm>)

| Enzymatic steps | Purification and isolation steps | |
|--|--|--|
| | MicroSAGE (SADE) | SAGE |
| (1) mRNA preparation | Affinity purification | Precipitation, selection with biotinylated oligo(dT) |
| (2) cDNA synthesis | – | Phenol extraction, precipitation |
| (3) cleavage with anchoring enzyme (digest with NlaIII) | – | Phenol extraction, precipitation |
| (4) 3' end cDNA isolation | – | Affinity purification |
| (5) Ligating adaptors to bound 3' end cDNA | – | |
| (6) Release of cDNA tags (digest with BsmFI) | Phenol extraction, precipitation | |
| (7) Blunt-ending of cDNA tags | Phenol extraction, precipitation | |
| (8) Ligating tags to form ditags | – | |
| (9) PCR amplification of ditags | Phenol extraction, precipitation | |
| | PAGE purification, gel extraction, precipitation | |
| (10) Adaptor removal (NlaIII digestion) and purification of ditags | Phenol extraction, precipitation | |
| | PAGE purification, gel extraction, precipitation | |
| (11) Ligation of ditags to form concatamers | PAGE purification, size selection, gel extraction, precipitation | |
| (12) Insertion into vector | Phenol extraction, selection by host | |

population of clones is serially sequenced to identify and tally each SST. Since each SST is derived from a defined position within a particular cDNA, a given tag can be cross-referenced through organism- and/or tissue-specific genome databases to a particular gene to give a profile of global gene expression. An important advantage compared with microarray technology is that unreferenced SSTs that arise out of the SAGE analysis can be used to identify previously unknown genes and aid in the completion of genome annotations for the organism under study (2,3,9–17).

The ability of SAGE to provide an accurate measure of gene expression profiles is dependent upon the extent to which the distribution of transcript abundances inferred through the sequenced set of amplified SSTs fully reflects the real distribution of the abundances of associated transcripts in the original mRNA population. This fidelity depends upon the accuracy of the sequencing method used to identify the SSTs (18) and on the depth of sequencing applied to the SAGE library (19,20). Less appreciated, however, is the extent to which losses and processing artefacts in each of the 12 enzymatic and 10 purification steps—or 7 in the microSAGE protocol—used to convert the starting mRNA sample into a SAGE library (Table 1) can skew the sequencing results away from the real distribution. To illustrate, if 5 µg of mRNA ($\sim 5 \times 10^{12}$ molecules of average length 2 kb) are used as starting material for the SAGE protocol, a 50% average yield in each processing step would result in an overall yield of 0.000024% (i.e. 0.5^{22}), such that the final sample ($\sim 1.2 \times 10^6$ molecules) would represent a minute fraction of the original. Such an overall yield would result in a form of sampling bias in SAGE analysis equivalent to the bias introduced by an insufficient depth of sequencing (19,20). Although inclusion of PCR steps in the SAGE protocol is intended to recover these losses, amplification after processing can only recover those ditags derived from targets that have survived the numerous enzymatic and purification steps. Clearly then, efforts to maximize yields and minimize artefacts introduced in each processing step are required to ensure the fidelity of SAGE.

Although a number of recent studies have resulted in the improvement of some of the purification steps in the SAGE

addressing the efficiencies of the enzymatic steps of the protocol. Given that the ability to generate a SAGE tag from a transcript is determined by the successful ligation of the SAGE adaptor to the anchored 3' end cDNA population, the yield in this step is likely to contribute significantly to the overall fidelity of the SAGE protocol. Here we demonstrate, using adaptors 1A/B of the current SAGE protocol (version 1e; <http://www.sagenet.org/protocol/index.htm>), that the yield of this ligation step is generally low due to a strong propensity of the anchored 3' end cDNA target to self-ligate. We then show that the addition of PEG-8000, traditionally used to favour the formation of linear ligation products (29–31), increases the yield of the desired adaptor-target heterodimer, but is unable to fully eliminate the formation of unwanted homodimer. Finally, we show that by using an alternative method of ligation, which we call 'directed ligation', a significant improvement in the SAGE protocol is achieved, increasing the efficiency of adaptor ligation and eliminating the irreversible formation of unwanted ligation products.

MATERIALS AND METHODS

Enzymes and constructs

A 956 bp clone homologous to rat liver α transcription factor (GenBank ID: X65948) from rat brain with a polyadenylated 3' end (58 bp), kindly provided by Dr Terry Snutch (Biotechnology Laboratory) in pBluescript SK⁻ (Stratagene), was propagated in *Escherichia coli* DH5 α (Invitrogen). Plasmids were isolated using the boiling miniprep method (32) from 3 ml Terrific broth (Sigma Aldrich) cultures in the presence of 100 µg/ml ampicillin (Sigma Aldrich) when required. Plasmids (~ 20 µg each) were linearized with EcoRV and further purified using the Qiagen Qiaquick purification kit according to the manufacturer's protocol (Qiagen). Orientation and identification of the insert were verified by sequencing of 100 ng of the purified plasmid at the Nucleic Acids and Peptide Synthesis Unit, University of British Columbia. *In vitro* RNA transcripts in the sense orientation were generated from 1 µg of linearized plasmid using the T3 MEGAscript kit (Ambion) following the manufacturer's protocol and stored at -70°C in diethyl-pyro-

Table 2. List of oligonucleotides used in this study to form various SAGE adaptors

| Oligo ID | Sequence (5'→3') | MW (g/mol) |
|--------------------|--|------------|
| 1A | TTTGGATTTGCTGGTGCAGTACAACCTAGGCTTAATAGGGACATG | 13657.06 |
| 1Am6A ^a | TTTGGATTTGCTGGTGCAGTACAACCTAGGCTTAATAGGGACA ^{m6} TG | 13670.95 |
| 1Am5C ^b | TTTGGATTTGCTGGTGCAGTACAACCTAGGCTTAATAGGGAC ^{m5} ATG | 13670.95 |
| 1Bphos | pTCCCTATTAAGCCTAGTTGACTGCACCAGCAAATCC-NH ₂ ^c | 11517.57 |
| 2A | TTTCTGCTCGAATTCAAGCTTCTAACGATGTACGGGGACATG | 12919.55 |
| 2Am6A ^a | TTTCTGCTCGAATTCAAGCTTCTAACGATGTACGGGGACA ^{m6} TG | 12933.58 |
| 2Am5C ^b | TTTCTGCTCGAATTCAAGCTTCTAACGATGTACGGGGAC ^{m5} ATG | 12933.58 |
| 2Bphos | pTCCCGTACATCGTTAGAAGCTTGAATTCGAGCAG-NH ₂ ^c | 11020.24 |

Oligonucleotides were obtained gel-purified and verified by mass spectrometry.

^aA^{m6}, N6-methyl-deoxyadenosine.

^bC^{m5}, 5-methyl-deoxycytosine.

^cNH₂, 3' C7 amino spacer.

study were incubated using an Eppendorf Mixmaster programmed for 3 s mixtures at 1400 rpm every 15 min.

Preparation of 3' end anchored cDNA

An aliquot of 5 µg (~16 pmol) or 0.1 µg (~0.3 pmol) of *in vitro* transcribed RNA was processed according to the regular SAGE protocol or the microSAGE protocol version 1e. Alternatively, *in vitro* transcribed RNA (0.6 µg or ~1.9 pmol) was annealed to 3.0 mg oligo(dT)₂₅ dynabeads (Dynal Biotech) in the presence of 600 U of SUPERase-In (Ambion). Annealed RNA was then processed according to the microSAGE protocol version 1e using components from a cDNA synthesis kit (Invitrogen) and scaled accordingly to a final volume of 600 µl with the following exception: after first strand synthesis, the reaction was cooled on ice, magnetized and 520 µl of the first strand reaction was replaced with 520 µl of a pre-chilled mixture of second strand synthesis reaction components and incubated for 16 h at 16°C. Anchored second strand products were then blunt-ended, washed and digested with NlaIII (New England Biolabs) as described. The resulting anchored 3' end cDNAs (~0.6 pmol/mg dynabeads) were stored at -20°C until ready for use.

Adaptors

Oligonucleotides corresponding to the adaptors and primers used in the SAGE and microSAGE protocols version 1e were obtained gel- or HPLC-purified (Qiagen) and are shown in Table 2. Stock concentrations (5 mM) of the following adaptors were prepared in 1 × NEB4 buffer (New England Biolabs) by mass dilutions: adaptor 1 (1A/1Bphos), adaptor 1m5C (1Am5C/1Bphos), adaptor 1m6A (1Am6A/1Bphos), adaptor 2 (2A/2Bphos), adaptor 2m5C (2Am5C/2Bphos) and adaptor 2m6A (2Am6A/2Bphos). Adaptors were annealed according to the annealing schedule described in the current SAGE protocols.

Standard ligation protocol used in SAGE

Ligation reactions using adaptor 1 at a final concentration of 80 nM were performed according to microSAGE protocol version 1e. Additional ligation reactions, scaled to a final volume of 10 µl (~0.075 pmol cDNA per 125 µg dynabeads) and containing varying amounts of adaptor 1 (0.038–38 pmol),

final adaptor concentration of 1 µM were also performed. All reaction samples were incubated for 2 h at 16°C or 25°C.

Directed ligation

Titration of T4 DNA ligase activity with NlaIII. Stock ligase mixture containing T4 DNA ligase (5 Weiss U/µl; Fermentas) were prepared with various amounts of NlaIII (120 U/µl; New England Biolabs) in a final buffer composition of 15 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 1 mM DTT, 200 mM KCl, 0.5 mg/ml BSA and 50% glycerol, and stored at -70°C. Oligo(dT)₂₅ dynabeads (125 µg) with anchored 3' end cDNA (0.075 pmol) were pre-incubated with adaptor 1, adaptor 1m5C or adaptor 1m6A (1 µM final) for 5 min at 37°C in 1 × NEB4 buffer supplemented with 1 mM ATP and 100 ng/µl BSA in a volume of 9 µl. The reactions were initiated by adding 1 µl from one of the stock enzyme mixtures described above, overlaid with mineral oil, and incubated for 2 h at 37°C.

Directed ligation protocol for SAGE. A stock enzyme mixture containing NlaIII (25 U/µl final) and T4 DNA ligase (2.5 Weiss U/µl final) was prepared as described above. Oligo(dT)₂₅ dynabeads (125 µg) with anchored 3' end cDNA (0.075 pmol) were pre-incubated with 2.5 pmol of adaptor 1m6A for 5 min at 37°C in 1 × NEB4 buffer supplemented with 100 ng/µl BSA and 1 mM ATP. After initiation with 1 µl of the stock enzyme mixture, reactions were spiked every 15 min with 2.5 pmol of adaptor 1m6A for a total incubation time of 1 h and a total addition of 10 pmol adaptor.

Analysis of anchored ligation products

The reactions were heat-inactivated for 20 min at 65°C in 200 µl of 1 × NEB4 supplemented with 100 ng/µl BSA, followed by two washes with the same buffer. Anchored ligation products were then cleaved off the dynabead support with 10 U DraI (New England Biolabs) in 30 µl of 1 × NEB4 supplemented with BSA. After incubation for 1 h at 37°C, products were resolved via PAGE (6% PAGE; Owl Scientific) for 3 h at 12.5 V/cm. SYBR-Gold (Molecular Probes) stained gels were visualized using a CCD-based gel documentation system (Alpha Innotech) using a SYBR-green filter set (Molecular Probes) at a sub-saturating aperture setting and recorded as TIFF files. When required, densitometric analysis was performed using publicly available software (tnimage-3.3.7a;

Preparation and PCR amplification of ditags

Adaptors 1 and 2 or adaptors 1m6A and 2m6A were ligated to anchored 3' end cDNA derived from 100 ng of *in vitro* transcripts as described above using the standard microSAGE protocol version 1e or the directed ligation protocol. After ligation, the anchored products were processed according to microSAGE protocol version 1e to form ditags. Ditag ligation mixtures (3 μ l) were brought up to a final volume of 20 μ l with LoTE buffer (2 mM Tris-HCl, 0.2 mM EDTA, pH, 8.0). One microlitre aliquots of 1 : 20 and 1 : 200 dilutions of the ligation mixture in LoTE were then used as a template for PCR amplification with Platinum *Pfx* thermophilic DNA polymerase (Invitrogen) supplemented with 0.5 \times PCR_X enhancer solution and 0.1 mM MgSO₄ according to the manufacturer's protocol in a final volume of 50 μ l. PCR amplification was performed in the presence or absence of template on an Eppendorf Mastercycler (Eppendorf) using primer 1 and primer 2 as described in the microSAGE protocol. After activation for 1 min at 95°C, 26 cycles were performed according to the following schedule: 95°C, 30 s; 55°C, 1 min and 72°C, 1 min. Upon completion, a 10 μ l aliquot was then resolved via 6% PAGE for 1 h at 12.5 V/cm and visualized as described above.

RESULTS AND DISCUSSION

The ability of SAGE to provide a truly quantitative picture of gene expression relies on the efficiency of each step required to generate the library of SSTs from the harvested mRNA starting material. Currently, two general approaches to generate SAGE libraries are utilized (Table 1), each customized towards the amount of starting material available to the researcher. The original SAGE protocol described by Velculescu *et al.* (2) uses 5 μ g of mRNA (~7.8 pmol mRNA of average length 2 kb) as starting material. After conversion into biotinylated cDNA, half of this sample is digested with the RE NlaIII, and the 3' end fragments are affinity purified via streptavidin-linked dynabeads (2 mg) to generate anchored 3' end cDNA (3.9 pmol/mg dynabeads). In contrast, the microSAGE protocol, a modification of the SADE (SAGE Analysis for Down-sized Extracts) protocol of Virlon *et al.* (33) and commercially available I-SAGETM kit from Invitrogen, is designed to process the RNA from 5×10^4 to 2×10^6 cells or up to 100 ng (~0.16 pmol mRNA of average length 2 kb) of starting mRNA. Oligo(dT)₂₅ dynabeads (0.5 mg) are used as an affinity support to directly harvest polyadenylated RNA from the sample. The anchored oligo(dT)₂₅ on the support is used to prime cDNA synthesis which is then digested with NlaIII to generate anchored 3' end cDNA (0.31 pmol/mg dynabeads).

When our *in vitro* RNA material was used as the starting material, we found that the amount of anchored 3' end cDNA recovered using the original SAGE protocol was similar to that obtained through the microSAGE protocol despite using 25-fold more starting material (data not shown). This observation is consistent with work by Virlon *et al.* (33) where 200-fold less anchored 3' end cDNA was recovered from microdissected renal tubules using the SAGE protocol compared to those recovered from their SADE protocol, which used half the amount of starting material and employed Sau3A I as the

due to the presence of four additional extraction and precipitation steps in the original SAGE protocol prior to adaptor ligation (Table 1), additional losses may arise from the presence of excess biotinylated oligo(dT)₂₀ primer used to prime first strand synthesis. Any such primer that survives the extraction and precipitation steps will compete with binding to the streptavidin support. This primer contamination is most probably small, however, as batch purification of biotinylated cDNAs using Qiaex II silica beads did not improve yields significantly.

After synthesis of the anchored 3' end cDNA library on either streptavidin-linked Dynabeads (i.e. SAGE) or oligo(dT)₂₅ Dynabeads (i.e. microSAGE), further processing towards generation of the SAGE library is essentially the same under the two protocols (Table 1).

Self-ligation of the anchored 3' end cDNA competes with ligation of the adaptor

Under standard microSAGE reaction conditions, we observe that the ligation of SAGE adaptors to the cohesive end of the anchored 3' end cDNA consistently produces two products. In the presence of T4 DNA ligase and the standard 80 nM concentration of adaptor 1, a relatively small fraction (<5%) of the anchored 3' end cDNA was found to ligate to adaptor 1 to form the desired adaptor-target cDNA hetero-ligation product (Figure 1). The bulk of the anchored cDNA underwent an undesired reaction to form a high molecular weight product (lane 3). Comparisons with the control reaction in which no T4

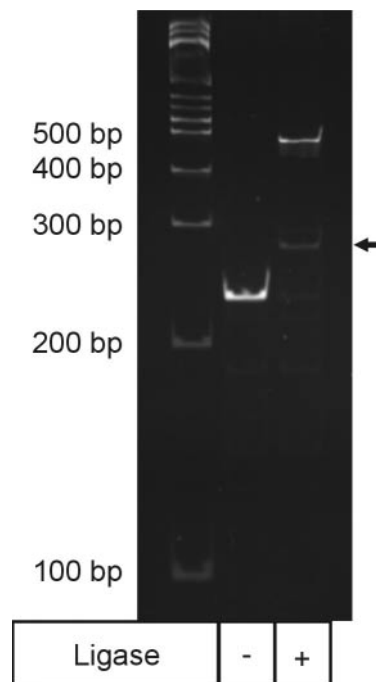


Figure 1. Ligation of SAGE adaptor 1A to anchored 3' end cDNA. An aliquot of 100 ng of *in vitro* transcribed polyadenylated product was processed under the microSAGE protocol and split into half. Lane 2 shows a control reaction in which T4 DNA ligase was not added to the ligation mixture. Lane 3 shows the formation of a small amount of the hetero-ligation product indicated by the arrow as well as a high molecular weight band corresponding to twice the molecular weight of the unligated cDNA. Ligations were performed as

DNA ligase was added (lane 2), and with a ligation reaction performed in the presence of NlaIII indicated that this high molecular weight product is a homodimer of the anchored 3' end cDNA. Identical experiments were also carried out on streptavidin anchored 3' end cDNA samples prepared by the original SAGE protocol and gave essentially the same results. Lower loading densities of *in vitro* RNA onto oligo(dT)₂₀ dynabeads or biotinylated cDNA onto streptavidin-linked dynabeads only marginally inhibited formation of the homodimer, suggesting that homodimer formation depends on both the distance of separation between anchored 3' end cDNA molecules on the surface of a given dynabead (intermolecular) as well as between those anchored on adjacent dynabeads (intramolecular). Formation of the homodimer was also observed when other *in vitro* RNA transcripts were utilized to generate anchored 3' end cDNA targets ranging from 132 to 355 bp in length. Thus, under the ligation conditions described, most of the desired hetero-ligation product is lost in favor of self-ligation of two anchored cDNA fragments.

The yield of the desired hetero-ligation product was found to depend on the amount of SAGE adaptor introduced into the ligation mixture, and increased with increasing adaptor concentration (Figure 2). However, even at very high concentrations of added adaptor (~500:1, lane 10), formation of the unwanted cDNA self-ligation product remained significant, resulting in a loss of approximately half of the starting cDNA material. Under homogeneous reaction conditions (i.e. all reactants present in the solution phase), mass-action should favour the formation of two products, the desired adaptor-cDNA heterodimer and the adaptor-adaptor homodimer at these high concentrations of added adaptor. However, tethering of the target cDNA to the polystyrene surface of dynabeads creates a heterogeneous reaction environment. The distribution of ligation products may therefore be controlled by mass transfer effects that limit the concentration of

adaptor in the solid-liquid interfacial region where the target cDNA is anchored and the reaction must take place. Consequently, adaptor-adaptor and cDNA-cDNA homodimers are produced preferentially, even in the presence of a large excess of the added adaptor.

Improving the yield of adaptor-cDNA heterodimer by increasing the adaptor concentration in the reaction mixture is impractical for large-scale SAGE projects. In addition to the high associated costs of preparing the adaptor, excess adaptor may have deleterious effects on subsequent steps in SAGE. High concentrations of adaptor promote the formation of a large number of adaptor dimers, which can interfere with subsequent PCR amplification steps or necessitate excessive washing of the anchored ligation product to remove unreacted adaptor and adaptor dimers. For this reason, some groups (33,34) have attempted to limit adaptor-dimer contamination of the ditag PCR mixture by reducing the concentration of adaptor used in the adaptor ligation step. However, our results show that lowering the added SAGE adaptor concentration below the standard concentration of 80 nM (i.e. lanes 4 and 5 of Figure 2) results in a significant reduction in the already low yield of the desired adaptor-cDNA hetero-ligation product. As the overall fidelity of SAGE to provide an accurate read of the distribution of transcript abundances will be affected by this sampling loss, there exists a need to develop cheaper and more effective methods to increase the yield of the desired hetero-ligation product by reducing or, better yet, eliminating the formation of self-ligation products.

Addition of macromolecular crowding agents increases the yield of adaptor modified anchored 3' end cDNA

Other changes in reaction conditions that alter the distribution of ligation products were therefore explored to improve the yield of the desired hetero-ligation product. For example, lowering the reaction temperature can be used to slow the ligation reaction to a point where the rate of mass transfer of the adaptor to the solid-liquid interface no longer limits the formation of the hetero-ligation product. In this case, however, a significantly increased incubation time is required, extending the already lengthy process involved in producing a SAGE library. Varying the rate of mixing during the reaction to decrease the hydrodynamic boundary layer and increase the surface concentration of the free adaptor was explored, but led to only a marginal improvement in the yield of the hetero-ligation product.

Adding co-solutes that act as macromolecular crowding agents (i.e. compaction agents) has been shown to dramatically affect the thermodynamics of reaction mixtures, generally favouring the formation of products with compact conformations and for some proteins, linear rod-like aggregates (35,36). For ligation reactions, addition of 15% (w/v) of the neutral polymer polyethylene glycol (PEG) has been shown to enhance by up to 100-fold the formation of intermolecular ligation products (i.e. linear concatamers) during the ligation of cohesive or blunt-ended DNA fragments in the solution phase (30,31,37). The influence of increased concentrations of PEG-8000 on the formation of the desired hetero-ligation product was therefore examined (Figure 3). At the

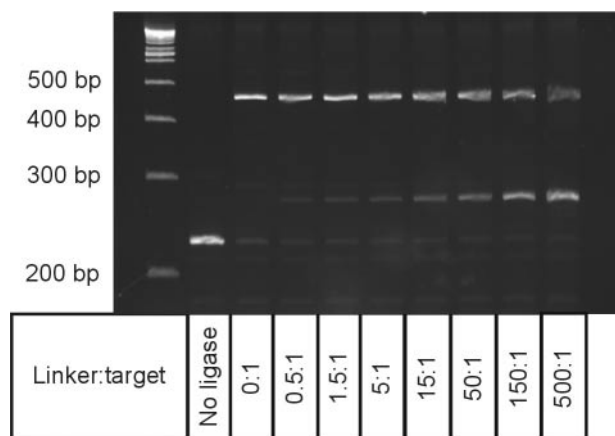


Figure 2. Influence of increasing adaptor:target molar ratios on the formation of adaptor-target heterodimer versus target homodimer. Increasing amounts of adaptor 1 (0–3.8 μ M final) were introduced into standard ligation reactions containing 0.075 pmol anchored target in a final volume of 10 μ l as described in Materials and Methods. In microSAGE, adaptors are introduced to a reaction mixture containing ~0.08 pmol anchored target at a final concentration of 0.08 μ M in a total volume of 20 μ l, corresponding to adaptor:target ratio of approximately 20:1. The classic SAGE protocol introduces a final adaptor concentration of 0.8 μ M to the ligation mixture containing ~1.95 pmol anchored target in a total volume of 40 μ l, corresponding to an adaptor:

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