

METHOD

An RNA ligase-mediated method for the efficient creation of large, synthetic RNAs

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ABSTRACT

RNA ligation has been a powerful tool for incorporation of cross-linkers and nonnatural nucleotides into internal positions of RNA molecules. The most widely used method for template-directed RNA ligation uses DNA ligase and a DNA splint. While this method has been used successfully for many years, it suffers from a number of drawbacks, principally, slow and inefficient product formation and slow product release, resulting in a requirement for large quantities of enzyme. We describe an alternative technique catalyzed by T4 RNA ligase instead of DNA ligase. Using a splint design that allows the ligation junction to mimic the natural substrate of RNA ligase, we demonstrate several ligation reactions that appear to go nearly to completion. Furthermore, the reactions generally go to completion within 30 min. We present data evaluating the relative importance of various parameters in this reaction. Finally, we show the utility of this method by generating a 128-nucleotide pre-mRNA from three synthetic oligoribonucleotides. The ability to ligate synthetic or *in vitro* transcribed RNA with high efficiency has the potential to open up areas of RNA biology to new functional and biophysical investigation. In particular, we anticipate that site-specific incorporation of fluorescent dyes into large RNA molecules will yield a wealth of new information on RNA structure and function.

Keywords: T4 RNA ligase; ligation; RNA; YOL047c; splint

INTRODUCTION

Site-specific incorporation of modifications into RNA molecules has been a powerful tool for both biochemical and biophysical investigations of RNA function (Moore and Sharp 1992; Wyatt et al. 1992; Heilek et al. 1995; Teigelkamp et al. 1995; Golden et al. 1996; Chavatte et al. 2001). Improved methods for the chemical synthesis of RNA have greatly facilitated such experiments by allowing a wide variety of nonnatural nucleotides to be placed at almost any position in the RNA (McGregor et al. 1996). The primary limitation of this method is that chemical synthesis becomes prohibitively inefficient beyond ~50 nucleotides (nt). Consequently, to generate larger RNAs with site-specific modifications it is necessary to join the

synthetic oligoribonucleotide either to other oligonucleotides or to longer, *in vitro* transcribed RNA.

The most widely used method for ligating RNA molecules employs a DNA splint to hold the RNA ends in apposition while DNA ligase catalyzes chemical bond formation (Moore and Sharp 1992). The design of DNA ligase-mediated ligation (henceforth referred to as “*D*ligation”) is shown in Figure 1A. DNA ligase recognizes the nicked, double-stranded substrate and catalyzes formation of a phosphodiester linkage between the 5′ phosphate of one oligo (generally referred to as the “donor” oligo) and the free 3′ hydroxyl of a second oligo (generally referred to as the “acceptor” oligo). Although this method has been used successfully in many experiments, it frequently produces a low yield of product, takes several hours to reach completion, and requires large amounts of DNA ligase (Moore and Query 2000). These limitations have impeded certain applications of the technique, notably those that require multiple ligation steps.

An alternative method for RNA ligation uses T4 RNA ligase. Like DNA ligase, this enzyme also requires a free 5′-phosphate on the donor substrate, a 3′ hydroxyl on the

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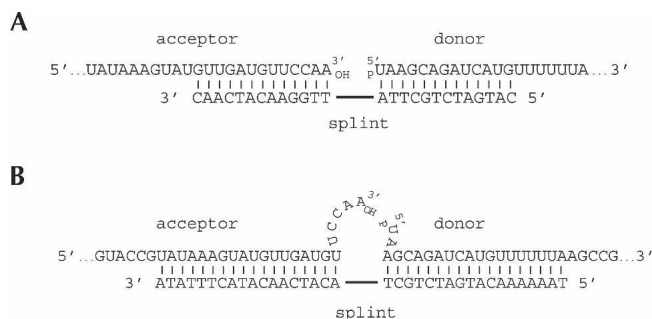


FIGURE 1. Templating schemes for (A) DNA ligase-mediated RNA ligation, and (B) RNA ligase-mediated RNA ligation (sequences shown are YOL047c, junction I). The solid line connecting the two halves of the splint indicates that they are part of a single oligonucleotide. Ligation occurs between the phosphate at the 5' end of the donor (indicated by “P”) and the 3' OH on the acceptor.

acceptor substrate, and exogenous ATP. However, unlike DNA ligase, RNA ligase is only active when the nucleotides at the splice junction are single stranded. Consequently, RNA ligase works effectively in a nontemplated fashion, indiscriminately joining any nucleotides with the correct 5' and 3' termini, a characteristic that has made it useful for techniques such as pCp labeling (England et al. 1980).

In an effort to impose sequence specificity on the reaction, a method was developed based on the observation that the natural substrate of T4 RNA ligase is the anticodon loop of tRNA^{Lys} (Amitsur et al. 1987). A DNA splint was designed to hold the donor and acceptor molecules near one another while leaving single-stranded regions (“linkers”) near the ligation junction (Bain and Switzer 1992; see Fig. 1B). Synthetic yields remained low, however, presumably due to the high efficiency of T4 ligase-mediated circularization (Gumport and Uhlenbeck 1981; Romaniuk and Uhlenbeck 1983), as well as non-optimal linker lengths.

We report a significant improvement on RNA ligase-mediated ligation (henceforth “Rligation”) achieved in part through the incorporation of the 5'-silyl-2'-acetoxy ethyl orthoester (2'-ACE) protecting group used in oligoribonucleotide synthesis (Hartsel et al. 2004), as well as through optimized linker lengths and chemically incorporated 5' phosphates on the donor strand. RNA oligos containing 2'-ACE protecting groups are far less susceptible to intramolecular circularization than are corresponding 2' OH RNAs. These reactions proceed rapidly, some within 5 min or less, and give high yields of product. Notably, the ligation yields are high enough that it is now possible to create RNAs through multiple ligation steps, allowing site-specific modification of large RNAs. As an initial example, we have used this technique to produce with high efficiency a 128-nt pre-messenger RNA from three chemically synthesized oligonucleotide precursors. We describe the important parameters necessary to apply this ligation method to virtually any RNA sequence, and anticipate that this will

facilitate the synthesis of a wide array of RNA molecules for detailed physico-chemical characterization.

RESULTS AND DISCUSSION

Designing an RNA ligation method

Many structure–function studies examining RNA biology are facilitated by the ability to insert site-specific modifications. Unsatisfied with the methods currently available for generating long RNA molecules containing site-specific modifications, we sought to optimize conditions necessary for producing such reagents. As a common problem in RNA ligase-mediated reactions is the production of side products, we sought a protocol that limited the number of possible reactions at each step to the single, desired reaction. Ideally, each step of the reaction should involve an acceptor molecule with a 3' OH and no 5' phosphate, and a donor molecule with a 5' monophosphate and no accessible 3' OH. In this way, circularization of either substrate molecule would be impossible, reducing the amount of side product at each step. By keeping the donor molecule in its 2'ACE form, its 3' OH is rendered largely inaccessible, minimizing its ability to circularize and, therefore, the number of equivalents required for complete conversion of the acceptor molecule. The product of this reaction, after removal of the remaining 2'ACE protecting groups, would be an RNA terminated at both its 5' and 3' ends with free hydroxyls, making it an ideal acceptor molecule for subsequent reactions. Because the donor molecule can be added in sufficient excess to drive all of the acceptor molecules into product, this design, when implemented in the 5' to 3' direction, can produce RNAs of considerable size with reasonable efficiency.

To ascertain the efficiency of this method, we tested four unrelated ligation junctions, including the MD1 test sequence, two different positions in the transcript of yeast ORF YOL047c (including junction I shown in Fig. 1B), and one in the yeast U6 snRNA. As shown in Figure 2, the RNA ligase-mediated reactions yielded significant amounts of ligated product in all four cases. These reactions were run with donor (labeled B) in excess, so 100% conversion of acceptor (A) to products would result in the disappearance of the A band, as in panels 1 and 3. Note that the reactions in panels 1 and 2 were treated with DNase I after ligation to remove the splint oligo, which runs at the same position as the acceptor in those reactions. Under optimal reaction conditions (see below and Materials and Methods), apparent reaction yields were typically in the range of 70%–80% conversion of acceptor molecule into product, with many reactions proceeding to $\geq 95\%$ conversion. Importantly, many reactions were complete within 5 min, the shortest time measured, which is considerably faster than other reported ligation methods. The reactions shown in Figure 2

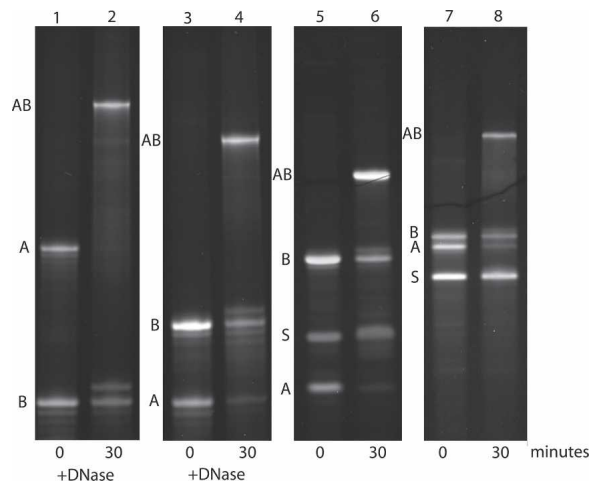


FIGURE 2. Rligation efficiency for four RNA junctions. Ligation reactions were performed as described in Materials and Methods and monitored by polyacrylamide gel electrophoresis (PAGE) followed by ethidium bromide staining. RNA used: (lanes 1,2) YOL047c junction I shown in Figure 1B; (lanes 3,4) YOL047c junction II; (lanes 5,6) MD1 RNA; (lanes 7,8) U6 snRNA. The corresponding splints are shown in Materials and Methods. The Rligation reactions in lanes 1–4 were treated with 1 unit of DNase I for 15 min at 37°C following ligation in order to remove the DNA splint, which comigrates with the donor (oligo B). The locations of the acceptor oligos (A), the donor oligos (B), and the ligated products (AB) are indicated for each of the reactions.

were visualized by staining with ethidium bromide after complete deprotection of the reactions. Because 2'ACE containing oligonucleotides are poorly stained by ethidium bromide, the donor oligo is only visible as a weak, fuzzy band, unless it has been deprotected.

Optimizing a ligation reaction

To facilitate application of this method to a variety of RNA sequences, we sought to optimize the reaction parameters. In general, we found that the most important determinant of efficient ligation is the capacity to form a complex between the splint and the two substrates. We have examined ligation reactions using both non-denaturing and denaturing gels and found a high correlation between the amount of splint:substrate complex formed on the native gels and the amount of ligated product formed. Consistent with the recent observations using DNA ligase (Kurschat et al. 2005), formation of a splint:substrate complex appears to be required, if not sufficient, for ligation. With this in mind, ligation junctions were chosen at locations in which predicted secondary structures were most likely to be compatible with complex formation.

Previous work demonstrated that RNA ligase is inefficient when the ultimate or penultimate nucleotide of the acceptor molecule is a pyrimidine (Wittenberg and Uhlenbeck 1985). In the context of these splint:substrate complexes, we have

examined multiple ligation reactions, including several containing 3'-terminal pyrimidines in the acceptor oligo, and see no sequence specificity in either the donor or acceptor substrates (data not shown). We surmise that in the geometry achieved with a splint, the chemical steps of ligation are fast enough that the reaction becomes insensitive to the identity of the terminal nucleotides. It is possible that bulky nucleotide modifications (e.g., fluorescent dyes) at or near the ligation junction might interfere with the reaction, although we have not tested this directly. Modifications distal to the junction are unlikely to interfere, however, as they would be far from the catalytic site of the enzyme.

Once the ligation junction has been selected, the splint must be designed. The tRNA substrate of T4 RNA ligase has linker lengths of 5 and 2 nt on the acceptor and donor molecules, respectively. While the optimum lengths may vary for different sequences, our experience suggests that the 5:2 (acceptor:donor) combination is generally effective. Using the MD1 sequence, we independently varied the length of the linkers. For the donor molecule, efficiency drops off sharply for lengths other than 1 or 2 nt (data not shown). For the acceptor molecule, maximum activity was observed with eight single-stranded nucleotides, but high levels of ligation were also obtained with four to seven single-stranded nucleotides. Having chosen the single-stranded portions, it is also necessary to ensure stable association between the splint and the RNA substrates. The splints used in the ligations for Figure 2 were designed such that the melting temperature (T_M) for each “half” of the splint was in the range of 40–45°C. Our initial analysis suggested that a $T_M < 40^\circ\text{C}$, especially between the splint and the donor, was particularly deleterious (data not shown).

When designing the splint, important consideration should be given to the possibility that the single-stranded linkers may inadvertently anneal to the splint. For example, it is important that the two linker nucleotides in the donor molecule (B) are not complementary to the last two nucleotides in the acceptor-binding region of the splint. Our original splint design incorporated the nucleotide purine, which is unable to participate in a stable base pair, between the donor- and acceptor-binding regions of the splint to prevent such base pairing. Splints containing nonstandard nucleotide spacers, such as purine, were highly active for ligation, but may slightly alter linker-length optima. While we have not used a spacer in most of our splints, it may nevertheless be an effective strategy for improving efficiency in cases where cross-hybridization may occur.

We initially hypothesized that 2'ACE-containing RNA splints might be more effective than DNA splints, as the 2'ACE groups prevent tertiary structure formation (Scaringe 2001), and would therefore maintain an RNA splint in a more accessible conformation for base pairing to the

substrates. In the cases where we have directly compared RNA and DNA splints, however, we see little or no difference (data not shown).

One potential disadvantage of Rligation compared to Dligation is the potential for heterogeneity at the ligation junction, resulting from the ability of the enzyme to accommodate a variety of lengths in the single-stranded region. This problem can be eliminated, however, by using chemically synthesized RNA. By their nature, the 3' ends of synthetic oligos have a negligible amount of heterogeneity, ensuring the quality of the acceptor side of the junction. In contrast, the 5' ends of synthetic oligos can be heterogeneous, resulting from truncated products of chemical synthesis. However, if the 5' phosphate on the donor molecule is added during chemical synthesis of the molecule (as opposed to adding it post-synthetically using T4 polynucleotide kinase), one ensures that only full-length donor molecules will be ligated, since truncated products are chemically capped to prevent extension, and therefore will not contain the 5' phosphate required for ligation. When using enzymatically produced RNAs, it will be important to ensure the purity of both the 3' and the 5' ends of the molecule (Pleiss et al. 1998) to guarantee junction fidelity as well as to maintain high yields. In such cases, sequencing the junction may be desirable.

Nontemplated ligation reactions can be detrimental to efficient ligation. Because donor molecules have the capacity to either circularize and/or concatamerize, we compared donor molecules in either the 2'ACE or 2' OH form. In initial experiments with the MD1 substrates, the ligation efficiency increased from 60% to 80% in the presence of the 2'ACE groups while reducing the number of visible side products (data not shown). This finding is consistent with our observation that acceptor molecules are inactive when in the 2'ACE form, presumably because of steric inaccessibility of the 3' OH. While we have not seen this magnitude of change in all of the reactions we have tested, donor molecules in the 2'ACE form can generally be accommodated in the ligation scheme. We expect other 2' protecting groups that render the 3' OH inaccessible to be equally effective in preventing side reactions. Other simple steps to avoid unwanted products include using splint oligos with a 3' dideoxy nucleotide and terminating the 3' terminal RNA oligo with a 2',3'-dideoxy nucleotide.

Proof of principle: Synthetic splicing reporter

To establish the utility of the Rligation strategy in a biochemical context, we designed a pre-mRNA transcript made entirely of synthetic oligonucleotides (Fig. 3). We used the yeast intron database (Spingola et al. 1999) to identify intron-containing genes with introns shorter than 80 nt so that our transcript could be made from three RNA oligos. We also looked for genes expressed at high levels in yeast, reasoning that these would be readily spliced, at least

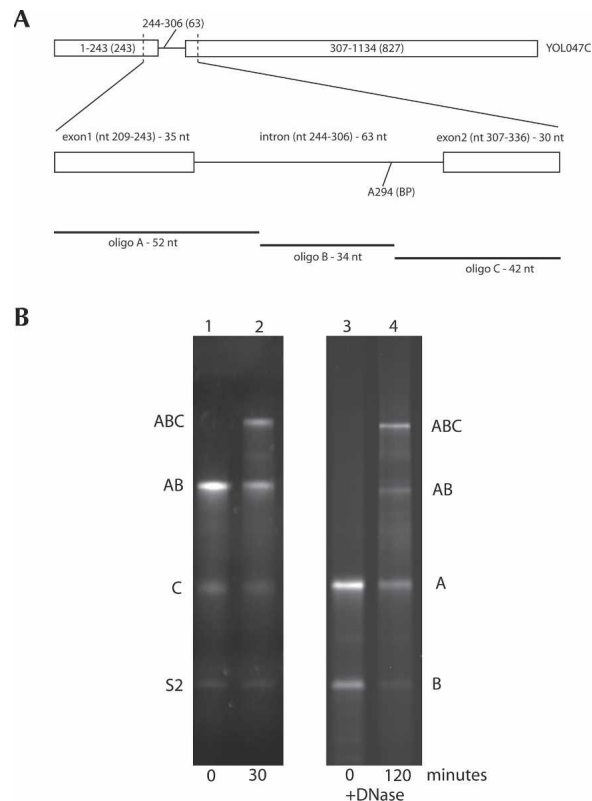


FIGURE 3. Ligation of 128-nt, synthetic pre-mRNA. (A) Strategy for making synthetic YOL047c transcript. The YOL047c gene contains a single 63-nt intron near the 5' end of the gene. Dashed lines indicate boundaries of the splicing reporter. The 128-nt splicing reporter contains 35 nt of exon 1, the entire intron (branch point nucleotide, BP), and 30 nt of exon 2. Synthetic oligoribonucleotides (labeled A, B, C) for generating the splicing reporter contain, respectively, the 5' splice junction, the branch point, and the 3' splice junction. (B) Two-step ligation of transcript (lanes 1,2). Gel-purified AB product (lane 1, "AB") was ligated with oligo C and splint S2, yielding full-length ABC product (lane 2, "ABC"). One-step ligation of transcript (lanes 3,4). All three RNA oligonucleotides and both splints were incubated in a single reaction. Reactions were treated with DNase I after ligation, so no splints are visible.

in vivo. Using these criteria, we selected yeast open reading frame (ORF) YOL047c, an ORF of unknown function (Fig. 3A). We chose a region containing the entire intron (63 nt), plus 35 nt of exon1 and 30 nt of exon2, as our minimal splicing substrate, which we had synthesized in three fragments (oligos A, B, and C; Fig. 3A).

We attempted to generate our full-length product in two different ways. Our initial attempt utilized a stepwise approach where we first generated the AB ligation product (YOL047c ligation I), using splint S1 in a large-scale reaction under conditions similar to those shown in Figure 2. The second ligation step (AB ligated to C) was then carried out using gel-purified, deprotected AB (Fig. 3B, lanes 1 and 2). For comparison, we attempted to synthesize the ABC product in a single-step, three-way ligation reaction simultaneously using all three oligos and both splints. For this

reaction, piece B was deprotected prior to the ligation, as it is required to act both as a donor and an acceptor. As seen in Figure 3B, lanes 3 and 4, the single-step reaction was as efficient as using gel-purified AB. Presumably, the efficiency of the three-way ligation suggests that piece B is not highly susceptible to deleterious side reactions, perhaps due to sequestration by the splints. While this may not be true for all three-way ligations, we have had success ligating several different sequences in this way (data not shown).

Our experience with this method suggests that this technique will prove useful for the ligation of RNA molecules of virtually any sequence. Indeed, the high efficiency of this technique, demonstrated by our synthesis of a 128-nt RNA from three synthetic oligoribonucleotides, opens the possibility of modifying multiple positions in still longer RNA sequences. While we have exclusively used oligonucleotides in our experiments, we expect that *in vitro* transcribed RNA could be accommodated in our reaction scheme, as long as care is taken to ensure the homogeneity of the ligation junctions. When combined with the variety of modified nucleotides that can be incorporated during chemical synthesis, one can imagine creating site-specifically modified RNAs for use in a variety of structure–function studies. We presume that the modifications would be less likely to interfere with ligation if the modification sites are not in the linker regions, although we have not tested this directly. We anticipate that an important application of this method will be for the incorporation of multiple fluorescent dyes into a single RNA molecule for use in fluorescence resonance energy transfer (FRET) experiments.

MATERIALS AND METHODS

RNA oligo design

Oligonucleotides were synthesized by Dharmacon using the 2'-ACE method on modified Applied Biosystems 380B synthesizers, using standard amidites. Potential RNA sequences were analyzed using m-fold (<http://www.bioinfo.rpi.edu/applications/mfold/old/rna/form1.cgi>; Zucker 2003; Mathews et al. 1999) to determine the extent of secondary structure. Ligation sites were chosen either in regions with little or no intramolecular base pairing or at naturally occurring exon/intron junctions, with the constraint that oligos should not be significantly longer than 50 nt. The longest oligo synthesized was 63 nt, but most were between 30 and 50 nt. Candidate junctions were analyzed for possible base pairing between the linker and the splint. The 3' ends of the acceptor (A) oligos were in general designed to terminate in two purine bases, as it was originally thought that this provided a better substrate for T4 RNA ligase. The donor (B) oligos were synthesized with a 5'-terminal phosphate.

DNA splint oligo design

DNA oligos were purchased from Operon. DNA splints for the Rligation were designed to have ~18 nt of homology with the

RNA oligos on both the acceptor and donor halves, with melting temperatures for each half approximately equal to each other and $\geq 40^\circ\text{C}$. T_M 's were calculated using nearest-neighbor thermodynamic parameters as implemented in IDT's OligoAnalyzer web tool (<http://scitools.idtdna.com/scitools/Applications/OligoAnalyzer/default.aspx>; Xia et al. 1998).

Rligation

For all two-part ligations, the acceptor oligo (A) was deprotected and the donor oligo (B) was phosphorylated and 2'-ACE protected. The DNA splint oligo was mixed with the A and B RNA oligos at a 1:1.5:2 ratio of oligoA:splint:oligoB. Oligos were annealed (65°C for 3 min followed by 25°C for 5 min) in the presence of 1× T4 RNA ligase buffer (NEB: 50 mM Tris-HCl at pH 7.8, 10 mM MgCl₂, 10 mM DTT, 1 mM ATP). Annealing in the absence of ligase buffer results in at least partial deprotection of the B oligo, probably due to the acidity of our water, increasing the risk of circularization. Final oligo concentrations were between 2 and 4 μM. T4 RNA ligase (NEB) was added at a final concentration of 1 U/μL and the reactions were incubated at 37°C, removing aliquots (5 or 10 μL) at the indicated times. Many reactions were complete in as little as 5 min.

Samples in which the splint and one of the RNA oligos were too close in size to resolve on a gel were then treated with 1 U DNase I (Ambion) for 15 min at 37°C, as indicated in the figure legends. All ligation reactions were stopped and deprotected by the addition of 100 μL 2'-ACE deprotection buffer (100 mM acetate adjusted to pH 3.8 with TEMED; Dharmacon), heated at 65°C for 30 min, evaporated in a speed-vac, and resuspended in urea sample buffer. Ligation products were analyzed on 12% TBE polyacrylamide/7 M urea gels and stained in EtBr (0.5 mg/mL) after running.

The three-part ligation performed in two steps was done in the same way as the two-part ligation, with the first step scaled up to generate enough product to gel purify. The final A and B oligo concentrations were 50 and 100 μM, respectively, in a 20-μL reaction, with 75 μM splint. The ligation was stopped after 30 min by the addition of an equal volume of formamide, denatured at 65°C for 5 min, and run on a 12% denaturing gel. The ligation products were isolated by UV shadowing and eluted from the gel. The purified AB product was deprotected as above to serve as the acceptor in the second step. The second step of the three-part ligation was then performed with the AB acceptor, C donor, and DNA splint 2 using equimolar amounts of the three components under the same conditions as for the original two-part ligation.

For the single-step three-part ligation the B oligo, as well as the A oligo, was deprotected, as it must serve as both a donor and an acceptor in this reaction. All five oligos were annealed to each other as above. Several different ratios of oligos were tested in an attempt to find optimal conditions. For the reaction shown in Figure 3B, the ratio of A:B:C was 2:1:2, with a B:splint ratio of 1:1.5.

Oligo sequences

YOL047c ligation 1

oligo A r020 5'-AGUCGUCGCACCAGAUAUCUGGUAUCC
GUAUAAAGUAUGUUGAUGUCCAA-3'

oligo B r021 5'-PUAAGCAGAUCAUGUUUUUAAGCCGUCA
UACUAA-3'
DNA splint oSDR171 5'-TAAAAACATGATCTGCTACATCAA
CATACTTTATA-3'

YOL047c ligation II

oligo A r028 5'-UAAGCAGAUCAUGUUUUUAAGCCGUCA
UACUAA-3'
oligo B r022 5'-PCCGCCUUUGAAGGGAAUUAGCGAAUCCG
UGGGAAACUCGGAU-3'
DNA splint oSDR172 5'-CTAATTCCTTCAAAGGCATGACGG
CTTAAAAACA-3'

YOL047c ligation III

oligo A r020 5'-AGUCGUCGCCAGAUCAUCUGGUAUCC
GUAUAAAAGUAUGUUGAUGUCCAA-3'
oligo B r021 5'-PUAAGCAGAUCAUGUUUUUAAGCCGUC
AUACUAA-3'
oligo C r022 5'-PCCGCCUUUGAAGGGAAUUAGCGAAUCC
GUGGGAAACUCGGAU-3'
DNA splint 1 oSDR171 5'-TAAAAACATGATCTGCTACATCA
ACATACTTTATA-3'
DNA splint 2 oSDR172 5'-CTAATTCCTTCAAAGGCATGAC
GGCTTAAAAACA-3'

MD1

oligo A r001 5'-UGACCCGUCGAGAGAG-3'
oligo B r004 5'-PGAGAGUAGCCCAUGCAAUGCGAGGA-3'
DNA splint oSDR183 5'-GCATGGGCTACTTCGACGGGTCA-3'

U6 snRNA

oligo A r006 5'-AUUUGAAACAAUACAGAGAUGAUCAGCAG
UUCCCCUGC-3'
oligo B r007 5'-PAUAAGGAUGAACCGUUUACAAAGAGAU
UUAUUUCGUUUU-3'
DNA splint oSDR210 5'-GTAAAAACGGTTCATCCTTGGAAT
GCTGATCATCT-3'

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