3′ Non-templated 'A' addition by *Taq* DNA polymerase: An advantage in the construction of single and double mutants

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The 3′-end non-templated 'A' addition by *Taq* DNA polymerase has been described as a disadvantage in the generation of site-specific mutants as this 'A' leads to a unplanned second mutation. Here we demonstrate the utility of this 3′ non-templated 'A' addition in the simultaneous construction of single and double mutants of serine hydroxymethyltransferase.

SITE-directed mutagenesis (SDM) has been widely used for research in molecular biology and protein engineering. Several methods for SDM using polymerase chain reaction (PCR) have been described[1–8]. Megaprimer method is one of the most rapid and universal, in which one mutagenic primer and two universal flanking primers are required. A possible problem associated with this method is the addition of an adenosine residue at 3′

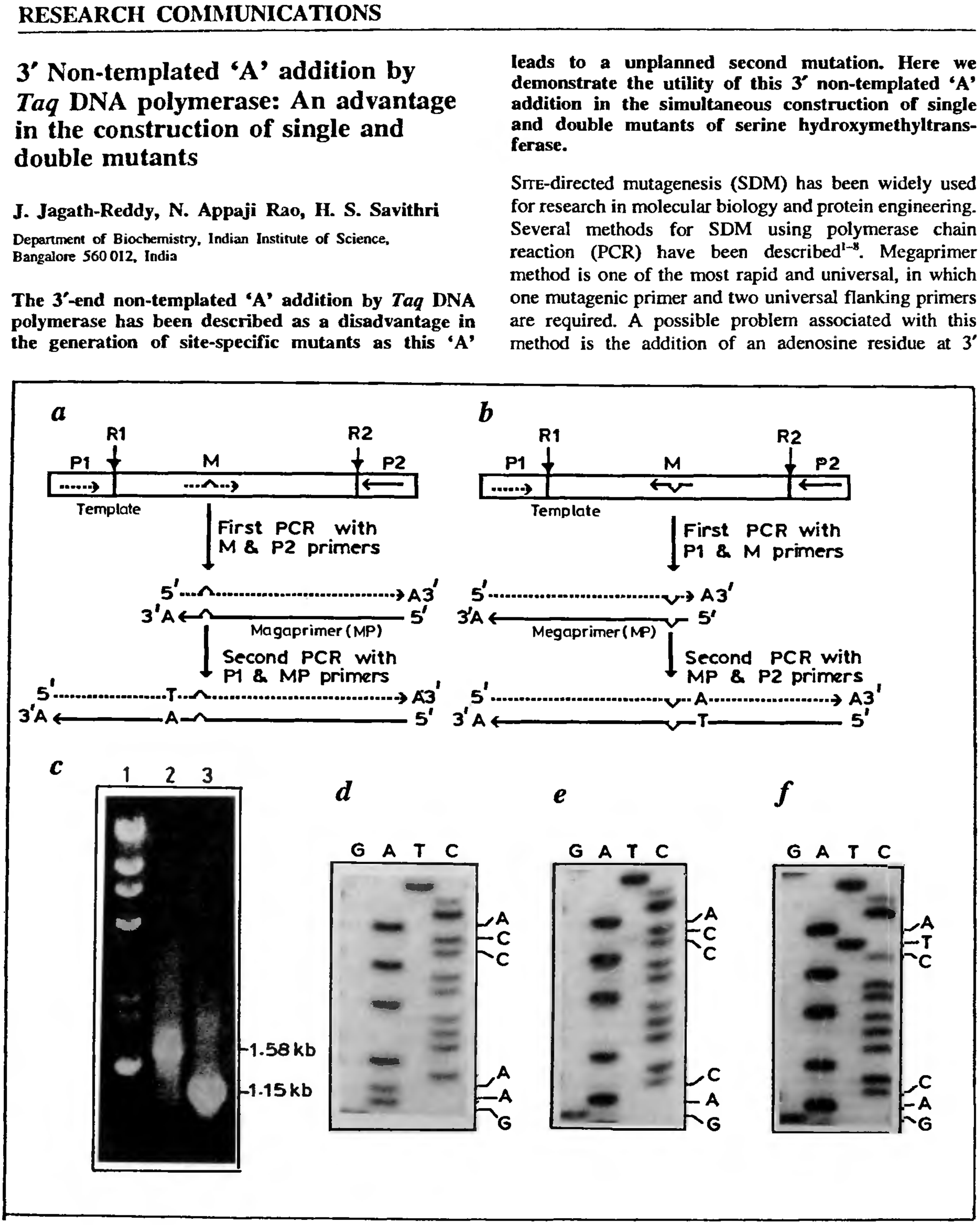

Figure 1 *a–c*. A megaprimer method for the construction of single and double mutants. ***a***, ***b***, Schematic diagram for the construction of single and double mutants. The coding strand of PCR product is shown as dotted lines and non-coding strand in solid lines. Sense and antisense primers are shown as (. . . .) and (←). ***c***, Agarose gel with lane 1. Lambda *Hind* III/pUC 19 *Hinf* I marker, lane 2. Final PCR product with expected size of 1.58 kb and lane 3. First PCR product (megaprimer) with an expected size of 1.15 kb. Part of sequencing gel confirming ***d***, wild type SHMT sequence; ***e***, single mutant (AAG → CAG); ***f***, double mutant (AAG → CAG and ACC → ATC).

end of the PCR product by template independent terminal transferase activity of *Taq* DNA polymerase[9], resulting in an unplanned mutation. Here we report an advantage of this 3′ non-templated 'A' residue addition in the generation of specific double mutants using a single mutagenic primer.

As shown in Figure 1 *a*, if a sense mutant primer is used it will anneal to the non coding strand and an 'A' residue will be added to the 3′ end of the PCR product during first PCR. This addition of an 'A' residue will eventually lead to a 'T' substitution in the coding strand upstream of the desired mutation. In contrast when an antisense primer is used, it will lead to an 'A' substitution in the coding strand downstream of the desired mutation (Figure 1 *b*). As apparent from the Table 1, by a proper design of the mutant oligos, double mutants can be obtained with varying amino acid substitutions as described in this study.

In our studies aimed at understanding the structure–function relationship of serine hydroxymethyltransferase (SHMT) from sheep liver, we were interested in the construction of single and double mutants of the SHMT gene. The protocol for this purpose involved, the first PCR amplification carried out in a total volume of 100 µl containing 75 pmoles of the 19-mer reverse universal primer (P2) and 20-mer mutagenic primer (M) (H148N mutagenic primer: 5′ G GAT GGG GGC AAC CTG CTG A 3′ or K257Q mutagenic primer: 5′ C ACC ACC CAC CAG ACC CTG C 3′) (Bangalore Genei Pvt Ltd., Bangalore, India), 2.5 units of *Taq* DNA polymerase (United States Biochemical, Cleveland, OH, USA), 0.2 mM deoxyribonucleoside triphosphate (dNTP, Amersham International plc, Buckinghamshire, UK), 2 mM $MgCl_2$, 50 ng of DNA template (pUCSH) and the buffer provided with the enzyme at 1x concentration. The reactions were amplified in a thermal cycler (COY Tempcycler II Model 110S, COY Laboratory Products Inc., Ann Arbor, MI, USA) with the initial 4 min denaturation at 94°C then for 30 cycles with 1 min denaturation (94°C), 1 min annealing (50°C), 1 min 30 sec elongation (72°C) followed by 10 min elongation at 72°C. Samples were analysed on 1x TAE agarose gel (Figure 1 *c*). PCR products were purified as described by low-melting temperature-agarose method[10]. Purified PCR products were quantitated on agarose gel by ethidium bromide fluorescence. A second PCR was set up using the conditions similar to those described above with 25 pmoles P1 (internal primer for SHMT gene: 5′T ATG GCA GCT CCA GTC AAC 3′) (National Biosciences, Plymouth, MN, USA) and 1 µg of purified first PCR product as primers and 50 ng of pETSH DNA as template. We have used different templates in the two PCR amplifications, i.e. pUCSH in the first PCR (1.2 kb SHMT cDNA clone lacking 227 bp at 5′ end in pUC19 vector) and pETSH in the second PCR (1.45 kb full length SHMT cDNA clone in pET 3c vector)[11] to obtain convenient restriction sites in the final PCR product. However, the same template can be used for both the PCRs. The final PCR product was gel purified, digested with KpnI, BamHI and ligated with KpnI, BamHI digested pUC 19. The ligation mixture was used to transform *Escherichia coli* DH5α cells. The mutations were confirmed by dideoxy double-stranded DNA sequencing[12] using sequense version 2.0 DNA sequencing kit (United States Biochemical).

In the design of a mutagenic primer, a Lys (AAG) residue has been changed to a Gln (CAG). The primer has been designed to begin with a cytosine (C) base corresponding to 3rd base of Thr (ACC) which is 3 amino acids upstream to this Lys in the SHMT gene. Since this is a sense mutant primer, a 'T' will be

Table 1. Anticipated amino acid changes due to 3′ non-templated 'A' addition by *Taq* DNA polymerase

		1st base		2nd base		3rd base	
Amino acids	Codon	T	A	T	A	T	A
Phe/Leu	TTN	–	Ile/Met	–	Tyr/*	Phe	Leu
Ser	TCN	–	Thr	Phe/Leu	Tyr/*	–	–
Tyr/*	TAN	–	Asn/Lys	Phe/Leu	–	Tyr	*
Cys/*/Trp	TGN	–	Ser/Arg	Phe/Leu	Tyr/*	Cys	*
Leu	CTN	Phe/Leu	Ile/Met	–	His/Gln	–	–
Pro	CCN	Ser	Thr	Leu	His/Gln	–	–
His/Gln	CAN	Tyr/*	Asn/Lys	Leu	–	His	Gln
Arg	CGN	Cys/*/Trp	Ser/Arg	Leu	His/Gln	–	–
Ile/Met	ATN	Phe/Leu	–	–	Asn/Lys	Ile	Ile
Thr	ACN	Ser	–	Ile/Met	Asn/Lys	–	–
Asn/Lys	AAN	Tyr/*	–	Ile/Met	–	Asn	Lys
Ser/Arg	AGN	Cys/*/Trp	–	Ile/Met	Asn/Lys	Ser	Arg
Val	GTN	Phe/Leu	Ile/Met	–	Asp/Glu	–	–
Ala	GCN	Ser	Thr	Val	Asp/Glu	–	–
Asp/Glu	GAN	Tyr/*	Asn/Lys	Val	–	Asp	Glu
Gly	GGN	Cys/*/Trp	Ser/Arg	Val	Asp/Glu	–	–

The table shows the possible amino acid changes which could arise by 'T' or 'A' substitution in the coding strand at 1st, 2nd or 3rd base of a given amino acid(s). Stop codon is shown as (*), (–) is shown for no change in the amino acid.

substituted for 'C' (i.e. 2nd position in the ACC codon) which will result in the mutation Thr → Ile (ATC), leading to the generation of a double mutant (Lys → Gln and Thr → Ile). Similarly, 'T' to 'C' substitution was observed in the case of *nisA* gene[6]. If the primer starts from the second base of the codon (ACC) then the 'T' to 'A' substitution will take place at first base of the codon resulting in the mutation Thr → Ser (TCC) (see Table 1). Thus by a proper design of mutagenic primers, specific double mutants can be generated. Some DNA molecules will be left without 3′ non-templated 'A' addition which would lead to a single mutant (Lys → Gln). We have sequenced 4 clones in which 2 of them are double mutants (**Ile**–Thr–Thr–His–**Gln**) and 2 of them are single mutants (Thr–Thr–Thr–His–**Gln**) (Figure 1 *d*). Similarly, we have constructed single and double mutants for the SHMT where Pro–Asp–Gly–Gly–His (wild type) had been converted to Pro–Asp–Gly–Gly–**Asn** (single mutant) and **Leu**–Asp–Gly– Gly–**Asn** (double mutant). In this case, we have sequenced 13 clones in which one of them is single and 12 are double mutants. These mutants were expressed and found to be present in the soluble fraction (Jagath–Reddy *et al.* unpublished results). The characterization of these mutant proteins is in progress.

The advantages of this protocol are: (i) To obtain single and double mutants simultaneously. This procedure is useful in the case of highly conserved proteins where there are too many amino acids to be screened from structure/function point of view. (ii) It is more economical as one would need a shorter oligonucleotide (20–25 bp) compared to other methods (30–35 bp) to generate double mutants in which the two mutation sites are separated by 10–15 bp. Although the second mutation in this case is restricted to certain amino acid replacements only, the method is useful for the generation of single and double mutants. The 3′ non-templated 'A' addition results only in substitution (confirmed by sequencing more than 20 different mutant clones) and will not result in a frame shift mutation as described[8]. Only two non-specific mutations were noted in more than 8 kb sequence determined for all the mutant clones.

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Direct and indirect somatic embryogenesis in teak (*Tectona grandis* L.)

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Apical and axillary buds from three-year-old teak (*Tectona grandis* L.) were used to initiate the cultures. Callus from apical buds of teak formed globular and heart-shaped somatic embryos on Murashige and Skoog medium supplemented with 6-benzylamino-purine (BAP) (0.1 mg/l) + 1-naphthalene acetic acid (NAA) (0.01 mg/l) and 3% sucrose. However, callus initiated from axillary buds was unable to form somatic embryos on semisolid Murashige and Skoog medium with different combinations of growth regulators. On the other hand, somatic embryos were readily formed from the same callus when transferred to half strength liquid medium containing BAP (0.1 mg/l) + NAA (0.1 mg/l). Somatic embryos were directly formed from axillary buds of teak inoculated in test tubes having filter paper bridges with half strength liquid medium containing BAP (1.0 mg/l) + 2iP (1.5 mg/l).

TEAK (*Tectona grandis* L.) is an important tree known for its high value timber. However, it is slow growing besides the low percentage of seed germination. Tissue culture is a faster method of propagation. The regeneration of teak plantlets by multiple shooting of nodal segments and shoot tips[1,2] and by organogenesis via callus culture of young and mature leaves[3] has already been reported. The present report is on somatic embryogenesis in teak which has not been reported so far.

Apical and axillary buds of three-year-old plants were inoculated on semisolid Murashige and Skoog[4] (MS) medium (pH 5.7 ± 0.1) and incubated 25 ± 2°C in dark for 72 h and then in 16/8 h photoperiod having 1200 lux light intensity. Callus initiated on MS + 6-benzyl-