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Novel properties of recombinant Sso7d-Taq DNA polymerase purified using aqueous two-phase extraction: Utilities of the enzyme in viral diagnosis

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ABSTRACT

Using Sso7d from *Sulfolobus solfataricus* as the DNA binding protein fused to Taq DNA polymerase at its amino terminus, we report the hyper-expression and a novel purification methodology of Sso7d-Taq polymerase (S-Taq) using aqueous two-phase extraction system followed by Ni-affinity chromatography. The utility of such a fusion enzyme in carrying out PCR of human genes from whole blood directly and in detecting hepatitis B virus from clinical samples is demonstrated in this article. We present data on the enhanced thermo-stability of S-Taq DNA polymerase over Taq DNA polymerase and also provide evidence of its higher stability with detergents in comparison to Taq polymerase. The purified S-Taq protein showed acceptable limits of host genomic DNA levels without the use of DNases and other DNA precipitating agents and shows promising potential for use in PCR based diagnostics, in-situ PCR's and forensic science.

1. Introduction

Mutants of Taq DNA polymerases that render them resistant to activity inhibition by blood components are available commercially. Kermekchiev et al. [1] have described the use of mutant Taq polymerase in the PCR-based tests of blood and soil samples that are widely used for diagnostics and forensic analyses. Miura et al. [2] have compared six different types of DNA polymerases for detecting *Plasmodium falciparum* genomic DNA in the presence of the blood components and reported variable efficiencies of the polymerases used.

A major problem with PCR-based diagnostic tests of blood samples is the possibility of seeing false-negative reactions caused by PCR inhibitors present in the blood. Since, PCR-based diagnosis of microbial infection, genetic disease, forensic analysis, and blood banking [3–5] and prenatal genetic diagnosis are extensively used using direct blood/plasma/serum as the template [6,7], having a DNA polymerase that is resistant to PCR inhibitors appeared an attractive option for researchers worldwide. AmpliTaq Gold [8], KlenTaq [2], novel Taq mutants [9], Phusion Blood Direct PCR Kit, Phire Hot Start DNA polymerase, and Phire Hot Start DNA polymerase and KAPA Blood PCR kit [10] are some Taq mutants reported. Al-Soud and Radstrom [11] observed that Hot *Tub*, *Pwo*, *rTth*, and *Tfl* DNA polymerases were able to amplify DNA in the presence of 20% blood without reduced amplification sensitivity, while AmpliTaq was sensitive to PCR inhibitors. Since no single chemistry performs the best across all of the different matrices evaluated, it is imperative that efforts to generate inhibitor resistant Taq polymerases is the hour of the need.

Zhang et al. [9] has reported the use of PCR cocktail to reduce the effect of PCR inhibition by the blood inhibitors and recently, a PCR protocol for amplifying DNA directly from whole blood without any preparative steps using FoLT (formamide low temperature) has been reported [12].

Chien et al. [13] described a 94 kDa thermostable DNA polymerase from the eubacterium *Thermus aquaticus*, whose natural habitat is hot springs of ambient temperatures of 70–75 °C. Lawyer et al [14] expressed this gene in *E. coli* and isolated the enzyme with its thermostable property where polyethyleneimine (PEI) was used to precipitate the A_{260} absorbing material and phenyl-sepharose CL-4B was used to remove the residual nucleic acids. Although *E. coli* proteins do not affect the activity of Taq Polymerase [15], it is well known that the residual DNA contamination in its preparations influences PCR and short DNA

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fragments may act as primers to produce false positive bands rendering the interpretation of the results difficult and challenging [16].

Pluthero purified the Taq polymerase using ammonium sulphate as the precipitating agent [17] while Chen et al. [18] used ethanol to precipitate the protein. The mutant Taq enzymes like KlenTaq have been purified by BioRex 70 and heparin-agarose chromatography [1] while some authors have included pre-treatment of Taq DNA polymerase with DNase I or purification on a $CsCl_2$density gradient [19].

Wang et al. [20] have described construction of various types of Taq fusion proteins and demonstrated that S-Taq, with the DNA binding protein, Sso7d, from *Sulfolobus solfataricus* at the amino terminus of the Taq gene, shows maximum processivity amongst all other types and allows the use of less enzyme and amplification in lesser time. This fusion protein was purified through multiple steps and appeared not cost-effective in large scale. Hence, we attempted purification of the Sso7d- Taq fusion protein (S-Taq) using aqueous two-phase extraction (ATPE) methodology.

ATPE system is a useful technique for separation and purification of biomolecules, such as proteins and antibodies. The method employs the use of either two different immiscible mixing polymers, or one polymer with salt, which are water-soluble in a certain concentration. Factors such as molecular weight/size of polymer and concentration of polymer, the ionic strength of salt and the addition of salts, such as NaCl affect the partitioning of biomolecules in ATPE systems. Furthermore, the partitioning of biomolecules can also be affected by pH and by the affinity of the macromolecule for the phase forming polymer [21]. We have reported the use of similar strategy for purification of native Taq polymerase [22] and show the usefulness of the ATPE method in achieving active Taq polymerase protein in this paper. The methodology of S-Taq purification using ATPE, described here, is simple, cost-effective and relatively easy to perform even in large scale.

2. Materials and methods

2.1. Patients, clinical samples and ethics statement

Patients who visited Kuppam Medical College, Andhra Pradesh, India for viral testing of HIV, HBV and HCV during the period of March 2016 to March 2017 served as the study material. Blood samples were collected in EDTA coated tubes and plasma was collected by centrifuging the samples at 2000 x g for 10 min. The plasma samples were stored at −20 °C until further use. The plasma samples were screened for Hepatitis B virus using HEPACARD that is a visual, rapid, sensitive and accurate one step immunoassay for the qualitative detection of Hepatitis B surface antigen (HBsAg) in human serum or plasma. The method detects all the 11 subtypes of HBsAg with an antigen sensitivity of 0.5 ng/ml. The confirmed positive samples were used for the HIV-1 and HBV test by PCR and Q-PCR that is described in this study. Patients were given oral and written information and signed an informed consent form and ethical approval was granted by the ethical committee of the P.E.S. Institute of Medical Sciences and Research, Kuppam, Andhra Pradesh, India.

2.2. General reagents

All chemicals were procured from Amresco, USA while restriction enzymes and DNA ligase were purchased from New England Biolabs, USA. Oligonucleotides were synthesized at BioServe Technologies, Hyderabad, India.

2.3. Cloning and expression studies

Taq DNA polymerase clone was generated by PCR using primers as indicated in Table 1. PCR amplification was carried out using pTrc99A-Taq polymerase plasmid as a template and the amplified product was ligated to Nde I/Hind III digested pET26b vector (Novagen, San Diego, USA). One of the clones of pET26b-Taq was designated as pCAN009 and used for further studies.

To generate an expression cassette of Sso7d -Taq polymerase gene fusion, the plasmid DNA of Sso7d-Pfu fusion construct was used as template in the PCR reaction using the primers as indicated (Table 1). The amplified Sso7d fragment was digested with Nde I and ligated to pCAN009 vector at the Nde1 site. The resulting construct was designated as pET26b-S-Taq (pCAN11)

Both pCAN009 and pCAN11 expression constructs were expressed in *E. coli* BL21 (DE3) cells in Luria Bertani (LB) broth at an OD_{600} of 0.5-0.8 by induction with 1 mM IPTG for 4 h at 37 °C as reported by Das et al. [23].

2.4. Purification of Sso7d-Taq (S-Taq) fusion by ATPE method and analysis

The induced pellet of pCAN11 from a 250 ml culture was taken and re-suspended in 50 ml of the resuspension buffer (50 mM Tris. HCl, pH 8.0, 150 mM NaCl and 100 μg lysozyme). Sonication was carried out for 10 min and contents were centrifuged at 26, 450 x g for 15 min to separate the soluble and the insoluble fractions.

The soluble fraction was treated at 80 °C for 30 min since several of the cytoplasmic *E. coli* proteins precipitate at this temperature [24]. The supernatant obtained after centrifugation at 26,450 x g for 10 min, was later subjected to ATPE. To every 45 g of the lysate, 4.5 g of solid PEG 4000 and 8 g of sodium sulphate was added with continuous stirring. The contents were allowed to dissolve completely and then two phases were separated by centrifugation at 7670*g* for 10 min. The salt phase (bottom) which contained the protein of interest was removed and then dialysed against 100 bed volumes of 50 mM Tris. HCl, pH 8.0 overnight in cold (4–10 °C). The dialysed protein was later purified on Ni-NTA column (Novagen, USA) using 50 mM Tris.HCl, pH 8.0 buffer and elutions were done using imidazole step gradients. The fractions containing the protein of interest were dialysed against 20 mM Tris.HCl, pH 8.0, 100 mM KCl, 1 mM DTT, 0.1 mM EDTA, 10% glycerol overnight in cold under stirring.

The purified Sso7d-Taq fusion protein was analysed by silver-stain gel as per the method of Nesterenko et al. [25] and the final storage buffer contained 20 mM Tris.HCl (pH 7.9), 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5% NP-40, 0.5% Tween 20 and 50% glycerol. The protein estimations were done using Bradford's

Table 1
Oligonucleotides usedin this study.

PCR primers (5' to 3') direction/Gene	Primer
CCGGAATTCCATATGGAGTTTGGGATGCTGCCCCTC	Forward/Taq pol
CCCGGGGCGGCCGCCTCCTTGGCGGAGA	Reverse/Taq pol
CCGGAATTCCATATGGCGACGGTGAAATTCAAATACAAAGG	Forward/Sso7d
CCGGAATTCCATATGTTTTTTCTGTTTTTCCAGCATTTGCAGC	Reverse/Sso7d
GTAAAACGACGGCCAGT	Forward M13
AACAGCTATGACCATG	Reverse M13
GCTACAATGGCGCATACAAA	Forward/16 s rRNA
TTCATGGAGTCGAGTTGCAG	Reverse/16 s rRNA
ACATCAAGCAGCCATGCAAAT	Forward HIV-1/gag
TACTAGTAGTTCCTGCTATGTC	Reverse HIV-1/gag
CCCCCACTGGCTGGGGCTTGGT	Forward HBV/ HBsAg
AGGACGTCCCGCGCAGGATC	Reverse HBV/HBsAg

method [26]. The same protocol was followed for the protein expressed from pCAN009 construct.

2.5. Properties of S-Taq polymerase

2.5.1. Thermal stability studies

We subjected the in-house purified Taq DNA polymerase and S-Taq purified by similar methods to 97.5 °C for 10 min and then cooled the heat treated enzymes and used suitable aliquots of each of the enzymes for PCR. PCR was tested for a 300 bp gene product of human HLA-DRB1 exon 2 gene cloned under a TA cloning pTZ57R/T vector (Thermo Fisher Scientific, USA) using primers designed of M13 of the vector used, unless mentioned otherwise (Table 1). The final extension step for all the PCR's was done at 72 °C for 10 min and suitable aliquots were loaded on 2% agarose gel for visualization.

The M13 PCR was carried out in a total volume of 25 μl using 1 X ammonium sulphate buffer, pH 8.3 with magnesium chloride, 5% DMSO, 0.5% BSA and 0.2% Tween-20. The PCR was carried out for 35 cycles with an initial denaturation of 5 min at 95 °C and 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 45 s. After a final extension of 7 min at 72 °C, suitable aliquots of the PCR amplicons were examined on Agilent 2100 Bioanalyzer.

2.5.2. Effect of variable magnesium ion concentration on PCR amplification by S-Taq

Magnesium is a required cofactor for thermostable DNA polymerases. The dsDNA gets stabilized by Mg^{2+} in the PCR mixture and hence is an important for controlling the specificity of the reaction. Insufficient Mg^{2+} concentration in a PCR mixture can causes failure of the reaction while excess magnesium (or the presence of manganese) causes the fidelity of DNA polymerases to be reduced [27] and increases the chances of appearance of non-specific products [28,29].

To examine, the effect of magnesium ions on the activity of S-Taq, we set up a series of PCR reactions containing 0–7 mM Mg^{2+} in the increments of 1 mM and examined the concentration of magnesium that produced highest yield of product of interest with minimal non-specific product. The template used for the PCR was as indicated in the Section 2.4.1.

2.5.3. Effect of detergents on S-Taq activity

Suitable ionic and non-ionic detergents were chosen for this parameter. PCR was carried out using Taq and S-Taq proteins at inhibitory concentrations of detergents such as 5% Tween 20, 5% Triton-X-100, and 5% NP-40.

2.5.4. E. coli *host genomic DNA (gDNA) estimation in the purified S-Taq protein*

The primer specific for the 16S rRNA gene (Table 1) of *E. coli* was designed as per Lee et.al. [30]. 25 μl of the reaction mix contained 1X Thermo Scientific Maxima SYBR Green master mix and primers at a final concentration of 5 pmol/reaction. Suitable amount of S-Taq fusion protein was taken in the reaction mixture to test the amount of residual *E. coli* gDNA in the purified protein preparation.

To generate quantification curves, purified 16 sDNA amplicon from *E. coli* gDNA was quantified using Qubit DNA quantitation kit (Life Technologies, USA). This DNA was subsequently diluted serially by copy number (calculated by molecular weight) and then used for PCR amplification of the gene using 16S rRNA specific primers by qRT-PCR assay. The concentration of the *E. coli* gDNA ranged from 10 fg-10 attg per PCR reaction in a 25 μl final reaction volume.

The thermal cycling protocol was as follows: initial denaturation for 10 min at 95 °C followed by 35 cycles of 5 s at 95 °C, 5 s at 60 °C, and 5 s at 72 °C. The fluorescence signal was measured at the end of each extension step at 72 °C. After the amplification, a melting peak analysis with a temperature gradient of 0.1 °C /s from 50 °C to 99 °C was performed to confirm that only the specific product was amplified.

2.5.5. Effect of LB broth on Taq and S-Taq activity

PCR was done using M13 primers for a suitable volume of an overnight culture (2 μl) of the recombinant TA clone as mentioned in the Section 2.4.1. Briefly, the PCR was carried out in a total volume of 25 μl with 1 X ammonium sulphate buffer, pH 8.3, 2.5 mM magnesium chloride, 0.2 mM dNTP's, 5% DMSO, 2% Tween-20, primers 0.4 μM M13 forward and reverse primers and suitable amount for S-Taq fusion protein with variable amounts of sterile LB from 0 to 7 μl for every 25 μl reaction volume. PCR was carried out for 35 cycles which included initial denaturation of 95 °C, 5 min, followed by 35 cycles of denaturation step of 95 °C, 30 s, 51.1 °C, 30 s and 72 °C, 45 s and a final extension of 10 min at 72 °C. Another set with variable amounts of LB with in-house purified Taq DNA polymerase served as a comparison control.

2.5.6. Higher efficiency rate of S-Taq over Taq polymerase

PCR for the *gag* gene of HIV-1 was carried out using SYBR Green dye and Taq and SYBR Green dye and S-Taq independently. Briefly, PCR reaction carried in a final volume of 25 μl contained 1X Thermo Scientific Maxima SYBR Green master mix and primers (Table 1) at a final concentration of 5 pmol per reaction. Suitable volumes of S-Taq fusion and Taq-Pol were added separately into the reaction mixture and 1 ng of plasmid DNA of pTZ19U-pGS3 δ ENV HIV-1 was added as template.

qPCR was performed in Qiagen qPCR machine (Qiagen, Germany), Conditions for qPCR were 45 cycles of PCR amplification each comprising of 95 °C for 5 min, 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 30 s. Data collection was done at 72 °C of each cycle. Following amplification, a melting curve analysis was performed to verify the authenticity of the amplified products by checking their specific melting temperatures (Tm). Experiments were done in duplicates.

For examining the rate of amplification rate of both Taq and S-Taq, PCR was done for a few selected HLA genes using human gDNA as template. The PCR mixture comprised of 1 X ammonium sulphate buffer, pH 8.3, 2.5 mM magnesium chloride, 0.2 mM dNTP's, 5% DMSO, 0.4 μM HLA primers as suggested by Itoh et al. [31], and suitable amounts of Taq and S-Taq enzyme. PCR was done with an initial denaturation of 98 °C for 3 min, followed by 40 cycles of denaturation step of 98 °C, 20 s, 57 °C, 20 s and 72 °C, 30 s and a final extension of 10 min at 72 °C.

2.5.7. Uses of S-Taq for PCR amplification of variable targets

2.5.7.1. Detection of DNA virus like HBV in clinical samples using S-Taq by PCR. Serum samples from HBV positive patients were obtained from Dr. C. Nagaraj, Kuppam hospital, India. All the samples were handled with adequate precautions in BSL-II facility of Cancyte Technologies Pvt. Ltd, Bangalore, India. IVD certified NucleospinDx virus kit (Macherey-Nagel, GmbH) was used to isolate HBV DNA from clinical samples as per manufacturer's instructions.

PCR for HBV detection in clinical samples was carried out in 25 μl reaction volume. The PCR mixture comprised of 1 X ammonium sulphate buffer, pH 8.3, 2.5 mM magnesium chloride, 0.2 mM dNTP's, 5% DMSO, primers 0.4 μM primers and suitable amount for S-Taq fusion protein. PCR was carried out for 40 cycles which included initial denaturation of 95 °C, 10 min, followed by 40 cycles of denaturation step of 95 °C, 30 s, 60 °C, 30 s and 72 °C, 45 s and a final extension of 10 min at 72 °C.

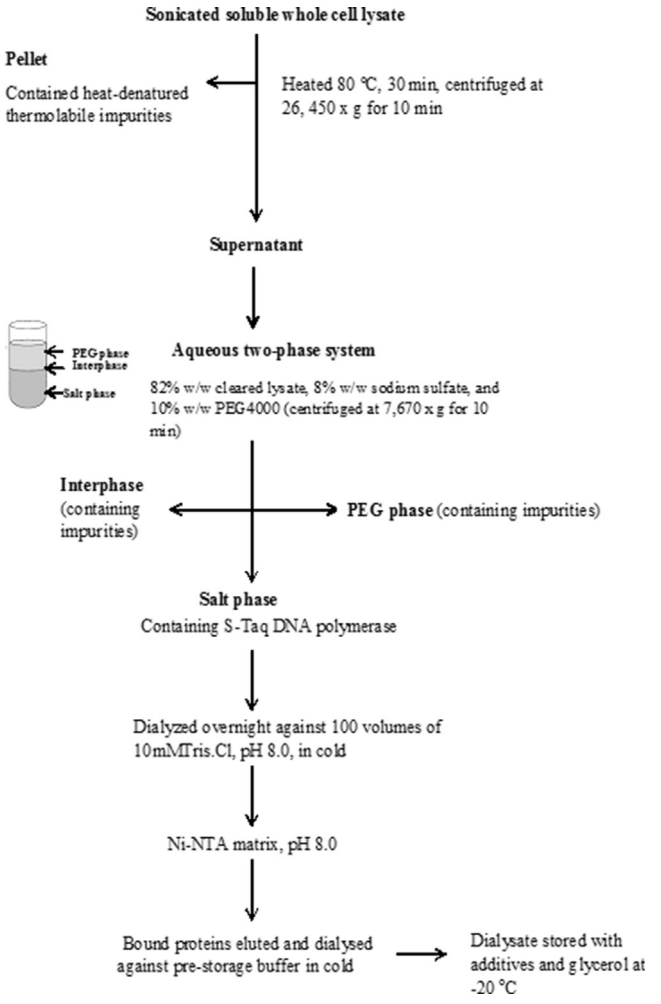

Fig. 1. Scheme of purification of S-Taq by aqueous two-phase extraction system. Same scheme was followed for purification of Taq polymerase.

2.5.7.2. Detection of a few human leucocyte antigen (HLA) exons from blood gDNA by PCR. HLA- class I and Class II antigens have been shown to be highly polymorphic in between individuals and are usually the panel used by researchers for HLA typing [32]. Human genomic DNA (100 ng/ml) was used as the template to assess the relative efficiency of the S-Taq polymerase in HLA PCR's of Class I genes namely exons 2 and 3 of HLA-A and HLA-B. PCR was carried out in 25 μl reaction volume that comprised 1 X ammonium sulphate buffer, pH 8.3, 2.5 mM magnesium chloride, 0.2 mM dNTP's, 5% DMSO, 0.2% Tween-20, 0.04% BSA and 0.4 μM HLA primers as suggested by Itoh et al. [31] and Taq and S-Taq separately. PCR condition included an initial denaturation of 95 °C, 5 min, followed by 40 cycles of denaturation step of 95 °C, 30 s, 60 °C, 30 s and 72 °C, 45 s and a final extension of 10 min at 72 °C.

2.5.7.3. S-Taq for PCR of HLA genes from whole blood directly without any processing. Sso7d fusion proteins are significantly more efficient in PCR amplifications and known to have higher and broader salt tolerances in PCR [20]. However, use of such a fusion polymerase for PCR's from blood directly has not been attempted, so far. Hence, we tested the efficiency of the S-Taq fusion protein to amplify a few PCR targets of HLA genes namely HLA-A and HLA-B, exons 2 and 3, from whole blood directly without any processing.

PCR was carried out in 25 μl reaction volume that contained 1 X ammonium sulphate buffer, pH 8.3, 2.5 mM magnesium chloride, 0.2 mM dNTP's, 5% DMSO and 0.4 μM primers (for HLA-A and B, exons 2 and 3) as per Itoh et al. [31], and 3 μl of S-Taq enzyme and 2 μl of (EDTA mixed) human blood as a source of DNA template. The initial denaturation of 98 °C, 3 min was followed by 40 cycles of denaturation step of 98 °C, 20 s, 57 °C, 20 s and 72 °C, 30 s and a final extension of 10 min at 72 °C.

3. Results

3.1. Cloning and expression studies

The plasmid map of pET26b-Taq and pET26b-Sso7d-Taq is depicted in supplementary Figures S1A and S1B respectively.

3.2. Purification of Taq and Sso7d-Taq fusion by ATPE method

The schematic diagram depicting the process flow for purification of Taq polymerase is shown in Fig. 1 while Fig.2A and B show the purified proteins as analysed on silver-stained gel. The histidine-tagged Taq polymerase was of expected ~95 kDa (Fig. 2A, lane 1) while the commercial Taq polymerase showed the expected molecular weight of 94 kDa (Fig. 2A, lane 2). The his-tag Sso7d-Taq polymerase protein showed an expected molecular weight of 101 kDa (Fig. 2B, lane 3). All the proteins showed>95% purity as judged by silver stained SDS-PAGE.

3.3. Characteristics of S-Taq polymerase

3.3.1. Thermal stability

The half-life of Taq DNA polymerase is reported to be 40 min when exposed to 95 °C and is only 5–6 min at 97.5 °C [33]. It is evident from the Agilent Electropherogram (Fig. 3) that the S-Taq protein has better half-life at 97.5 °C in comparison to Taq DNA polymerase under similar experimental conditions since the heat treated Taq polymerase lost 50% activity in comparison to untreated preparation supporting literature reports (Fig. 3A and Fig.3B), the S-Taq showed almost similar activity before and after the heat treatment (Fig. 3C and Fig. 3D).

3.3.2. Effect of magnesium ions on activity of S-Taq

From the supplementary Fig. S2, it is clear that the S-Taq polymerase activity was negligible when the Mg^{+2} ion concentrations was less than 1.5 mM in the reaction mix. While S-Taq showed strong PCR signals at other Mg^{+2}concentrations like 2.5–

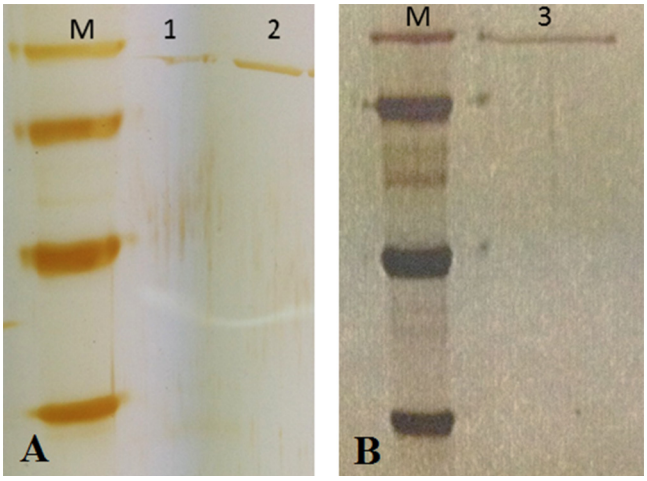

Fig. 2. SDS-PAGE and silver stained gel for purity of Taq and S-Taq. Panel A shows M : molecular weight marker (29 kDa–97 kDa); lane 1; Taq (in-house); lane 2: Commercial Taq polymerase while panel B shows purified S-Taq in lane 3.

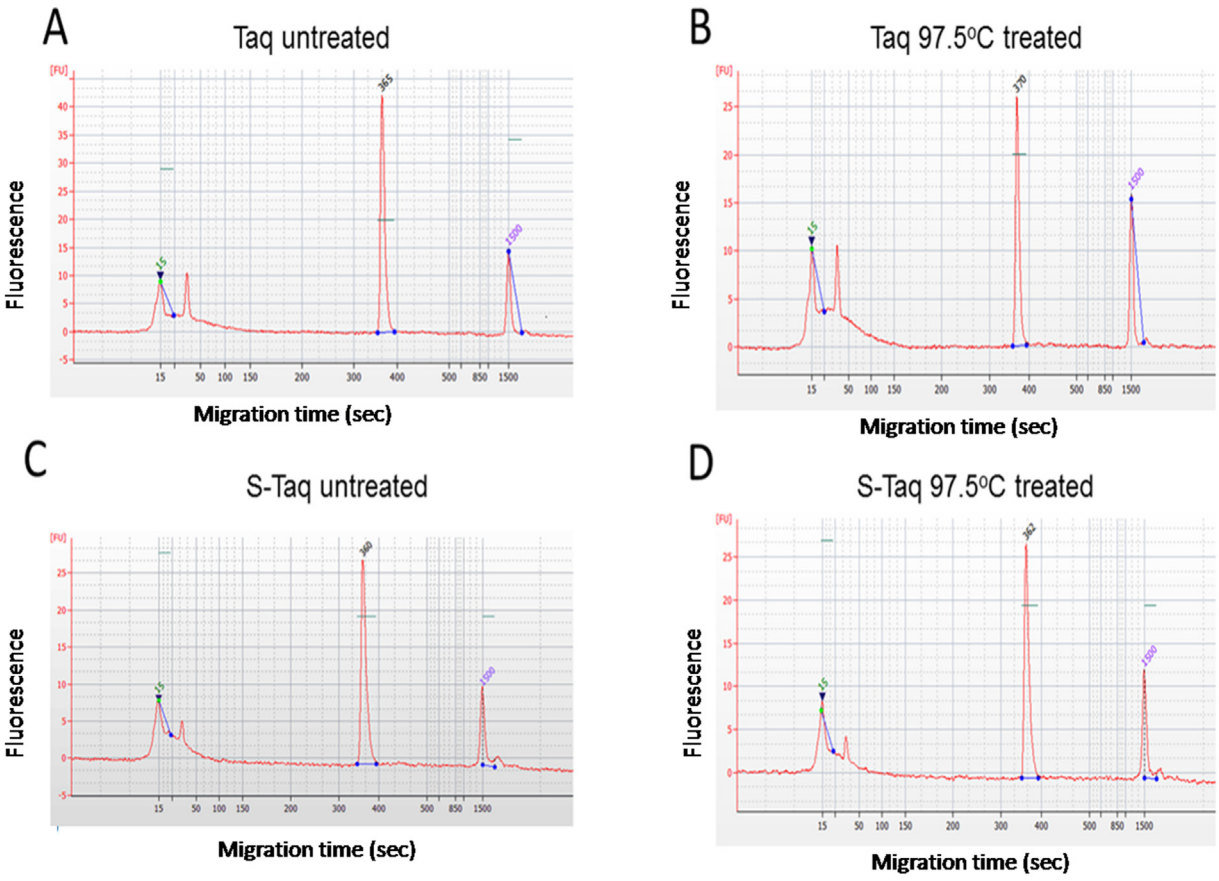

Fig. 3. Comparison of thermal stability of Taq and S-Taq: Suitable amounts of Taq and S-Taq purified proteins were subjected to 97.5 °C for 10 min and then after cooling, the aliquots were taken for PCR of a 300 bp gene product of human HLA-DRB1 Exon 2 gene cloned in a pTZ51R/T vector. Panel A shows untreated Taq polymerase protein while panel B shows Taq treated with heat. Panel C shows S-Taq untreated while panel D shows heat treated S-Taq. Note the differences in the fluorescence intensity between untreated and treated samples. While Taq polymerase showed nearly 50% loss in its PCR activity, the S-Taq showed no loss in PCR activity after being exposed to 97.5 °C for 10 min. The migration time is indicated in seconds [sec] on the abscissa, while the arbitrary fluorescence units [FU] are indicated on the ordinate axis.

7 mM $MgCl_2$, Taq DNA polymerase showed reduced amplification of products beyond 3.5 mM magnesium chloride.

3.3.3. Effect of detergents on S-Taq activity

Concentrations of DMSO greater than 10% is known to inhibit Taq DNA polymerase and presumably other DNA polymerases as well [34] and non-ionic detergents such as Tween-20, Triton X-100 and NP-40 at 5% final concentration or greater are detrimental to Taq polymerase activity [35]. Our data shown in Fig. 4 clearly shows that S-Taq shows PCR amplification of the gene of interest even in the high concentration of the non-ionic detergents tested (Fig. 4B, D, F) while no amplification was observed for the same gene with Taq polymerase (Fig. 4A, C, E). Suitable untreated controls (Fig. 4G and H) showed the expected PCR product of the right size.

3.3.4. Effect of LB on Taq and S-Taq activity

The reported observations of S-Taq being more tolerant to salts [20] made us to extrapolate this data to test if the S-Taq fusion polymerase works in presence of LB, a routinely used growth medium in regular molecular biology labs. The S-Taq showed higher tolerance to LB in comparison to Taq polymerase, known for its sensitivity to salts and growth medium (Fig. S3). Hence, one could safely use the S-Taq polymerase for screening clones directly in overnight grown cultures and avoid the cost of making plasmid DNA preparations of the cultures for further work like PCR.

3.3.5. Higher PCR efficiency rate of S-Taq over Taq polymerase

From the Fig. 5, it is clear that the Ct values obtained for the reaction with S-Taq was faster (Fig. 5A) by 2 cycles (~1 log) in comparison to the reaction which contained Taq alone. The melt curve analysis also substantiated the Ct data (Fig. 5B). These data clearly reflects that S-Taq is able to incorporate more number of nucleotides per binding event, and hence the replication rate of the template with S-Taq is higher in every PCR cycle in comparison to that achieved with unmodified Taq polymerase. This is purely due to higher processivity of S-Taq over Taq alone.

When a polymerase has a higher processivity, it means one would achieve more PCR signals with a shorter extension time for a target of interest. Wang et al. [20] have shown that Sso7d fused with truncated Taq has higher processivity but such a data for a S-Taq fusion is missing. Hence, we carried out PCR of a few HLA Class I genes using S-Taq and Taq polymerase using a PCR protocol with shortest extension time. The results indicated in Fig. 6A show that PCR signals with shorter PCR protocol are obtained only with S-Taq and not with Taq polymerase under similar experimental conditions.

3.3.6. Amount of host DNA in S-Taq purified protein

The recombinant Taq polymerase purified from *E. coli* is known to have contaminating bacterial DNA [36]. Větrovský and Baldrian [37] have shown that the information on 16S rRNA copy numbers and genome sizes of genome-sequenced bacteria can be used as an

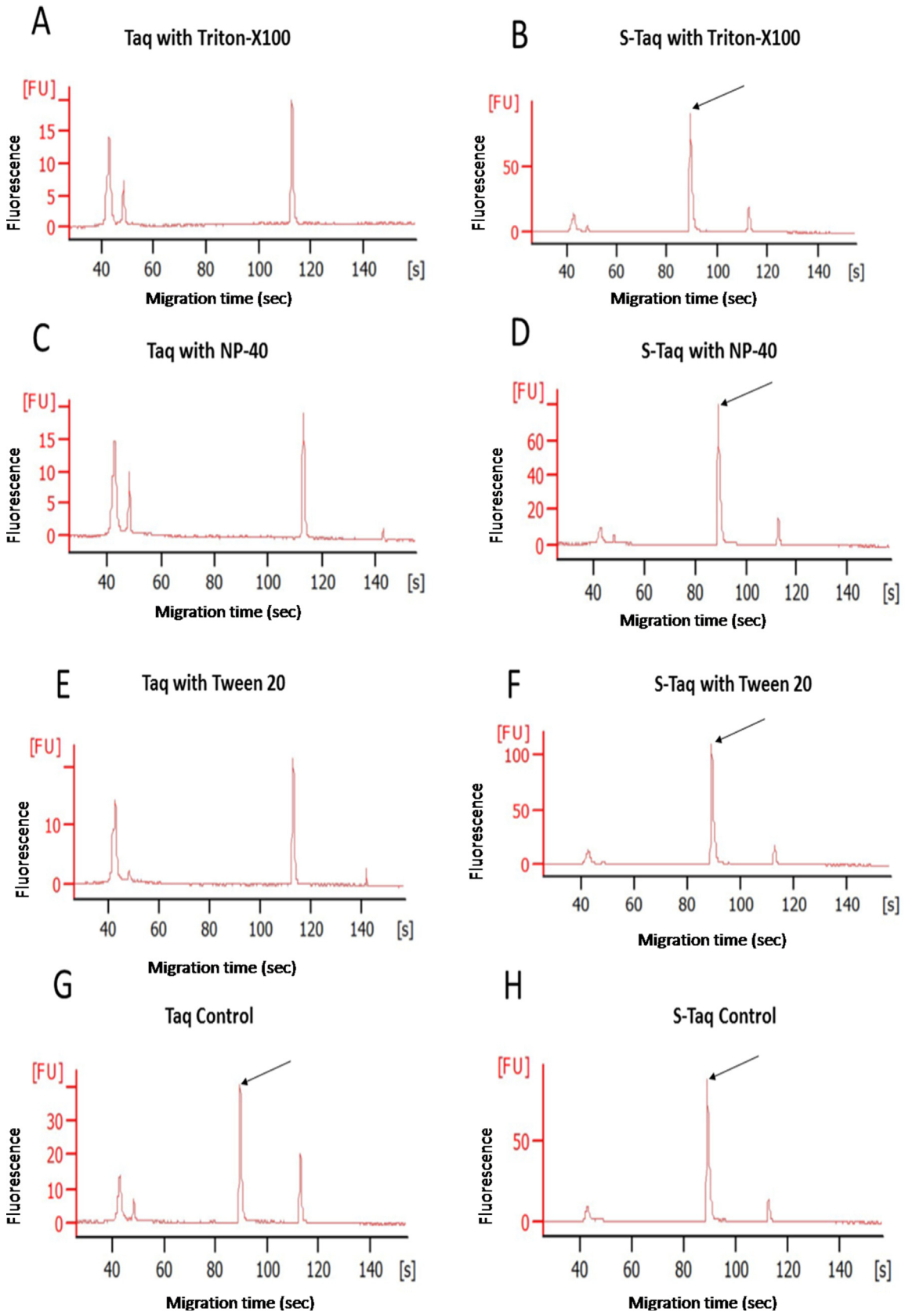

Fig. 4. Effect of detergents on Taq and S-Taq activity: Three non-ionic detergents namely Triton X-100, Tween-20 and NP-40 were chosen for this study and included in the PCR mixture at 5% final concentrations. PCR was carried out as described in M & M section. Panels A, C and E shows no PCR signals when the PCR was carried using Taq polymerase in the presence of the above detergents while panels B, D and F shows prominent PCR signals of the band of interest (marked by arrow) when the PCR was carried out with S-Taq in presence of the same detergents. Suitable controls of untreated Taq (panel G) and S-Taq (panel H) were included as controls. The migration time is indicated in seconds [sec] on the abscissa, while the arbitrary fluorescence units [FU] are indicated on the ordinate axis.

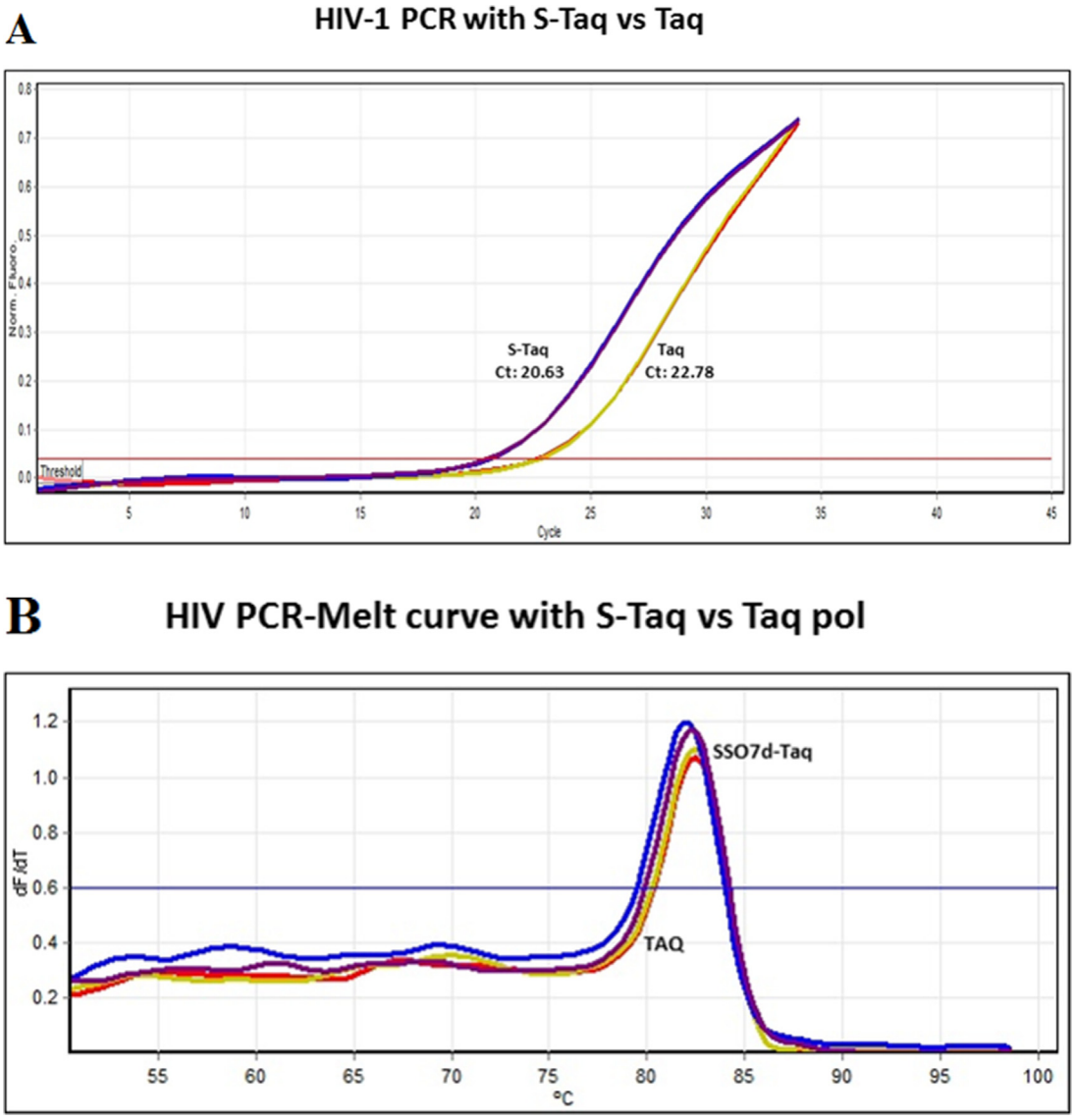

Fig. 5. PCR efficiency rate of S-Taq over Taq polymerase: A plasmid carrying HIV-1 genome (pTZ19U-pGS3 δ ENV HIV-1) was used as the template at appropriate dilutions and Q-PCR was done using SYBR dye with Taq and S-Taq separately. Fig. 5B shows that the Ct values achieved with S-Taq (20.63) was at least 1 log faster in comparison to that achieved using Taq (22.78). It is also clear from Fig. 5B that the melt curve of the products is similar in both the cases. Red and yellow colored lines are products obtained with Taq while blue and black lines represent products obtained with S-Taq preparations. Experiments were done in duplicates. In Fig. 5A, the cycle threshold (Ct) time is indicated on the abscissa, while the arbitrary fluorescence units are indicated on the ordinate axis while the Fig. 5B shows the derivative of the function "fluorescence vs. Tmelting" (dF/dT) which represents the rate of the fluorescence variation in the reaction where Y axis indicates fluorescence variation and the x-axis represents the temperature (°C).

estimate for the closest related taxon in an environmental dataset to calculate alternative estimates of the relative abundance of individual bacterial taxa in the test samples. The S-Taq protein accounted for 1200 genome equivalents which is close to the reported values of 10–1000 genome copies per enzyme unit [38].

3.3.7. Use of S-Taq for PCR amplification of variable targets

3.3.7.1. Detection of DNA virus like HBV in clinical samples using S-Taq. It is clear from Fig. 6B that HBV signal for the positive clinical samples were detected by S-Taq. The differences in the intensity of the HBV band seen with different samples as seen in Fig. 6B can be attributed to the variable viral loads in these samples which have been tested by Q-PCR in our lab using SYBR Green dye and HBV specific primers (data not shown).

3.3.7.2. PCR of HLA genes from whole blood directly without any processing. It is evident from Fig. 6C that S-Taq successfully amplifies tested HLA genes directly from blood without the need of isolation of genomic DNA's. Taq polymerase failed to give any PCR signals under similar conditions.

4. Discussion

Purification of Taq polymerase described earlier either employ solvents [18] or salts followed by affinity chromatography [20]), nucleotide-mimetic affinity chromatography [39] or ananion exchange resin [40]. Although, the protocols claim the purified Taq to be of high purity with appreciable yield (61,500 units/mg), not all methods yields the enzyme preparations of devoid of contaminating proteins and not all methods are cost-effective in large scale.

The Sso7d fragment is a 7 kDa protein from *S. solfataricus*, capable of covalently binding to dsDNA without any preference for specific sequences [41]. In vitro studies have shown that Sso7d promotes the annealing of complementary DNA strands [42],

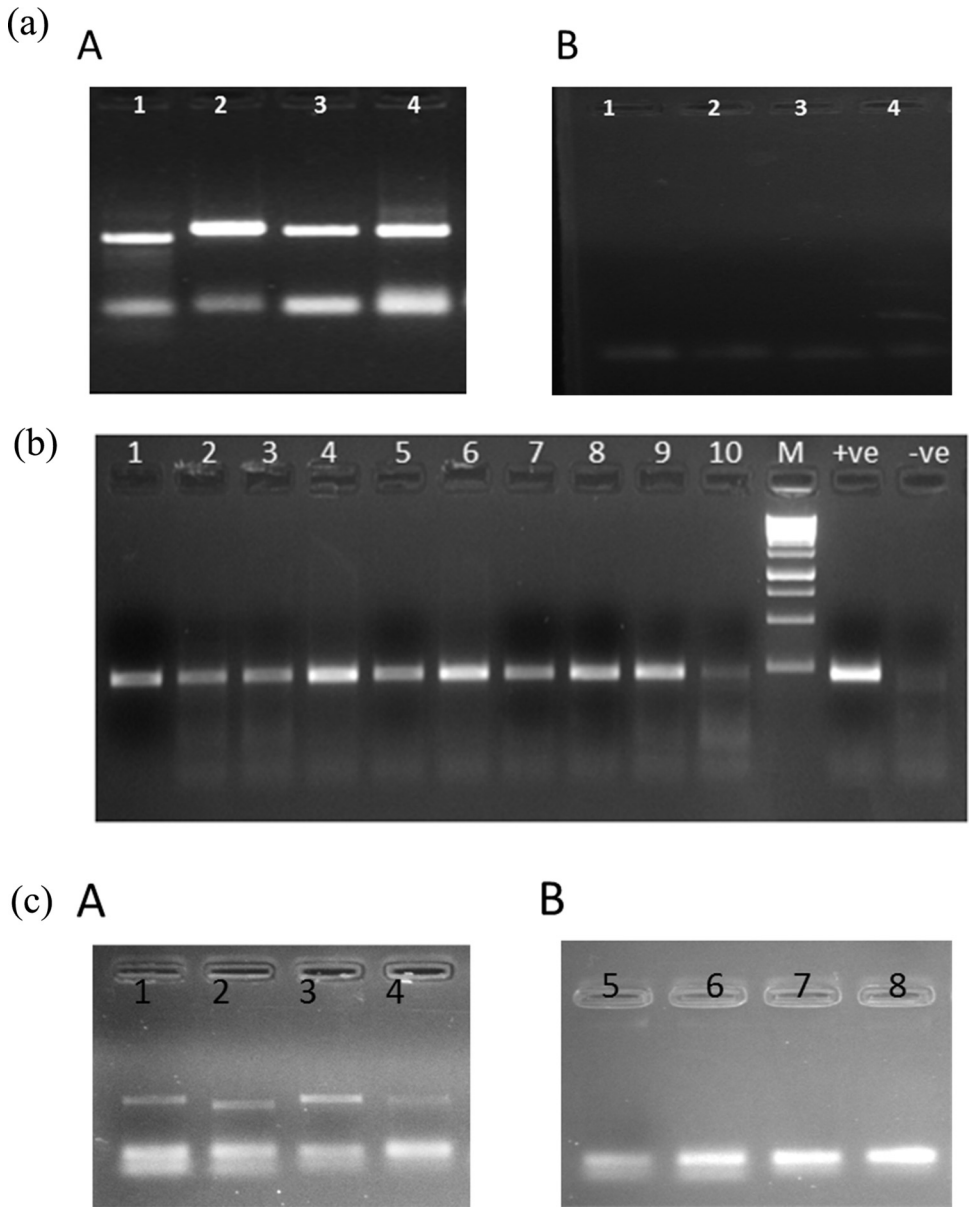

Fig. 6. PCR of a few HLA Class I genes using S-Taq and Taq: HLA-A-Exon 2, HLA-A-Exon 3, HLA-B-Exon 2 and HLA-B-exon 3 genes were PCR amplified using primers as described by Itoh et al. described in M & M section. Fig. 6A clearly depicts positive PCR signals with S-Taq for all the genes while no products were seen with Taq when the PCR protocol had lesser extension time. While Fig. 6B depicts HBV PCR for 10 HBV ELISA positive samples using S-Taq as described in M &M section, Fig. 6C shows PCR achieved from direct blood samples using S-Taq (lanes 1 to 4) while no products were seen in panel B when Taq was used as the source of DNA polymerase (lanes 5 to 8). Lane 1: HLA-A-Exon 2 (363)bp; lane 2: HLA-A-Exon 3 (315 bp); lane 3: HLA-B-Exon 2 (366 bp); lane 4: HLA-B-Exon 3 (359 bp); lane 5: HLA-A-Exon 2; lane 6: HLA-A-Exon 3; lane 7: HLA-B-Exon 2; lane 8: HLA-B-Exon 3.

induces negative supercoiling [43], and chaperones the disassembly and renaturation of protein aggregates in an ATP hydrolysis-dependent manner [44]. The stabilized S-Taq is known to prevent the polymerase from dissociating during amplification process, thereby increasing the processivity of the polymerase and hence the efficiency of a PCR/QPCR reaction.

Purification of S-Taq fusion protein as described by Wang et al. [20] is time-consuming since it involves several steps that include precipitation of with ammonium sulfate followed by purification on a Ni-NTA matrix, heparin-agarose matrix and a second Ni- NTA matrix before dialyzing in the storage buffer.

We have successfully purified recombinant Taq DNA polymerase using an aqueous two-phase extraction method earlier [22] and applied the same methodology for the purification of Sso7d-Taq fusion protein and the results are described in this paper.

The total yield of S-Taq from the present clone was 53,000 units of enzyme/litre of LB. This process can be easily implemented in large scale manufacturing and such a process would be cost-effective for research labs working on using such enzymes for diagnostics and other assays. This purification methodology represents a fast and easy to use system, which could surely be adapted for other DNA polymerases as well. Also, the specific activity of most commercial preparations of Taq is ~80,000 units/mg of protein [38] and it is interesting to see that we achieved almost 88,000 units/mg protein by the methodology described here. Since no universally applicable method for removing DNA from Taq polymerase preparations has been established [45], our report on a high specific activity of the enzyme with host DNA within the specified limits appears encouraging.

An aqueous two-phase system is an aqueous, liquid–liquid, biphasic system which is obtained either by mixture of aqueous solution of two polymers, or a polymer and a salt. There are several advantages of using ATPE for purifying proteins which include fewer number of initial downstream steps and clarification and concentration [46–52].

The observations of S-Taq being more tolerant to detergents throws light on the novel property of the Sso7d fragment to offer detergent tolerance to the molecule is hitherto unreported.

The most common cause of viral liver disease, cirrhosis and hepatocellular carcinoma, accounting for over 1 million deaths annually, is due to Hepatitis B virus [53]. Hence, measurement of levels of HBV DNA in the serum could guide the clinicians for the beginning and ending of treatment to access liver damage [54]. Since S-Taq has improved properties over regular Taq DNA polymerase as shown in this study, it is tempting to speculate that one could use this enzyme for a cost-effective diagnostic assay for HBV.

The use of in situ PCR requires higher Mg^{2+} concentrations (approximately 4.5 mM versus the normal 1.5–2.5 mM) as described before [55,56]. Hence, the present observations of the S-Taq being active in higher concentrations of magnesium ions make it an ideal enzyme candidate for in-situ PCR's also. Hu et al. [57] have shown the critical importance right concentration of Mg^{+2} ions for proper allele typing of HLA, hence our current observations of S-Taq capable of tolerating higher concentrations of Mg^{+2} reflects the possibility of its safe usage in HLA PCR's also.

The results indicated in Fig. 6B shows that PCR amplification of a few tested HLA genes with S-Taq shows promising results while Taq polymerase failed to show any amplification under similar conditions. This data clearly shows that due to higher processivity, the S-Taq is capable to generate more number of template copies in comparison to Taq polymerase.

The resistance of S-Taq with LB will be of immense use to molecular biologists and our results appears similar to the reported robust Taq DNA polymerase called MyTaq from Bioline (www.bioline.com) which is also stable with 28% LB in the reaction mixture.

Our observations of successful amplification of HLA genes using S-Taq from the human blood directly is very promising and opens up new avenue for this polymerase as a valuable tool for medical diagnosis and forensic science where sample availability is minimal. We believe that the enhanced salt tolerance of the S-Taq property is responsible for its successful use in PCR's from the wholeblood directly without any processing.

Studies have shown that SYBR Green I and other fluorescent dyes used recently in qPCR exhibit some inhibitory effect on Taq enzyme [58,59]. The SYBR Green dye does not seem to impact the activity of S-Taq as depicted in Fig. 6 as confirmed by the melting curve analysis (Fig. 6B). A gene specific amplification was still observed in the sample with S-Taq versus sample with Taq alonewhere the Ct was found to be more with the latter sample (Fig. 6). These data demonstrate that the S-Taq does not negatively interfere with the fluorescence of SYBR Green, which is interesting.

Our observation that the S-Taq has higher thermo-stability over Taq makes it an attractive enzyme for PCR related diagnostics. It is tempting to speculate that one could explore the use of S-Taq in the detection of common DNA viruses such as SV40, HPV, Herpesvirus, Parvoviruses etc.

Conflict of interest

We the authors of the research paper titled "Properties of recombinant Sso7d-Taq DNA polymerase purified using aqueous two-phase extraction" state that we have no conflict of interest associated with this publication.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btre.2018.e00270.

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