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Emulating a crowded intracellular environment *in vitro* dramatically improves RT-PCR performance

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Abstract

The polymerase chain reaction's (PCR) phenomenal success in advancing fields as diverse as Medicine, Agriculture, Conservation, or Paleontology is based on the ability of using isolated prokaryotic thermostable DNA polymerases *in vitro* to copy DNA irrespective of origin. This process occurs intracellularly and has evolved to function efficiently under crowded conditions, namely in an environment packed with macromolecules. However, current *in vitro* practice ignores this important biophysical parameter of life. In order to more closely emulate conditions of intracellular biochemistry *in vitro* we added inert macromolecules into reverse transcription (RT) and PCR. We show dramatic improvements in all parameters of RT-PCR including 8- to 10-fold greater sensitivity, enhanced polymerase processivity, higher specific amplicon yield, greater primer annealing and specificity, and enhanced DNA polymerase thermal stability. The faster and more efficient reaction kinetics was a consequence of the cumulative molecular and thermodynamic effects of the excluded volume effect created by macromolecular crowding.

Keywords: Macromolecular crowding; Excluded volume effect; Macromolecule; Polymerase chain reaction; DNA polymerase; Reverse transcriptase; Reverse transcription; Sensitivity

Biochemical reactions in cells function in a carefully controlled intracellular environment which biologists have, to a certain extent, reproduced *in vitro* by controlling factors such as pH, ionic strength, temperature, and supply of cofactors which constitute the buffer system. However, the biophysical effect of macromolecular crowding has not been transferred to the *in vitro* setting and has gone largely unnoticed and underappreciated [1]. In fact, all DNA modifying enzymes that are commonly used today (e.g. polymerases, nucleases, ligases) have evolved to function efficiently within the crowded interior of cells. For example, the total concentration of protein and RNA inside bacteria (e.g. *Escherichia coli*) is in the range of 300–400 g/l [2] and this level of crowding is also present in eukaryotic cells [1]. Biological crowding occurs in the range of 5–40% w/v solute content [1,3,4] which translates to even higher excluded volume [5]. This high solute content, colloquially termed crowding, results from no single molecule species being present at a high concentration however, collectively, the consequence is expressed in the principle of the Excluded Volume Effect (EVE). It states that the volume of a solution that is excluded to a particular molecule in question is the result of the sum of non-specific steric hindrances (size and shape) and electrostatic repulsions (charge) of the other macromolecules [6]. This results in molecules constantly interacting non-specifically with an assortment of diverse macromolecular species which is responsible for a

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spectrum of molecular thermodynamic effects namely, reaction rate/kinetics [7], molecular assembly [8], and protein folding [9]. It has been postulated that macromolecular crowding is a key factor responsible for the phenomenally high rates of reactions and molecular interactions *in vivo* while seemingly relatively low amounts of reactants are present, at least when compared to their *in vitro* use [10,11].

Our aim was to more closely emulate the intracellular biophysical environment of the bacterium in the *in vitro* setting and thus enhance reverse transcription (RT) and polymerase chain reaction (PCR). Herein, for the first time with the addition of inert macromolecules we demonstrate significant improvements in all aspects of RT-PCR, including sensitivity, specificity, processivity, yield, and thermal stability of Taq DNA polymerase.

Materials and methods

General materials. All reactions were performed on the real-time Mx3000P (Stratagene, CA, USA). Macromolecules: Ficoll™ (Fc) 70 kDa (Fc70) and Fc400 kDa (Fc400) (Amersham Pharmacia, Uppsala, Sweden); trehalose (Fluka–Sigma–Aldrich, Singapore); proline (Sigma–Aldrich); and polyethylene glycol (PEG) 4 kDa. Additives were dissolved in nuclease-free water as a concentrate and added freshly to the reaction buffers each time.

RNA extraction. RNA was extracted from human WI-38 fibroblasts (American Tissue Culture Collection, VA, USA) from which complementary DNA (cDNA) was prepared for all PCR assays except for aP2 (fatty acid binding protein 2) which used RNA from adipocytes differentiated from human mesenchymal stem cells. Extractions were performed with RNAqueous™ (Ambion Inc., TX, USA) according to the Manufacturer's protocol.

Reverse transcriptase. Complementary DNA synthesis was carried out according to the Manufacturer's protocol for SuperScript II reverse transcriptase with oligo(dT) primers with the following modifications when macromolecules were used. Fc70 (7.5 mg/ml) was added to the annealing buffer and mixture of Fc70/400 (7.5 and 2.5 mg/ml) was added to the polymerization step.

Polymerase chain reaction. Two microliters of cDNA was used as target for all PCRs in a final volume of 20 μl and all samples were run in duplicates. Reactions as follows unless otherwise stated: 1 U Platinum Taq DNA polymerase in 1× reaction buffer, 300 nM primers and 2.5 mM $MgCl_2$. The thermal cycling program for all PCRs was the following, unless otherwise stated: 94 °C/5 min, 94 °C/30 s, 56 °C/30 s, 72 °C/30 s, for (collagen I set 1, 30; GAPDH, 35; aP2 and M13, 40; collagen I set 2, 42) cycles with a final dissociation step of 60–94 °C at 1.1 °C/s. The annealing temperature for collagen I set 1 and set 2 was 55 °C. Fluorescence was detected with SYBR Green I (Molecular Probes–Invitrogen). Primer sequences were: GAPDH, gtccactggcgtcttcacca, gtggcagtgatggcatggac; collagen I set 1, agccagcagatcgagaacat, tcttgtccttggggttcttg; aP2, tactgggccaggaatttgac, gtggaagtgacgaatttcat; M13, ttgcttccggtctggttc, caccctcagagccaccac; collagen I set 2, gtgctaaaggtgccaatggt, ctcctcgctttccttcctct. Oligonucleotides poly-adenine ($oligo(dA)_{20}$) and poly-thymine ($oligo(dT)_{20}$) (both 20-mer) at 10 μM were combined in the presence of reaction buffer, 2.5 mM $MgCl_2$ and SYBR Green 1 and thermal cycled through 94, 50, and 72 °C for 30 s each followed by a dissociation step 50–94 °C.

Processivity experiments. The single-stranded M13 (ssM13) processivity assay for Taq DNA polymerase was modified from Bambara et al. [12]. Briefly, 100 nM of primer (gtaaaacgacggccagt) was added to 100 nM ssM13mp18 DNA (New England Biolabs Inc., MA, USA) in buffer with 1 U Taq DNA polymerase in the absence or presence of Fc400 (2.5 mg/ml). The samples were heated to 94 °C/5 min, cooled to 55 °C/1 min followed by 72 °C for 1 and 3 min, respectively. For the reverse transcriptase processivity assay, a standard RT was performed without and with the macromolecules Fc70/Fc400 mixture, as above. Reaction products were separated on a denaturing 0.6% agarose gel.

Agarose gel electrophoresis. Reaction products were either resolved in 1XTAE agarose (Seakem, ME, USA) gels or in formamide-denaturing agarose gels [13] at the stipulated concentrations of 0.6% or 2.0%. The molecular weight markers were 1 kb (Promega Corporation, WI, USA), 50 and 100 bp (Invitrogen) DNA ladders. Post-staining was done with SYBR Gold (Molecular Probes–Invitrogen), images were captured with a Versadoc™ (Bio-Rad), and analysed using Quantity One v4.5.2 (Bio-Rad).

Calculation of the area-under-the-curve and late phase PCR efficiency. The method of Rasmussen et al. [14] which uses the NCSS™ software was used to calculate the area-under-the-curve from the PCR dissociation curves raw data values derived from the Stratgene software MxPro v3.20. The late-phase efficiency of PCR amplification was calculated according to the method of Liu and Saint [15].

Results

Sensitivity

Total RNA (1000 and 50 ng) was reverse transcribed in the presence and absence of a macromolecule mixture (Fc70 and Fc400) followed by amplification with collagen I PCR assay in the presence and absence of a single macromolecule (Fc400), respectively. Crowding resulted in a reduction of greater-than 3 Ct (threshold cycle) (green) compared to standard (i.e. non-crowded) RT-PCR samples (orange) (Fig. 1A; taken from the amplification plots Fig. 1B). This translates to enhanced sensitivity of >10-fold. The dissociation curves (Fig. 1C) in conjunction with the agarose gel electrophoresis (Fig. 1D) confirm amplification of the specific target. Complementary DNA was prepared from 500 ng of total RNA under standard condition (i.e. non-crowded) and subjected to amplification with GAPDH PCR in the absence or presence of macromolecule mixture Fc70/400 (7.5/2.5 mg/ml) or PEG 4 kDa at 2.5, 5 or 10 mg/ml concentrations. Unlike the macromolecules which enhanced an already optimized PCR by 2 Ct (i.e. 4-fold increase), PEG inhibited sensitivity by greater-than 4 Ct (i.e. 16-fold decrease) which was dose-dependent (Fig. 1E). In addition, the presence of PEG caused the amplification of a smaller, non-specific product, apparent by a shoulder on the left of the dissociation curves (Fig. 1F) and ~200 bp band on the agarose gel (Fig. 1G).

Specificity

We were unable to amplify a particular collagen I template target region through standard RT-PCR due to its long distance form the olig(dT) priming site (~4390 bp; NM_000088). However, in the presence of a mixture of Fc70 and Fc400 the specific product was obtained with the lower range of primer concentrations (100–300 nM) (Fig. 2A). Although higher primer concentrations resulted in high background the specific product was still present and dominated the amplicons that were generated in the presence of macromolecules. In contrast, non-crowded reactions yielded only non-specific products. In fact, we

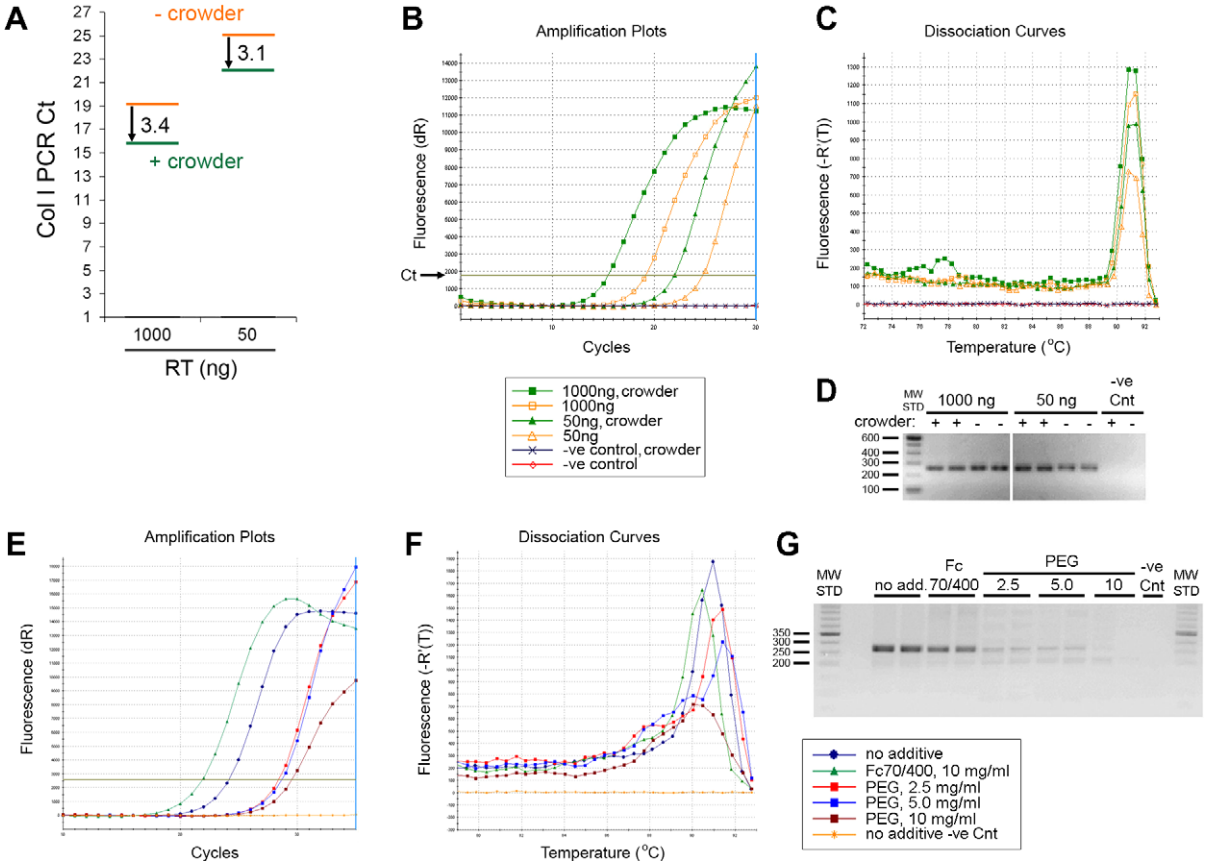

Fig. 1. Macromolecular crowding enhances the sensitivity of RT and PCR assays. (A) The average Ct (threshold cycle) values from samples amplified with the collagen I set 1 PCR in the presence (green) and absence (orange) of Fc400 from cDNA prepared in the presence and absence of mixed crowders (Fc70 7.5 and Fc400 2.5 mg/ml), respectively. The amount of total RNA used for the RT was 1000 and 50 ng. (B) Amplification plots and (C) dissociation curves of the PCR samples. (D) Composite of the same agarose gel (2%) demonstrating a specific 250 bp collagen I amplicon. (E) Amplification plots and (F) dissociation curves of the GAPDH PCR showing the relative performance of macromolecular mixture Fc70/Fc400 (7.5/2.5 mg/ml), PEG 4 kDa at either 2.5, 5 or 10 mg/ml, and standard conditions (without additives). (G) Agarose gel (2%) demonstrating a specific 261 bp GAPDH amplicon. All the graphs show one replicate per PCR sample for display clarity. −ve Cnt = PCR template-free control: no add = no additive. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

demonstrate that the presence of macromolecules directly enhances primer annealing. The total amount of duplex formation consisting of oligos of adenine and thymine was quantified with SYBR Green 1 dye. This primer configuration was chosen to avoid secondary structures and self-annealing. There was an average increase of 1.8-fold in specific duplex formation in the presence of macromolecules (Fig. 2B).

Processivity

In order to assess the ability of macromolecules to enhance processivity of Taq DNA polymerase, we performed a classical ssM13 assay in the absence and presence of macromolecules. The presence of Fc400 resulted in an average increase in DNA product of 15% (Fig. 3A) and longer DNA fragment lengths after 1 and 3 min of extension time (Fig. 3B). Figs. 3A and B are based on the intensities and relative migration profiles of the bands from the denaturing agarose gel (Fig. 3C). The enhanced processivity induced by crowding was tested with a long PCR assay with limited extension time of 40 s. The addition of macromolecule mixture Fc70/Fc400 enabled the amplification of the correct amplicon (1547 bp) under these limiting experimental conditions (Fig. 3D). In contrast, the reaction carried out in the absence of crowding did not amplify the correct and long amplicon. Total RNA was use to test the effect of crowders on the processivity of reverse transcriptase. We carried out cDNA synthesis in the absence and presence of crowding additives (Fc70/Fc400). Densitometric analysis of the denaturing agarose gel of reaction products (Fig. 3E) demonstrated an increase in total cDNA of 86% (Fig. 3F) and overall longer cDNA products under crowded condition (Fig. 3G).

PCR product yield

Decreasing amounts of Taq DNA polymerase were used to amplify a specific aP2 product from cDNA in the absence and presence of Fc400. For all Taq DNA polymerase concentrations (units of activity (U)/reaction) the presence of a crowding agent resulted in > 2-fold yield of

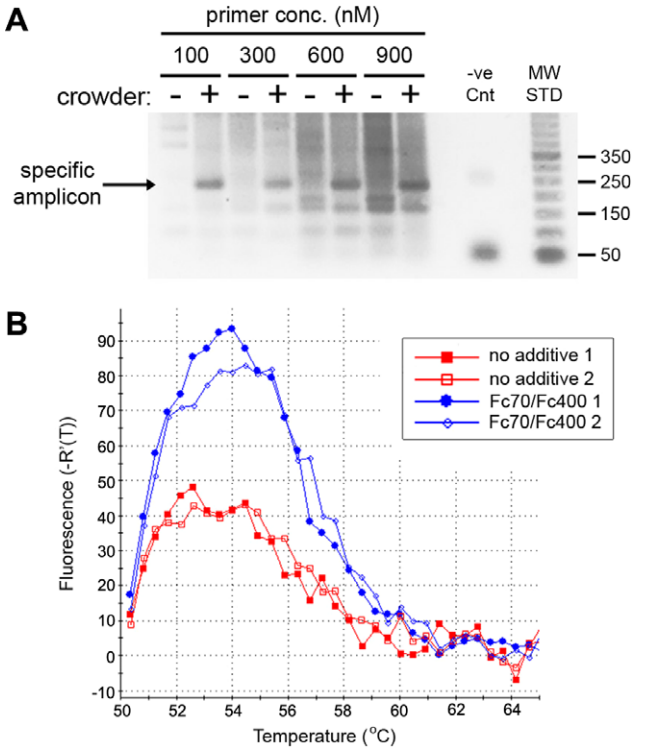

Fig. 2. Macromolecular crowding increased primer binding and specificity. Agarose gel of RT-PCR samples amplified with the collagen I set 2 PCR in the absence or presence of the macromolecule mixture Fc70/Fc400 (15/5 mg/ml) with increasing concentrations (conc.) of primers. The specific target is indicated at 228 bp. The cDNA was prepared from 250 ng total RNA. The −ve Cnt (control) was the PCR template-free control. (B) Dissociation curves of the hybridized oligonucleotide duplex between oligo(dA)$_{20}$ and oligo(dT)$_{20}$ in the absence (no additive) and presence of a mixture of macromolecules Fc70/Fc400 (15/5 mg/ml).

specific amplicon (Fig. 4A). These data were derived from integrating the area under the dissociation curves (Fig. 4C). In order to assess the relative reaction rates in the presence and absence of macromolecular crowders, we calculated the slopes of the amplification plots (Fig. 4D) at the late exponential phase for the above samples run with 1 U of enzyme (Fig. 4B). The presence of Fc400 resulted in a 2-fold greater value for the slope and an additional cycle in the exponential phase demonstrating faster reaction kinetics.

Thermal stability

We tested the thermal-protective property of macromolecules (i.e. Fc400) for Taq DNA polymerase against trehalose and proline, known, small molecules that have been shown to work as thermoprotectants. The enzyme was heat-stressed (95 °C for 45 min) in the absence and presence of the individual additives following which it was used to amplify a specific amplicon in the presence of the same additive. Only the presence of Fc400 and trehalose preserved the Taq DNA polymerase's enzymatic activity (Fig. 4E). As expected, trehalose protected Taq while proline did not prevent the complete loss of activity.

Discussion

Macromolecular crowding has important thermodynamic consequences which influence reaction kinetics [2], however it has been neglected in biochemical and biological *in vitro* settings [1]. We have shown herein that reintroducing this parameter *in vitro* culminates in enhanced enzymatic properties expressed in dramatically more sensitive, specific and productive RT-PCR assays. Using molar concentration and hydrodynamic radii of the macromolecular additives, measured by Dynamic Light Scattering [16], all of which are hydrophilic, we have introduced fraction volume occupancies ranging from 5% to 15% based on steric repulsion, well within the accepted range of biological crowding [1]. However, the key to the success of crowding with macromolecules at relatively low concentrations is that the actual volume exclusion would be far greater as there is a non-linear relationship between macromolecular crowding concentration and excluded volume, which essentially has a magnifying effect due to steric exclusion of like-size molecules [5].

Although the addition of non-reacting molecules to improve RT and PCR is not new, the addition of inert "macromolecules" certainly is. Other studies have either been restricted to small molecules classified as compatible solutes or small molecular size polymers, such as PEG 4 kDa, with limited success. However, neither of which are classical macromolecules, defined by John R. Ellis [1]. In addition, PEG does not fit the description of EVE-causing models typically attributed to macromolecules because it displays hydrophobic interactions with proteins [1]. Their mode of action has been loosely referred to as molecular crowding but in actual fact their effects are due to improved hydration around substrate molecules. This is certainly true for trehalose [17], betaine and proline [18] which are classified as compatible solutes. They build water structures (kosmotropic effect) causing preferential hydration of other molecules like proteins [19]. They are able to stabilize the structures of protein/enzymes even at high temperatures [19,20]. We replicated this effect of trehalose and could show that the macromolecule Fc400 had the same protecting effect on Taq DNA polymerase.

Sensitivity and specificity are particularly crucial for diagnostic applications when the target is in low abundance (e.g. viral load in serum) or poor quality as found in archival sources. In using specific macromolecules as buffer additives we demonstrated dramatic increases in sensitivity up to 10-fold. Of note, the addition of PEG 4 kDa, in the same concentration range as macromolecules, to an optimized PCR assay was actually detrimental to the reaction with regards to sensitivity and yield, which was dose-dependent. Conversely, the addition of macromolecules still improved sensitivity of this assay. We were also able to specifically demonstrate enhanced primer specificity

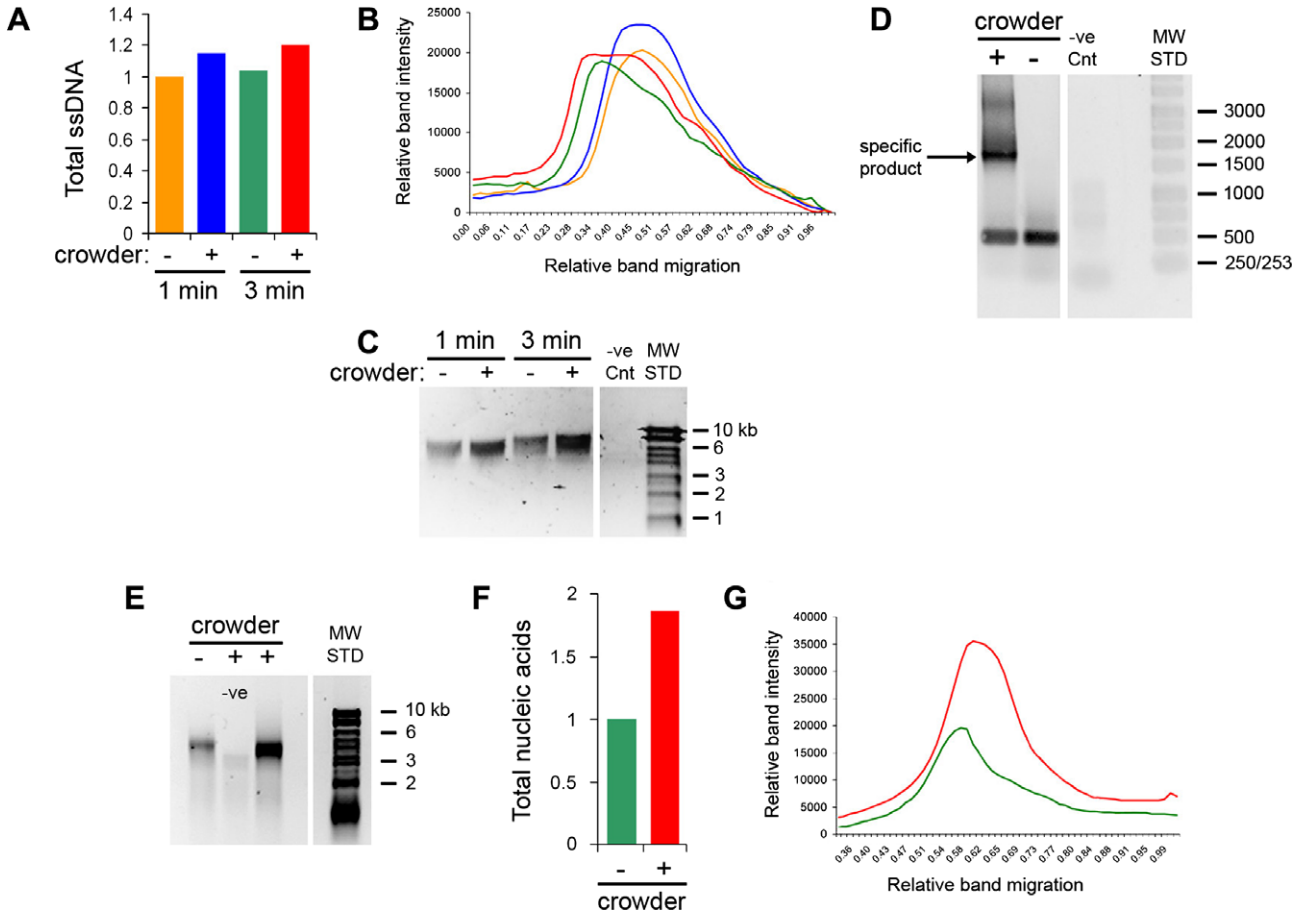

Fig. 3. Macromolecular crowding enhances enzyme processivity. The ssM13 processivity assay for Taq DNA polymerase was performed in the absence and presence of Fc400. (A) Densitometric analysis of the total amount of ssDNA products and (B) their relative migration through a (C) denaturing 0.6% agarose gel. The −ve Cnt was the enzyme-free negative control. (D) An agarose gel of the long M13 PCR products amplified in the absence and presence of macromolecule (mixture of Fc70 15 mg/ml and Fc400 5 mg/ml). One nanogram of ssM13 was used as target and the extension time was limited to 40 s. The −ve Cnt was without template. The arrow indicates the specific target which is 1547 bp. (E) A standard RT reaction was preformed in the absence (green) and presence (red) of Fc70/Fc400 with 500 ng of total RNA and the subsequent reaction products were separated in a denaturing 0.6% agarose gels. (F) Densitometric analysis of total reaction products and (G) their relative migration through (E). "−ve" is the enzyme negative control. Gel images are composites of the respective gels omitting irrelevant sections. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

under crowded conditions, which in turn would result in increased sensitivity. Furthermore, we have shown that macromolecules cause a greater proportion of primer annealing. The usefulness of trehalose in improving sensitivity of PCR has been limited to the case of difficult cDNA templates with GC-rich regions [17]. Its effect is to reduce the melting temperature of these secondary structures. With regards to adding trehalose and betaine to RT reactions, an increase in the sensitivity was detected in the subsequent PCR but only when they were used at very high concentrations [21].

The increase in processivity, defined as greater product amount and length, which we attained with the addition of macromolecules would have been the direct consequence of both increased number of enzyme-nucleic acid initiation events and longer read-through of the enzymes, respectively. This is particularly significant for RT in faithfully generating enough copies of long cDNA molecules and for PCR in amplifying long amplicons. In fact, it has been shown that a range of different molecular weight PEGs and dextrans were able to enhance the integrity and/or stability of the DNA-polymerase complex for *E. coli* T4 DNA polymerase [22,23]. However, they were not able to attain improved processivity. It has been reported that PEG destabilizes enzymes at high temperatures due to the inherent activity of its hydrophobic nature [24]. This may therefore hinder its application to PCR and the reason for the poor performance of PEG in our experiments and may have been responsible for the observed inability to improve processivity [22]. In comparison to an earlier study which used compatible solutes to enhance RT reactions [21], we employed high molecular weight macromolecules and attained an increase of both cDNA product and increased fragment length. However, in contrast to Spiess et al. [21] we achieved increased processivity at 50× lower additive concentrations. At these low mg concentrations viscosity was close to that of water (~1 centipoise) and therefore of no concern [16]. Conversely, the very high concentration

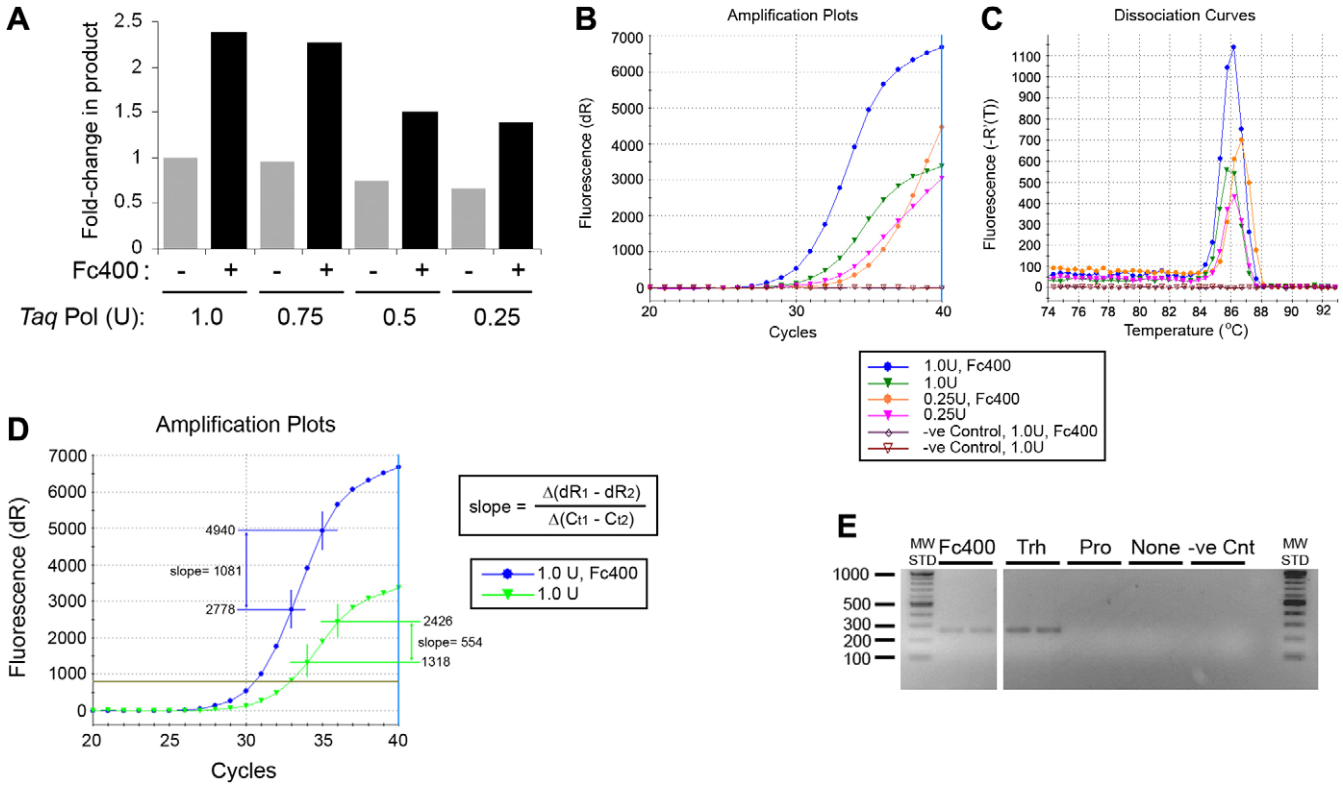

Fig. 4. Macromolecular crowding enhances activity of Taq DNA polymerase and protects it against thermal denaturation. (A) A range of Taq DNA polymerase concentrations (1–0.25 U/reaction) were used to amplify the aP2 product in the absence and presence of Fc400 (2.5 mg/ml). (B) Amplification plots and (C) dissociation curves for the PCR samples performed with 1 and 0.25 U of enzyme are only shown, for display clarity. (D) The slope of the late exponential phase was calculated for the samples amplified with 1 U of enzyme above. (E) Taq DNA polymerase was thermally stressed in the absence (None) and presence of 2.5 mg/ml Fc400, 100 mg/ml trehalose (Trh), or 113 mg/ml proline (Pro) and then the enzyme was used to amplify GAPDH PCR amplicons. Two replicates per treatment are shown on a 2% agarose gel demonstrating the presence of discrete bands of the correct size, 261 bp. The −ve Cnt (control) was without template.

required for compatible solutes to have an appreciable effect resulted in high viscosity to the point that it may have started acting like a "molecular brake" [21] and adversely affect other parameters of the reaction mixture and could possibly interfere with subsequent downstream processing of the products.

We attribute the success of the application of macromolecules to *in vitro* reactions in more closely emulating the intracellular environment of cells such as bacteria whence these enzymes were derived or naturally function in. This was clear from the overall better performance of the Taq DNA polymerase. Under these conditions we were able to reduce the amount of enzyme by 75% and still attained more reaction product due to faster reaction kinetics. We attribute these results to the cumulative molecular and thermodynamic effects of EVE created by macromolecular crowding, that is, lowering the entropy of the reaction and thus increasing the free energy of the reactants. We demonstrate that these gains were a consequence of or combination of enhanced enzyme thermal stability, more primer annealing to its target and greater specificity, and enhanced enzyme-nucleic acid complex formation and stability (i.e. processivity). This improvement did not necessitate the employment of a genetically upgraded DNA polymerase, many of which are currently on the market, but by using low-cost additives. We believe this study comprehensively demonstrates the importance and potential that macromolecular crowding holds for *in vitro* enzymatic settings with far-reaching consequences to the fields of Biochemistry, Molecular Biology and Biotechnology in general.

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