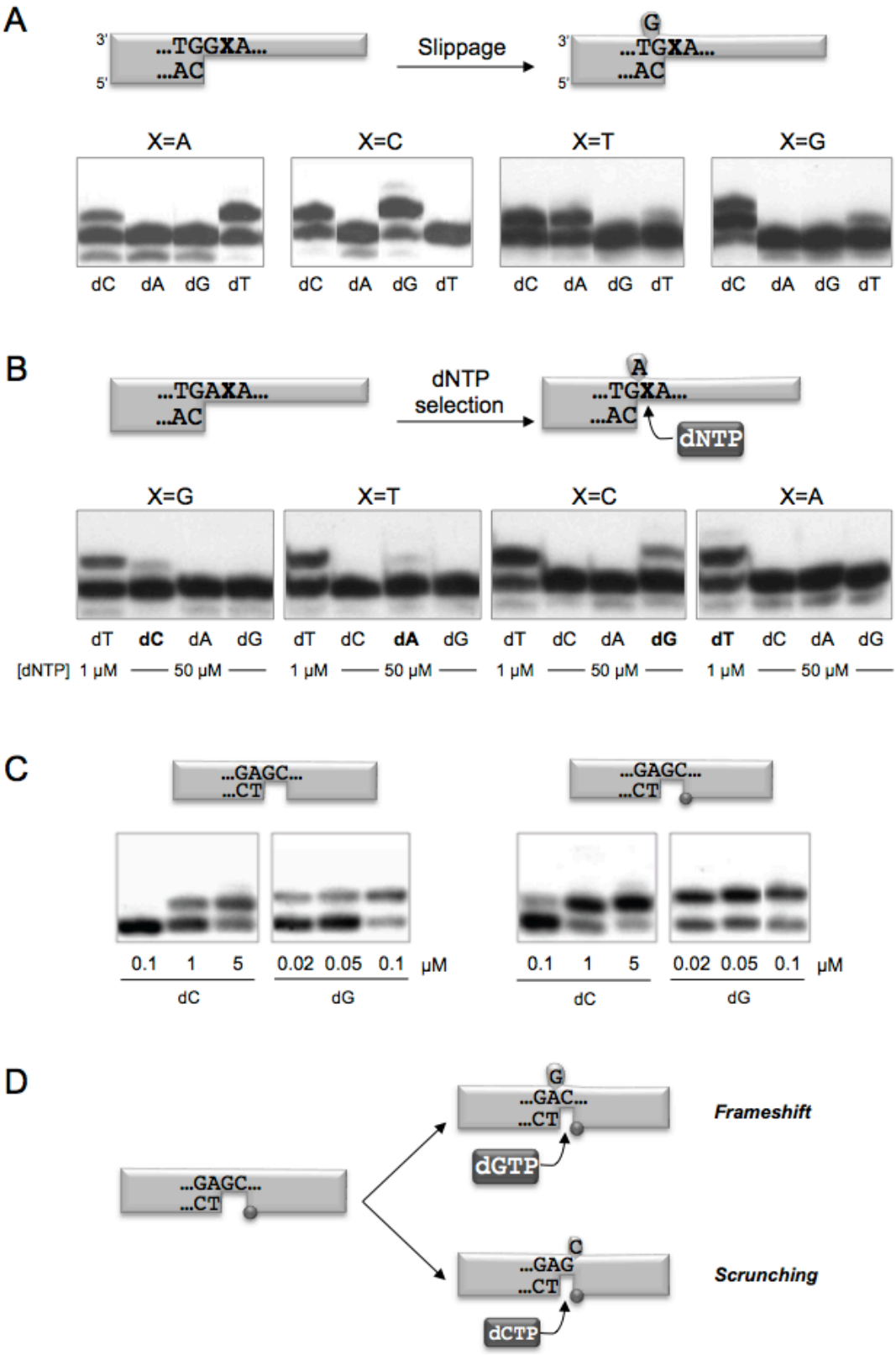
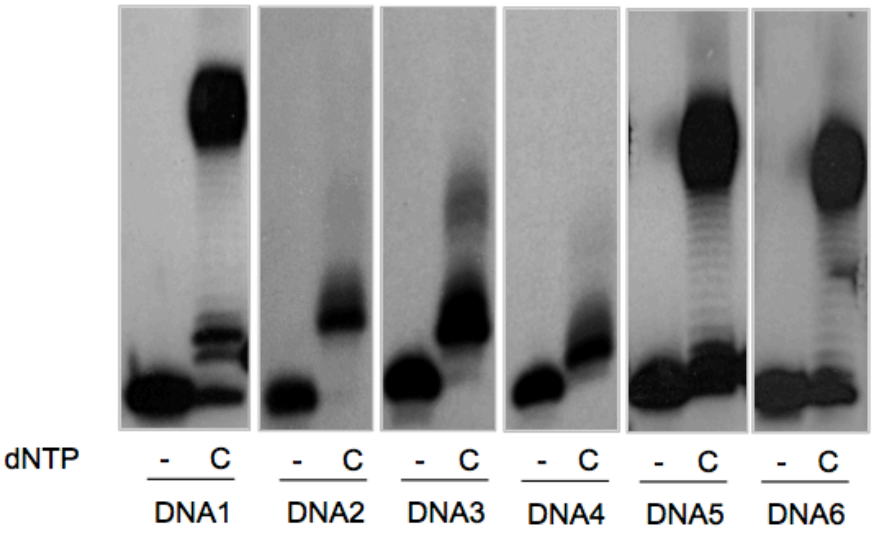
Supplementary Figure 1



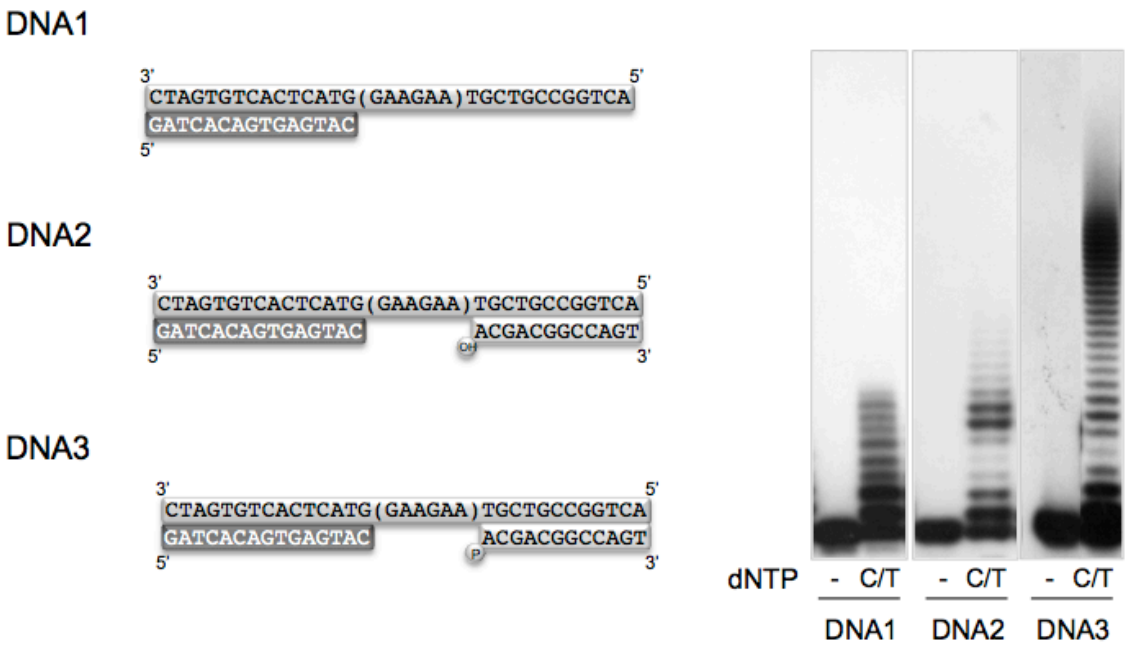
Supplementary Figure 1. Mechanisms of Polμ-mediated dislocation: "primer slippage-mediated" *versus* "dNTP-selection mediated". A) *Slippage-mediated dislocation.* The propensity of Polμ to dislocate the template strand is such that, when presented with a 2 nt gapped substrate in which the first template nucleotide is complementary to the end of the primer, it preferably incorporates the nucleotide complementary to the n+2 position of the template in each case (X in the scheme). This happens because the primer terminus is realigned opposite the n+1 position. In all cases studied, both insertions (in front of the n+1 and n+2 templating bases) can be alternatively detected on this type of substrate, but the insertion produced by dislocation is even more efficient than the incorporation obtained when the template is not relocated. The polymerization reactions were carried out as described in Materials and Methods, in the presence of the indicated DNA substrate, 5 μM dNTP, 2 mM $MgCl_2$ and 100 nM Polμ. Reactions were incubated for 30 min at 37°C and the extension of the primers was analyzed by denaturing gel electrophoresis (20% polyacrylamide/8 M urea) and subsequent autoradiography. **B)** *dNTP-mediated selection dislocation.* In order to observe Polμ-mediated dislocation, it is not strictly necessary that the n and n +1 bases are identical. There is a dislocation model stabilized by the incoming dNTP, whereby a misaligned intermediate (with a non-repetitive nucleotide-containing template strand) could be stabilized by stacking interactions with the dNTP, or with certain residues of the polymerase at the ternary complex level. In this case, the n and n +1 bases of the template strand are different. The X in position n+2 was changed to each of the four bases to provide all possible template options. Direction by the n+2 base (X) would occur if the template "dA" in position n+1 could be relocated outside of the enzyme active site without blocking its ability to polymerize. Thus, an incoming nucleotide complementary to the X position (n+2) of the template would be responsible for stabilizing the dislocation of the "A". As shown in the experiment, although the major incorporation corresponds to the insertion of dT (complementary to the n+1 position of the template) there is also a minor insertion of each of the nucleotides complementary to the X position. That indicates that Polμ is able to achieve this mechanism of "dNTP-selection mediated" dislocation, although not as efficiently as in the case of "slippage-mediated" dislocation. Polymerization reactions were performed as in A). **C)** *Increased efficiency of dislocation by Polμ in gapped substrates.* The efficiency of the "dNTP-selection mediated" dislocation performed by Polμ on a template-primer substrate, can increase sharply to be comparable or exceed the "correct" dNTP entry in some sequence contexts, and especially when the substrate is a small gap. Polymerization reactions were carried out as in A. The figure shows the incorporation of both the nucleotide complementary to the position n+1(dG) as well as that complementary to position n+2(dC), on a 2 nt-gap in which the n(dA) and n+1(dG) templating bases are different. In this case, dG is preferentially inserted as a result of "dNTP selection-mediated" dislocation (see scheme in D), at the various range of nucleotide concentrations used. From this result we can conclude that the "dNTP selection-mediated" dislocation capacity of Polμ is strongly stimulated in the presence of a gap, which emphasizes the importance of a stable binary complex for this reaction to occur. D) Scheme of the two possible outcomes when filling a 2nt-gap by human Polμ: 1) a dNTP selection-mediated distortion, leading to a -1 frameshift; 2) correct copying of the first templating base, via "scrunching" (*Garcia-Diaz, M, Bebenek, K, Larrea, A.A., Havener, J.M., Perera, L., Krahn, JM, Pedersen, L.C., Ramsden, D.A., Kunkel, TA (2008) Template strand scrunching during DNA gap repair synthesis. Nat. Struct. Mol. Biol. 9, 967-972).*

Supplementary Figure 2



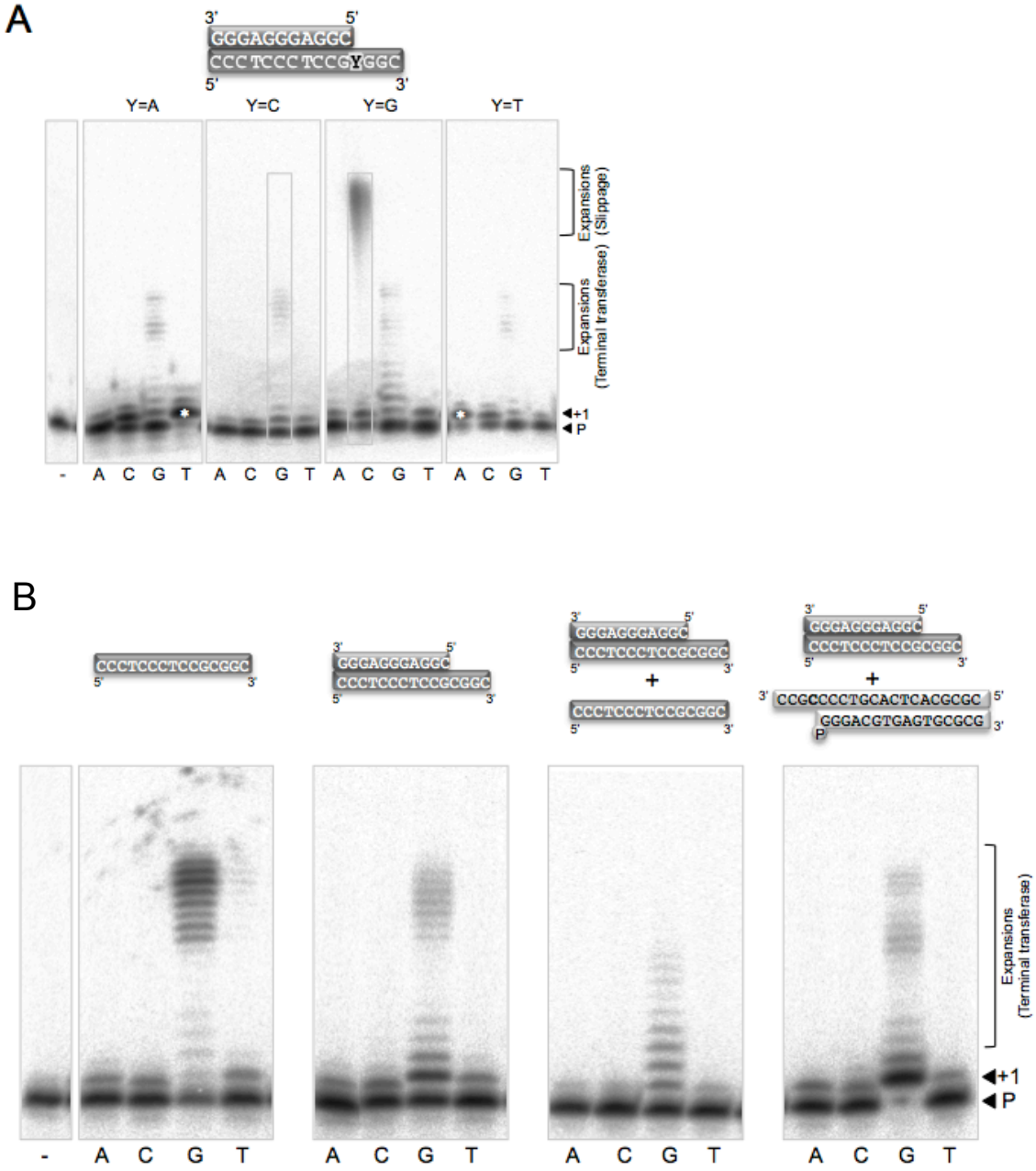
Supplementary Figure 2. Origin of the distortion: polymerase-mediated? The importance of an initial dislocation was analyzed in DNA substrates (progressively short gaps) with different primers that produce: a fully matched primer preceding a 3, 2 or 1 nt gap (DNA1, DNA2, DNA3) or a nick (DNA4), a 1 nt gap with a mismatch at the -1 position (DNA5), or a nicked substrate with a mismatch at the -3 position (DNA6). DNA1 contains the templating sequence 3'-CGG as a positive control of expansion. As shown in the autoradiogram, by providing only dCTP, Polμ was able to produce expansion on the DNA1, DNA5 and DNA6 substrates, confirming that the mechanism that produces these expansions is triggered by the presence of a pre-existing distortion upstream to the polymerization site. Polymerization reactions (described in Materials and Methods) were performed in the presence of 4 nM of the indicated DNA substrate in each case, 2 mM $MgCl_2$, 270 nM Polμ and 50 μM dCTP. After 1 h of incubation at 30ºC, polymerization products were analyzed by electrophoresis on 20% polyacrylamide / 8 M urea gels and autoradiography.

Supplementary Figure 3



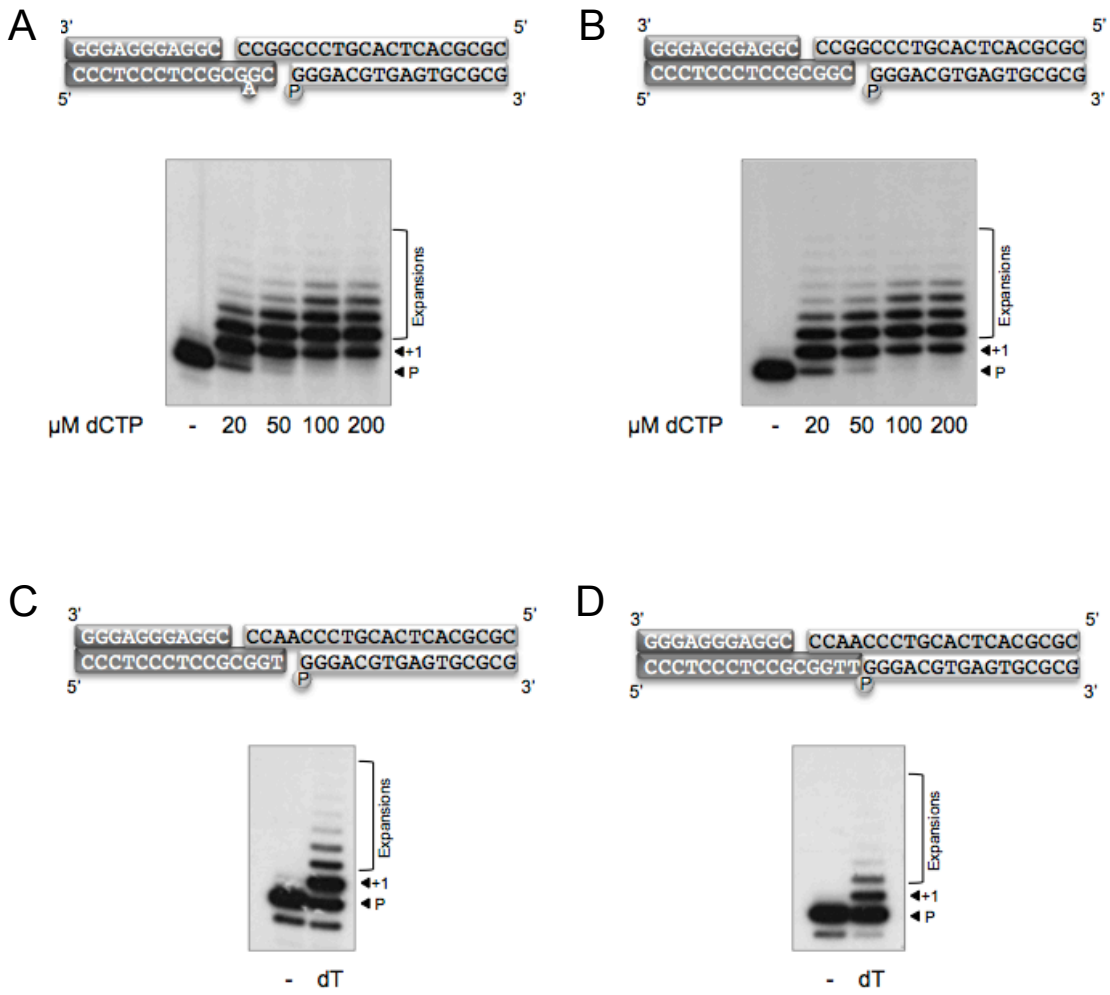
Supplementary Figure 3. Importance of the 5'P group. Polμ-driven nucleotide expansion depends on the presence of the downstream oligo at the 3' end of the gap, containing a 5' phosphate group. The schemes correspond to the different substrates used (DNA1: template/primer; DNA2: gap lacking 5'-P; DNA3: gap bearing 5'-P group). Polymerization reactions (described in Materials and Methods) were performed in the presence of 4 nM of the indicated DNA substrate in each case, 2,5 mM $MgCl_2$, 270 nM Polμ and 50 μM dNTP. After 1 h of incubation at 30°C, polymerization products were analyzed by electrophoresis on 20% polyacrylamide / 8 M urea gels and autoradiography.

Supplementary Figure 4



Supplementary Figure 4. Sequence expansions versus terminal transferase additions during NHEJ. A) The reiterative addition of dGTP catalyzed by Polμ in the studied sequence contexts (see figure 5) is due to pure terminal transferase activity, since control experiments in which the template-providing end is not present, also showed this outcome (second panel, Y=C, and also other sequence contexts such as Y=A and Y=T). We can also clearly differentiate between the terminal transferase additions obtained in each case with dGTP and the template-directed sequence expansions, which are slippage-mediated and thus only occur when the template contains a homo-dinucleotide (third panel, Y=G). The substrates used here are formed by hybridization of oligonucleotides D3YA (5´-CCCTCCCTCCGAGGC), D3YC (5´-CCCTCCCTCCGCGGC), D3YG (5´-CCCTCCCTCCGGGGC) or D3YT (5´-CCCTCCCTCCGTGGC) and D1. **B)** To help differentiate between pure terminal transferase additions and templated reiterative incorporations we used a new set of substrates: a labelled single-stranded heteropolymeric substrate (D3), a labelled double-stranded 3'-protruding substrate (D3 hybridized to D1), this same dsDNA together with the cold versions of the ssDNA and the original bipartite NHEJ substrate (labelled D3 hybridized to D1 and cold D4 hybridized to D2). Reaction on the ssDNA substrate resulted in the most efficient generation of ladder products. This outcome is greatly inhibited by the presence of a hybridized strand and further by the presence of cold ssDNA, suggesting that these products are the result of pure terminal transferase additions. On the other hand, the strongest +1 reaction was observed in the last case, indicating that this reaction is preferentially template directed, In all cases, polymerization reactions were performed in the presence of 200 nM Polμ. When indicated, dNTPs were added separately at 100 μM in the presence of 2.5 mM $MgCl_2$. After incubation for 1 h at 30ºC, reactions were stopped and loaded on 20% PA-8M urea gels. Labeled DNA fragments were detected by autoradiography.

Supplementary Figure 5



Supplementary Figure 5. Sequence expansions catalyzed by Polμ during NHEJ. A&B) The amount of nucleotide required by Polμ to produce nucleotide expansions during NHEJ is very low (20 μM), indicating that this process is highly efficient. Polymerization reactions were performed in the presence of the indicated range of concentrations of dCTP, and using the following substrates: the labelled substrate (dark gray) is formed by hybridization of oligonucleotides D3BB2 and D1 (C) or D3 and D1 (D), while the cold substrate (light gray) is formed by hybridization of D4 (X=G) and D2. **C&D)** Nucleotide expansions occurring during NHEJ were also generated when the template dinucleotide closer to the 5´-P was formed by a pair of adenines, either forming a 1 nt gap, or even forming a nick neighbour to the 5´-P, indicating that this mechanism is very robust and independent of the sequence context. Polymerization reactions were performed in the presence of 100 μM dTTP, and with the following substrates: the labelled substrate (dark gray) is formed by hybridization of oligonucleotides D3+T and D1 (E) or D3+TT and D1 (F), the cold substrate (light gray) is formed by hybridization of D4AA and D2. In all cases, polymerization reactions were performed in the presence of 200 nM Polμ. When indicated, dNTPs were added separately at the indicated amounts in the presence of 2.5 mM $MgCl_2$. After incubation for 1 h at 30ºC, reactions were stopped and loaded on 20% PA-8M urea gels. Labeled DNA fragments were detected by autoradiography.