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*Review*

# Effect of different divalent cations on the kinetics and fidelity of DNA polymerases

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**Abstract:** DNA polymerases (DNA pols) are essential for accurately copying genomes of all organisms. The polymerase and exonuclease activities associated with DNA pols require the presence of two divalent cations which occupy the A and B metal ion sites. Even though the two-metal ion mechanism is generally applicable for all DNA pols, a third metal ion was proposed to be essential for phosphoryl transfer reaction. The metal ion in the A site is coordinated by six ligands including the 3′ hydroxyl group of the primer, the carboxylates of two aspartic acid residues, as well as water molecules and the metal ion exhibits a distorted octahedral geometry. This metal ion plays a crucial role in lowering the p$K_a$ of the 3′ hydroxyl group of the primer increasing its nucleophilicity for attack on α phosphorous atom of the incoming dNTP. The metal ion occupying the B site stabilizes the transition state and is coordinated to the non-bridging oxygen atoms of the incoming dNTP and carboxylates of aspartic acid residues along with carboxyl oxygen of an adjacent peptide bond. In a similar fashion, two divalent cations are required for the 3′–5′ exonuclease activity of DNA pols. Analogous to their role in the polymerase active site, one divalent cation lowers the p$K_a$ of the water molecule making it a more potent nucleophile and the other cation helps to stabilize the transition state, assisting in excision of the 3′ terminal nucleotide. These divalent cations affect the various fidelity checkpoints along minimal kinetic scheme for DNA pols. The effect of different divalent cations on various steps in the kinetic scheme, including their influence on ground-state binding affinity, base selectivity, efficiency of extension past a mismatch and the exonuclease activity are discussed. We have also attempted to explain why only certain divalent cations act as cofactors for various DNA pols based on their properties including ionic radii, coordination geometry, and their ability to lower the p$K_a$ of the 3′ hydroxyl group of primer strand.
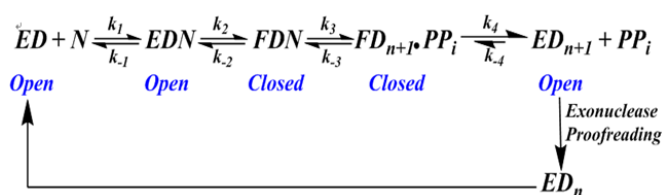
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**Abbreviations:** AMV: Avian myeloblastosis virus; 2AP: 2-aminopurine; BST: *Bacillus stearothermophilus*; dNTP: deoxynucleoside triphosphate; Dpo4: DNA polymerase IV from *Sulfolobus solfataricus*; exo⁻: DNA polymerase lacking the exonuclease proofreading activity; exo⁺: DNA polymerase exhibiting both the polymerase and exonuclease activities; $k_{obs}$: observed rate constant; $k_{pol}$: maximum rate of dNMP incorporation; $K_{d,app}$: apparent equilibrium dissociation constant for [dNTP] that supports the half-maximal rate of dNMP incorporation; $K_{d,g}$: ground-state equilibrium dissociation constant for an incoming dNTP from a DNA pol:DNA:dNTP ternary complex; pol: polymerase; RB69pol: bacteriophage RB69 DNA polymerase; tm: triple mutant of RB69 pol containing L561A, S565G, and Y567A mutations in the active site

## 1. Introduction

DNA polymerases are responsible for faithfully copying the genome during replication and repair [1–5]. DNA pols require two divalent metal ions $Mg^{2+}$ or $Mn^{2+}$ for primer extension and for removing the misincorporated dNTPs via the 3′–5′ exonuclease activity associated with certain DNA pols [6–10]. A two metal ion mechanism is used by all DNA pols to catalyze nucleotide addition to a growing primer strand [11]. Although DNA polymerases employ the physiologically relevant $Mg^{2+}$, other divalent metal ions can substitute for $Mg^{2+}$ though they tend to reduce the fidelity of DNA replication [12–15]. The effect of metal ion cofactors on the fidelity of DNA replication has been studied for various DNA pols including *E. coli* DNA pol I [16], AMV DNA pol [17], Klenow fragment of *E. coli* DNA pol I [18], T4 pol [12], T7 pol [12], human pol α [12], pol β [12] and Dpo4 [13]. Some metal ions have been shown to be mutagens and carcinogens probably because they reduce the base selectivity of DNA pols [12,13,16–20]. Different divalent cations influence fidelity check points in the minimal kinetic scheme for the nucleotidyl transfer reaction (Scheme 1). Cations that can substitute for $Mg^{2+}$ affect DNA pols by: 1) altering the ground-state binding affinity of incoming dNTPs to pol:DNA binary complexes [21]; 2) decreasing base selectivity by promoting misincorporation during primer-extension [13]; 3) decreasing the rate of base excision [22]; 4) altering primer-extension past a mismatch at the primer-template (P/T) terminus [22]. This review will address the way in which various metal ions increase misincorporation based on their physical properties. Our emphasis will be on the effect of different cations on the behavior of DNA pols [12,13,16–18,23]. Previous reviews have summarized the effect of different divalent cations on the structure and function of pol β [24–26].

$$\ddot{E}D + N \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EDN \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} FDN \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} FD_{n+1} \bullet PP_i \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} ED_{n+1} + PP_i$$

*Open*      *Open*      *Closed*      *Closed*      *Open*

Exonuclease Proofreading → $ED_n$

**Scheme 1.** Minimal kinetic scheme for DNA polymerases depicting various fidelity checkpoints along the reaction pathway. Additional details are provided in text.

## 2.   The two metal ion mechanism for nucleotidyl transfer reaction

All DNA pols known to date have a basic requirement of two divalent metal ions in order to catalyze primer extension [11]. The metal ion present in the "A" site assists in lowering the p$K_a$ of the terminal 3′-OH group on the primer and coordinates both 3′-OH of the primer strand and the α-phosphate of the incoming dNTP which facilitates its nucleophilic attack on the incoming dNTP's α-phosphorous atom [11]. The metal ion, occupying the "B" site, coordinates the α-, β-, and γ- non-bridging phosphate oxygens of the incoming dNTP, helping to neutralize the developing negative charge as the ternary complex approaches the transition state in the nucleotidyl transfer reaction, and assists in the departure of the PP$_i$ product. Yang et al. [27] have shown that for pol β, both A and B metal ions are required to prepare the active site for nucleotidyl transfer and formation of binary dNTP/metal ion complex in the "B" site is unable to induce fingers closing, a necessary step for the phosphodiester bond formation to take place. They also showed that release of the catalytic metal ion triggers the opening of the fingers. Bakhtina et al. [28] have replaced Mg:dNTP binary complex with Rh(III)dNTP (an exchange-inert complex) and selectively studied the effect of filling the "A" site with Mg. Their results showed that for pol β, fingers closing could take place even in the absence of the "A" metal ion but the "A" metal ion is readily able to diffuse into the A site even with fingers in the closed state [28]. In contrast to the results obtained with pol β, similar studies carried out on RB69pol showed that, analogous to pol β, the fingers can close in the absence of an "A" metal ion but unlike pol β the metal ion A is unable to diffuse into the "A" site [21]. The fingers must reopen before the A metal ion can diffuse into the "A" site. Moreover, catalysis can take place only upon the binding of both A and B metal ions. Nakamura et al. [29] used time-resolved X-ray crystallography with a ternary complex containing human pol η, DNA, and dATP and observed that once the metal ions in the A and B site are bound, the 3′-OH group and the α-phosphate of the incoming dNTP get aligned for catalysis followed by the formation of a new bond. Interestingly, these authors showed that a third $Mg^{2+}$ ion appears after the nucleotidyl transfer reaction was initiated with $Mg^{2+}$ but before release of the products [29]. Yang et al. proposed that the third $Mg^{2+}$ ion plays a crucial role in stabilizing the transition state by neutralizing the negative charges built up in the transition state and is likely involved in facilitating the protonation of the pyrophosphate leaving group [29]. The third metal ion was shown to be coordinated to four water molecules in addition to a bridging oxygen atom between the α- and β-phosphorous atoms and to the non-bridging oxygen of the α-phosphate [30]. Freudenthal et al. [31] reported similar results with pol β whereby they confirmed the presence of a third metal ion in the "C site" and showed that this presence is short lived. Recent studies by Gao et al. have clearly showed the occupancy of the C site with a $Mn^{2+}$ ion and that the third metal ion was proposed to be required for catalysis by pol η [30].

## 3.   The nature of metal ion coordination complexes with various DNA pols

A number of crystal structures have been solved with DNA pols bound to the incoming correct or incorrect dNTPs as well as the DNA substrate and different metal ions [11,14,30,32–34]. Xia et al. obtained the structure of the ternary complex of RB69pol in the presence of $Mn^{2+}$ using dUpNpp which is a non-hydrolyzable dNTP analogue using the triple mutant (tm) variant (L561A/S565G/Y567A) [11]. The ternary complex structure showed that $Mn^{2+}$ bound in both the "A" and "B" sites has octahedral geometry (Figure 1). Even though $Mn^{2+}$ is in octahedral geometry in both sites, $Mn^{2+}$ present in the "B"

site is present in perfect octahedral geometry and is coordinated by the carboxylate side-chains of D411 and D623, and the backbone carbonyl oxygen of L412 along with the triphosphate tail of the incoming dUpNpp. On the other hand, $Mn^{2+}$ bound in the "A" site, is present in a highly distorted octahedral geometry as $Mn^{2+}$ in this structure is coordinated with the 3′-OH group of the primer, the oxygen of the incoming dNTP's α-phosphate and the two carboxylate side-chains of D411 and D623. In this structure, the distance between the Pα of the incoming dUpNpp and the 3′-OH group is too large to allow phosphodiester bond formation [11]. These authors proposed that as the reaction proceeds to the transition state, $Mn^{2+}$ present in the "A" site helps to reduce the distance between the 3′-OH and the Pα of the incoming dNTP, allowing bond formation to occur [11]. In the same study, Xia et al. showed that when $Mn^{2+}$ was replaced with $Mg^{2+}$, $Mg^{2+}$ present in the "B" site was present in perfect octahedral while the $Mg^{2+}$ occupying the "A" site was present in a distorted octahedral geometry, results were very similar to those obtained with $Mn^{2+}$ [11].
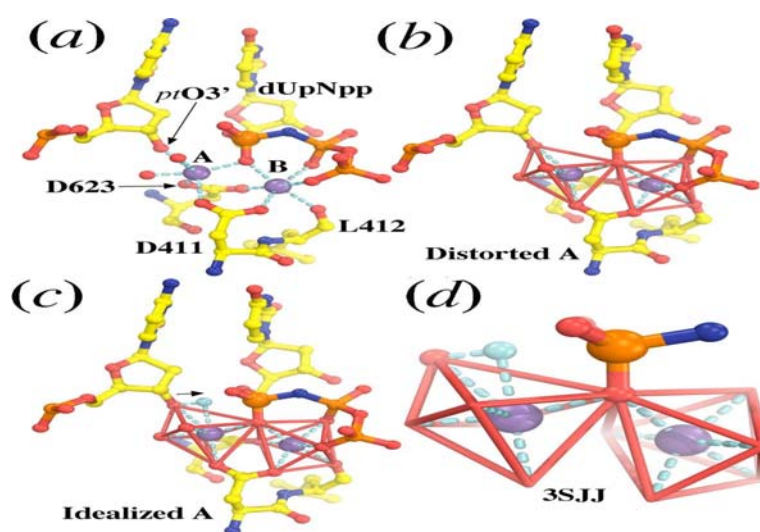


**Figure 1.** The structure of the (L561A/S565G/Y567A) tm RB69pol ternary complex (PDB accession 3SJJ). (a) Ternary complex showing dUpNpp bound in the active site with metal ions A and B. (b) Closeup of the tm RB69 pol active site showing the B metal ion in perfect octahedral geometry and the A metal ion in a distorted octahedral geometry. (c) Predicted position of 3′-OH group in an idealized octahedron during the transition state. (d) A close-up of the coordination complex showing the two metal ions, the 3′-OH group, and the alpha phosphate of the incoming dNTP.

Johnson et al. have determined the crystal structures of mismatched base-pairs with BST pol (an A family pol) in the presence of $Mg^{2+}$ and $Mn^{2+}$ [35]. Besides BST pol, the structures of all 12 mispairs have also been reported for RB69pol (a B family pol) in the presence of $Mg^{2+}$ [32]. These authors used a quadruple mutant (L415A/L561A/S565G/Y567A) to obtain the ternary complexes containing all 12 mispairs. In addition to RB69pol [11,36], T7 pol [37,38], Klenow fragment [39–41], and Dpo4 [13,15,42], pol β has also been extensively studied [14,24–26,28]. In summary, structural studies using various DNA pols shows that all DNA pols from different families have the same coordination geometry of the A and B metal ions in the active site.

## 4. DNA Polymerases can use different metal ions for catalyzing phosphoryl transfer

Sirover et al. [20,43] screened a number of metal ions for their ability to act as cofactors for AMV DNA pol lacking the exonuclease activity. Following this, they also studied the effect of these metal ions on single base substitutions directly measuring how these metal ions affect the fidelity of AMV DNA pol. They utilized poly(A).oligo(dT) as the DNA substrate for their assays and found that of all the metal ions tested, eight metal ions including $Ag^+$, $Be^{2+}$, $Cd^{2+}$, $Co^{2+}$, $Cr^{3+}$, $Mn^{2+}$, $Ni^{2+}$, and $Pb^{2+}$ decreased the fidelity of AMV DNA pol by 30% or more [20]. These metal ions were identified as being mutagenic or carcinogenic [20]. Subsequent studies carried out by these authors showed that $Mn^{2+}$ and $Co^{2+}$ could activate *E. coli* DNA pol I [16,44]. Despite their ability to act as cofactors, these divalent cations enhanced misincorporation. For example, the efficiency of incorporation of dGMP opposite poly[d(A-T)] DNA substrate was invariant at various $[Mg^{2+}]$ while the efficiency was 2–3 fold higher at all concentrations of $Co^{2+}$. When $Mg^{2+}$ was replaced with $Mn^{2+}$, the efficiency was 3–5 fold higher suggesting that $Mn^{2+}$ readily permits misincorporation. Further studies by Seal et al. on human pol α and pol β [45] showed that both these pols can be activated by $Mn^{2+}$, and $Co^{2+}$ but at activating concentrations, these divalent cations enhance the frequency of misincorporation. Interestingly, at higher concentrations these metal ions actually inhibit the incorporation of the complementary nucleotide further affecting the fidelity of these pols. Later studies by Snow et al. showed that $Ni^{2+}$ activates several different pols including T4 pol, Klenow fragment, AMV pol, human pol α, and T7 pol [12]. In the same study, they found that the base selectivity of different pols could be affected to different extents in the presence of $Ni^{2+}$ [12].

Pelletier et al. [14] have crystallized pol β in the presence of blunt-end DNA, incoming dNTP and various metal ions. The structures of these ternary complexes in the presence of several different metal ions showed primer extension in crystals in the presence of $Mn^{2+}$, $Cd^{2+}$, and $Zn^{2+}$ suggesting that these divalent cations were able to activate pol β. Egli et al. [42] carried out detailed steady-state kinetic studies using Dpo4 pol and tested a number of divalent cations and found that apart from $Mg^{2+}$, only $Mn^{2+}$ and $Ca^{2+}$ could support primer extension with Dpo4, other divalent cations tested including $Sr^{2+}$, $Ba^{2+}$, $Zn^{2+}$, $Cu^{2+}$, $Ni^{2+}$, and $Co^{2+}$ failed to show any extension products. Later studies by Vashishtha et al. tested the competence of several divalent cations to determine their ability to activate RB69 pol [36]. These studies showed that, apart from $Mg^{2+}$ and $Mn^{2+}$, $Co^{2+}$ and to a lesser extent $Ni^{2+}$, were the only divalent cations which could support both pol and exo activities [36]. Recent work by Vashishtha et al. on BST pol showed that $Mn^{2+}$, $Co^{2+}$ and $Cd^{2+}$ could replace $Mg^{2+}$ in supporting the polymerase activity [46]. These studies showed that BST pol and pol β [14] are the only exceptions among all DNA pols which have the ability to utilize $Cd^{2+}$. The metal ion preferences for different DNA pols are summarized in Table 1. From these studies it can be concluded that DNA pols from different families have the ability to utilize different metal ions for catalysis, but they do not follow a pattern that can be predicted from their physical properties.

**Table 1.** Summary of metal ion preferences of different DNA polymerases.

| DNA Polymerase (Pol Family) | Metal Ion | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | $Mn^{2+}$ | $Co^{2+}$ | $Fe^{2+}$ | $Ni^{2+}$ | $Zn^{2+}$ | $Cd^{2+}$ | $Sr^{2+}$ | $Ba^{2+}$ | $Cu^{2+}$ | $Cr^{3+}$ | $Ca^{2+}$ |
| DNA pol I (A) | + | + | - | + | - | - | - | - | - | - | - |
| Human pol α (B) | + | + | - | - | +[d] | - | - | - | - | - | - |
| pol β (X) | + | +[b] | - | - | + | + | - | - | - | - | - |
| AMV DNA pol (RT) | + | + | - | + | - | - | - | - | - | - | - |
| T4 pol (B) | + | + | - | + | - | - | - | - | - | - | - |
| T7 pol (A) | + | + | - | + | - | - | - | - | - | - | - |
| RB69pol (B) | + | + | - | + | - | - | - | - | - | - | - |
| BST pol (A) | + | +[c] | - | +[c] | -[c] | +[c] | - | - | - | -[c] | - |
| Dpo4 pol (Y) | + | +[b] | - | - | - | - | - | - | - | - | + |

[a] Metal ion which activate the respective DNA polymerase are shown with + and those that are not able to support the polymerase activity are shown with -.

[b] Studies by Pelletier et al. and Egli et al. previously claimed that human pol β and Dpo4 were not able to utilize $Co^{2+}$ as cofactor however, recent studies by Vashishtha et al. showed that both pol β and Dpo4 can catalyze primer extension in the presence of $Co^{2+}$ as explained in the text.

[c] Vashishtha et al. [46]

[d] Zhang et al. [64]

## 5.   Metal ions affect various fidelity checkpoints during DNA replication

The various steps involved in the phosphoryl transfer reaction involve binding of DNA pol to DNA to form the binary ED complex followed by addition of the incoming dNTP (correct or incorrect) to give the open ternary EDN complex (fingers open). This open complex then undergoes conformational changes to give the FDN closed complex (fingers closed) followed by the phosphoryl transfer reaction resulting in products $FD_{n+1}$ and $PP_i$. In this minimal kinetic scheme (Scheme 1), there are various fidelity checkpoints (steps in the minimal kinetic scheme) which the DNA pols employ to ensure accurate copying of template DNA minimizing mutations in the resulting DNA. These fidelity checkpoints include: 1) the conformational change of polymerase from open to a closed conformation when a correct incoming nucleotide is detected in the nascent base-pair binding pocket. 2) The chemistry step involving the formation of the phosphodiester bond which is much faster for a correct incoming nucleotide as compared to an incorrect incoming nucleotide and 3) removal of the incorrect dNMP incorporated in the growing primer strand via the exonuclease activity. Divalent metal ions can potentially affect these fidelity checkpoints and alter the fidelity of DNA replication by: 1) altering the ground-state binding affinity of incoming dNTPs (correct and incorrect) to DNA pol/P/T binary complexes [21]; 2) enhancing misincorporation by increasing the rate of incorporation of incorrect incoming dNTPs during primer-extension [13]; and 3) affecting the exonuclease activity [22]. In following sections, we will discuss the effect of divalent cations on these fidelity checkpoints.

## 6.   The effect of metal ions on ground-state binding affinity of incoming dNTPs for pol:P/T binary complexes

Scheme 2 shows the various steps along the reaction pathway for the nucleotidyl transfer

reaction. $K_{d,g}$ indicates the ground-state binding affinity involving all the steps up to the formation of the closed FDN complex. A lower $K_{d,g}$ value indicates higher binding affinity of the incoming dNTP towards the ED binary complex and *vice versa*. Typically, the $K_{d,g}$ values are higher for an incoming incorrect dNTP as compared to the correct incoming dNTP in the presence of a given divalent cation [47]. Different divalent cations can potentially affect the $K_{d,g}$ values for incoming dNTPs (correct or incorrect). Zhang et al. measured the $K_{d,g}$ for dTTP binding opposite 2AP (which represents a correct nucleotide binding event) as the templating base (2AP at the n position) using a dideoxy-terminated P/T with RB69pol and they reported a $K_{d,g}$ value of 9 µM in the presence of $Mg^{2+}$ [47]. The basis of this equilibrium binding assay was the fact that 2AP in the ED binary complex exists in the unstacked form (high fluorescence state) and during the equilibrium fluorescence titration involving the formation of the EDN complex, 2AP becomes stacked resulting in quenching of the fluorescence signal [48,49]. The quenching of 2AP fluorescence is a function of [dNTP] and follows a hyperbolic function [48]. When 2AP was shifted to the n + 1 position, the fluorescence signal did not change during the titration of dCTP opposite dG as the templating base [47], consequently the $K_{d,g}$ for binding of the incoming dNTPs could not be determined. Wang et al. [21] also carried out a similar study using RB69pol in the presence of $Ca^{2+}$ with ddP/T containing 2AP at the n position and reported a $K_{d,g}$ value of 53 nM for dTTP (correct). In contrast, the $K_{d,g}$ value for the incorrect incoming dCTP was found to be 53 µM, representing a difference of 1000-fold in the ground-state binding affinities of the correct and incorrect dNTPs. These data suggest that binding of the incoming dNTP in the ground-state acts as a crucial fidelity checkpoint. Similar studies on T4 pol carried out by Hariharan et al. [48] in the presence of $Mg^{2+}$ showed that the $K_{d,g}$ value for dTTP binding opposite 2AP was 31 µM, very similar to that reported for RB69pol [21]. Vashishtha et al. [36] carried out detailed studies deciphering the effect of various divalent cations on ground-state binding affinity for incoming dNTPs. They reported the $K_{d,g}$ values in the presence of different divalent cations which were shown to activate RB69pol including $Co^{2+}$, and $Mn^{2+}$, apart from $Mg^{2+}$. $K_{d,g}$ values were also reported in the presence of $Ca^{2+}$. Their data clearly showed that the identity of the divalent cation has a dramatic effect on the $K_{d,g}$ values of correct and incorrect incoming dNTPs. The order of binding affinities for incoming dTTP opposite 2AP observed with different cations was: $Ca^{2+} > Mn^{2+} > Co^{2+} > Mg^{2+}$ suggesting that the binding affinity was highest in the presence of $Ca^{2+}$ and lowest in the presence of $Mg^{2+}$. The $K_{d,g}$ values showed an identical pattern when dTTP was replaced with dCTP as the incorrect incoming dNTP.

$$ED + N \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EDN \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} FDN \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} FD_{n+1} \bullet PP_i$$

**Scheme 2.** Minimal Kinetic scheme for DNA polymerases depicting the ground-state binding affinity ($K_{d,g}$) and apparent binding affinity ($K_{d,app}$) for an incoming dNTP. EDN represents the open conformation of the ternary collision complex whereas FDN represents the closed conformation.

In a recent study by Vashishtha et al. [46] where they measured the $K_{d,g}$ values in the presence of different divalent cations with BST pol, showed contrasting behavior as compared to their previous study carried out using RB69pol [36]. For example, with RB69pol, the $K_{d,g}$ value for dTTP binding opposite 2AP was 5-fold lower as compared to $Mg^{2+}$ but with BST pol, this value was 3-fold higher as compared to $Mg^{2+}$ suggesting that with RB69pol dTTP binds much more tightly in the presence of $Co^{2+}$ as compared to $Mg^{2+}$ but surprisingly this trend is reversed with BST pol. When dTTP was replaced with dCTP (incorrect incoming nucleotide), $K_{d,g}$ values were enhanced by 1400-fold in the presence of $Mg^{2+}$, a behavior very similar to RB69pol but the difference in the $K_{d,g}$ values for the correct and incorrect incoming dNTPs is much more pronounced with BST pol as compared to RB69pol (a difference of 1400-fold with BST pol compared to 40-fold for RB69pol) suggesting that in the presence of $Mg^{2+}$, BST pol possesses a higher discrimination factor between the correct and incorrect incoming dNTPs as compared to RB69pol. Interestingly, the $K_{d,g}$ value in the presence of $Cd^{2+}$ was in between that of $Mg^{2+}$ and $Mn^{2+}$ [46]. These authors observed a very different behavior with $Co^{2+}$ and $Mn^{2+}$ where the $K_{d,g}$ values for the correct and incorrect incoming dNTPs differed by only 8-fold and 3-fold with $Co^{2+}$ and $Mn^{2+}$ respectively. Overall, the $K_{d,g}$ values obtained with BST pol showed a varied pattern as compared to similar values with RB69pol except with $Mn^{2+}$ where the $K_{d,g}$ values were substantially lower than $Mg^{2+}$ for both pols. The variation in trends observed with these pols can be rationalized based on the fact that BST pol belongs to the A-family of DNA pols while RB69pol belongs to the B-family of DNA pols and the sequence diversity of these two DNA pols could be accountable for the difference in their ground-state binding affinities.

## 7. Effect of metal ions on base selectivity

Studies in the past have shown that different divalent cations have a profound effect on the base selectivity of various pols and this effect varies with the nature of the pol as well as the identity of the divalent cation [18,19,50–53]. In general, when $Mg^{2+}$ is substituted by $Mn^{2+}$ the fidelity of DNA replication decreases, and this has been shown to be true for numerous DNA pols including T4 pol [19,54], T7 pol [37], *E. coli* DNA pol I [18,55], AMV DNA pol [52] and pol β [14]. Interestingly, the effect of $Mn^{2+}$ on the fidelity of replication varies as a function of the metal ion concentration for example, studies by Beckman et al. showed that the fidelity of DNA replication is similar to that observed with $Mg^{2+}$ at very low [$Mn^{2+}$] (< 1 μM), while the fidelity decreased as the concentration of $Mn^{2+}$ was elevated (< 100 μM) [55]. These authors proposed that the plausible reason for this dependence of fidelity on the metal ion concentration was that at elevated [$Mn^{2+}$], $Mn^{2+}$ could potentially bind to the DNA template affecting fidelity. Sirover et al. carried out similar studies using AMV DNA pol and showed that $Co^{2+}$ and $Ni^{2+}$ also affect the fidelity as a function of the metal ion concentration [17]. Studies by Hays et al. using T4 DNA pol showed that replacement of $Mg^{2+}$ by $Mn^{2+}$ enhanced the rate of dNMP incorporation opposite an abasic site by 11–34 fold [54]. Johnson et al. determined the structures of all 12 mismatched base-pairs for BST pol in the presence of $Mg^{2+}$ and $Mn^{2+}$ [35]. Moreover, in the presence of $Mn^{2+}$ some mismatches were more readily incorporated than others leading to primer extension in crystals. Similar results were obtained by Bebenek et al. in solution [5]. Vashishtha et al. [36] used pre-steady-state kinetic studies to determine the effect of various divalent cations on the fidelity of replication of RB69pol which is a B-family pol and showed that apart from $Mg^{2+}$, $Co^{2+}$ and $Mn^{2+}$ could act as cofactors for RB69pol catalyzed reactions. These authors showed that as compared to $Mg^{2+}$ the incorporation efficiency for dTMP incorporation

opposite dA was 5-fold higher in the presence of $Co^{2+}$ and 3-fold higher in the presence of $Mn^{2+}$ respectively. In the same study, the effect of divalent cations on base selectivity was studied using pyrimidine:pyrimidine, purine:pyrimidine and purine:purine mispairs. Surprisingly, base selectivity was also lower when $Mg^{2+}$ was replaced with $Co^{2+}$ but this decrease was still much less pronounced as compared to when $Mn^{2+}$ replaced $Mg^{2+}$ [36].

Recent studies by Vashishtha et al. [46] with BST pol which is an A-family pol showed that apart from $Mg^{2+}$, $Mn^{2+}$, and $Co^{2+}$, $Cd^{2+}$ could also serve as a cofactor for the polymerization reaction which is in contrast to similar studies using RB69pol [36] where $Cd^{2+}$ failed to support nucleotidyl transfer reaction. On the other hand, $Ni^{2+}$ which poorly activated RB69pol was not able to support nucleotidyl transfer with BST pol. They reported that the incorporation efficiency for the correct nucleotide incorporation (dTMP opposite dA) was 6-fold higher in the presence of $Co^{2+}$ as compared to $Mg^{2+}$ while this value was 8-fold higher with $Mn^{2+}$ and slightly higher with $Cd^{2+}$. Irrespective of the variation in $k_{pol}$ values, the $K_{d,app}$ values were very similar irrespective of the identity of the divalent cation. Interestingly, the incorporation efficiency with both these pols representing two different pol families is much higher with $Co^{2+}$ as compared to $Mg^{2+}$.

The effect of different divalent cations on base selectivity was also studied using pyrimidine:pyrimidine and purine:pyrimidine mispairs. In contrast to RB69pol [36], the incorporation efficiencies for incorrect incoming nucleotides were ~10–50 fold higher when $Mg^{2+}$ was replaced with $Co^{2+}$ suggesting that the base discrimination is greatly impacted with $Co^{2+}$. Interestingly, the $K_{d,app}$ values in the presence of $Co^{2+}$ were lower as compared to $Mg^{2+}$ with BST pol but the $K_{d,app}$ values did not follow a clear trend with RB69pol [36]. A drastic impact on base selectivity was observed when $Mg^{2+}$ was replaced with $Mn^{2+}$ ($k_{pol}/K_{d,app}$ values were 13–1300 fold higher as compared to $Mg^{2+}$). Interestingly, replacement of $Mg^{2+}$ with $Cd^{2+}$ resulted in a decrease in base selectivity [46]. These studies in conjunction with previous studies using long DNA substrates clearly show that among all divalent cations, $Mn^{2+}$ is the most highly mutagenic metal ion and this behavior is a direct result of a sharp increase in the rate of incorporation ($k_{pol}$ or $V_{max}$) accompanied by a simultaneous decrease in the $K_{d,app}$ or $K_m$ values for incorrect incoming dNTPs [19,36].

## 8. Effect of metal ions on extension past a mismatched P/T

During primer extension, if an incorrect nucleotide gets incorporated in the growing primer strand, the associated exonuclease activity removes the incorrect nucleotide. If the incorrect nucleotide is not removed, this results in errors being preserved during DNA replication. Loeb et al. and Snow et al. carried out extensive studies on the effect of metal ions on misincorporation with various DNA pols but they did not study the effect of metal ions on the efficiency of bypass past a mismatch [12,20,43]. Vashishtha et al. carried out comprehensive studies using RB69pol and determined the effect of different divalent cations on the ability of this pol to bury a mismatch [36]. They used DNA substrates containing either a dA/dC mismatch (representing a purine:pyrimidine mismatch which can form hydrogen bonds) or a dA/dG mismatch (representing a purine:purine bulky mispair incapable of forming hydrogen bonds between bases). In the presence of $Mg^{2+}$, there was a substantial increase in the $K_{d,app}$ value from 56 μM to 800 μM while the $k_{pol}$ value decreased sharply from > 300 $s^{-1}$ to 1 $s^{-1}$ for extension past the dA/dC mismatch, suggesting that the addition of a correct incoming nucleotide past the mismatch was very slow. With $Mn^{2+}$, the $K_{d,app}$ was similar to $Mg^{2+}$, while the $k_{pol}$ was 17-fold higher, as compared to $Mg^{2+}$. In the presence of $Co^{2+}$, the $K_{d,app}$

value was much higher than those obtained with $Mg^{2+}$ and $Mn^{2+}$. They observed similar trends with DNA containing the dA/dG mismatch in the presence of $Mg^{2+}$, $Mn^{2+}$, and $Co^{2+}$, except that the $K_{d,app}$ values were quite similar with all three divalent cations (300−500 μM). In general, the $k_{pol}$ values were 40−380-fold lower than those obtained with a dA/dC mispair.

Recent studies by Vashishtha et al. using BST pol examined the efficiency of mismatch bypass extension by this pol in the presence of $Mg^{2+}$, $Mn^{2+}$, $Co^{2+}$ and $Cd^{2+}$ [46]. They utilized a duplex DNA containing a dA/dC mismatch. In the presence of $Mg^{2+}$, the $k_{pol}$ value dropped by 200-fold and the $K_{d,app}$ value increased by 24-fold compared to the extension past a DNA containing matched DNA. This results in ~4800-fold decrease in the efficiency of incorporation when a mismatch is encountered at the P/T terminus. When $Mg^{2+}$ was replaced with $Co^{2+}$, the incorporation efficiency past the mismatched DNA was higher than that obtained in the presence of $Mg^{2+}$. Interestingly when $Mg^{2+}$ was replaced by $Mn^{2+}$ in the assay, the incorporation efficiency was 100-fold higher compared to $Mg^{2+}$. Majority of this enhancement was a direct result of a sharp increase in the $k_{pol}$ value accompanied by a substantial decrease in the $K_{d,app}$ value. Surprisingly, when $Cd^{2+}$ was used in the assay, the incorporation efficiency past a dA/dC mismatch was very similar to that obtained with $Mg^{2+}$ suggesting that $Cd^{2+}$ does not promote misincorporation past a mismatch and behaves in a similar fashion to $Mg^{2+}$ [46]. Based on the results obtained with RB69pol (a B-family pol) and BST pol (an A-family pol), in general, $Co^{2+}$ and $Mn^{2+}$ would be expected to increase the ability of DNA pols to bury a mismatch during DNA replication.

## 9. Effect of metal ions on the exonuclease activity

Similar to the polymerase active site, the exonuclease site also requires divalent cations to catalyze the 3′–5′ exonuclease activity associated with several DNA pols [5,36,38,41,56,57]. Divalent cations are required but have a different effect on the exonuclease activity. Results with RB69pol [36] showed that, compared to $Mn^{2+}$ and $Co^{2+}$, $Mg^{2+}$ was most effective in promoting base excision but the exo rates varied only slightly among these three metal ions. $Ni^{2+}$ on the other hand caused a dramatic decrease in exo activity (33-fold with $Ni^{2+}$ vs. $Mg^{2+}$). Similarly, the rates of base excision were reported to be nearly identical for *E. coli* DNA pol I with $Mg^{2+}$, $Mn^{2+}$ and $Co^{2+}$ [16].

## 10. Rare tautomer hypothesis for mutagenesis when $Mn^{2+}$ is present

High fidelity DNA pols usually possess high base selectivity preventing misincorporation events during primer extension. Despite this, sometimes they do incorporate an incorrect nucleotide but the mechanism for this misincorporation is still unclear. In order to answer this question as to how DNA pols actually incorporate an incorrect incoming nucleotide, several groups have successfully crystallized ternary complexes containing the mismatched bases mimicking the cognate base-pairs [58,59]. Bebenek et al. have used a deletion mutant of pol λ which is an X-family DNA pol and crystallized the pol λ:DNA:dGTP ternary complex using a non-hydrolyzable analog of dGTP ($dGM_PC_{PP}$) opposite dT [59]. In this structure, the A and B metal ions overlay quite well with the corresponding structures containing cognate base-pairs. Also, the G:T primer-terminal base-pair occupies the same position corresponding to the correct base-pair with the exception of a minor twist in the template base position.

Tautomerization is a well-known phenomenon among certain base-pairs during the nucleotidyl

transfer reaction [59–61]. Replicative DNA pols occasionally incorporate incorrect dNMPs [5] during the tautomerization reaction leading to the formation of high-energy tautomers [59–61]. Interestingly, structural evidence for these rare tautomers has been provided by Beese et al. whereby they observed these tautomers using a D598A/F710Y double mutant of BST pol in the presence of $Mn^{2+}$ [58]. These authors showed that when ternary complex formation (BST pol:DNA:dNTP) takes place in the presence of $Mn^{2+}$, the C/A mismatched base pair at the primer terminus adopts a tautomeric cognate base-pair shape, that is virtually indistinguishable from the canonical, Watson-crick base-pair in double stranded DNA at the insertion site [58]. Moreover, in the presence of $Mn^{2+}$, the triphosphate tail was also properly aligned for catalytic reaction to take place, as well as BST pol was present in the "closed" conformation normally observed during correct nucleotide incorporation facilitating the misincorporation. When the reaction was studied in the presence of $Mg^{2+}$, contrasting results were obtained as compared to $Mn^{2+}$. For example, the C/A mismatch formed a non-cognate wobble base pair, and the BST pol was found to be in an "ajar" or partially closed conformation which hindered the incorporation of the incorrect dNMP. In addition, the triphosphate tail was also distorted misaligning the geometry required for the attack on the alpha phosphate of the incoming dNTP by the 3′-OH group of the primer. Together, these factors prevent the incorporation of an incorrect incoming nucleotide into the growing primer strand in the presence of $Mg^{2+}$ [58].

Recent studies by Vashishtha et al. using wild type BST pol provided kinetic evidence for this rare tautomer hypothesis whereby they observed enhanced rates of incorporation of dAMP opposite dC in the presence of $Mn^{2+}$ as compared to $Mg^{2+}$ ($k_{pol}$ value was 130-fold higher in the presence of $Mn^{2+}$) [46]. The formation of cognate base-pair mimicking the Watson-Crick base pairing along with the proper alignment of the triphosphate tail for nucleophilic attack as shown by Beese et al. could account for these enhanced rates obtained with incorrect dNMPS in the presence of $Mn^{2+}$ as opposed to $Mg^{2+}$ where the A/C mismatch forms a wobble base-pair and the pol is present in the Ajar confirmation resulting in misalignment of the residues involved in catalyzing the nucleotidyl transfer. The enhanced $k_{pol}$ values observed for dAMP incorporation opposite dC in the presence of $Co^{2+}$ and $Cd^{2+}$ could similarly be explained based on the speculation that the A/C mismatch adopts a tautomeric cognate base-pair shape and the triphosphate tail is also likely present in the proper alignment for nucleotidyl transfer. This proposal awaits further confirmation which will be tested once the $Co^{2+}$ and $Cd^{2+}$ bound ternary crystal structures become available. Indeed, the formation of cognate base-pair in the presence of these metal ions could explain the lower $K_{d,app}$ values obtained with these divalent cations as opposed to $Mg^{2+}$. These results provide a structural rationale for the mutagenic behavior of $Mn^{2+}$.

## 11. All DNA pols can utilize $Co^{2+}$ as cofactor

In addition to $Mg^{2+}$ most other DNA pols can also use $Mn^{2+}$ and $Co^{2+}$, albeit with reduced fidelity [16–18,20,36]. Recent studies by Vashishtha et al. on BST pol have shown that this pol is also able to utilize $Co^{2+}$ to extend the primer terminus [46]. Pelletier et al. and Egli et al. have shown that pol β [14] and Dpo4 [42] are the two known exceptions where the DNA pols cannot be activated by $Co^{2+}$. In contrast, Vashishtha et al. [36] showed that these DNA pols can actually catalyze primer extension in the presence of $Co^{2+}$. The apparent conflicting results can be rationalized based on the fact that different assay conditions were used by each of these groups. For example Pelletier et al. [14] used blunt-ended DNA with pol β, while Vashishtha et al. [36] used a P/T with a four base overhang 5′

to the templating base. In the Egli et al. experiments [42] 2 mM DTT was included in their assay with Dpo4 which reduced $Co^{2+}$ to $Co^{1+}$ ($E^0 = -0.33$V for DTT vs. $-0.28$V for $Co^{2+}$). DTT was omitted by Vashishtha et al. in their assays of Dpo4 so cobalt remained as $Co^{2+}$ [36]. Based on these results it appears that $Co^{2+}$ can support catalysis for all DNA pols that have been studied to date.

## 12. Properties of Divalent cations that can activate DNA polymerases

Several studies have shown that DNA pols from different families can potentially utilize various divalent cations as cofactors in order to carry out nucleotidyl transfer reaction [14,18,19,37,52,54,55,62]. It is quite surprising and puzzling as to why different DNA pols are able to utilize only certain divalent cations despite the fact that all DNA pols share a common active site containing the metal ion occupying the "A" site in distorted octahedral geometry while the one occupying the "B" site in perfect octahedral geometry [6,8–10,19–21,23,30–36,63].

Certain properties of divalent cations including their coordination geometry, their ionic radii, and their ability to lower the p$K_a$ of the 3′-OH group help to determine if a given divalent cation could serve as a cofactor for DNA pols (Table 2). A crucial factor in this regard is the ability of the given divalent cation to make the 3′-OH group of the primer strand more nucleophilic by lowering its p$K_a$ and facilitating its attack on the α-phosphate atom of the incoming dNTP. By comparing the ability of different divalent cations to lower the p$K_a$ of bound water (and analogously the p$K_a$ of the 3′-OH group) it appears that the p$K_a$ value is lowest for $Fe^{2+}$ (8.4), similar for $Cd^{2+}$, $Co^{2+}$, and $Zn^{2+}$ (9.8, 9.4, and 9.6 respectively), slightly higher for $Ni^{2+}$, and $Mn^{2+}$ (10.6 and 10.1), and much higher for $Ca^{2+}$ and $Mg^{2+}$ (12.8 and 11.4) (Table 2). Based on these values, $Fe^{2+}$ is expected to support catalysis with DNA pols but surprisingly, $Fe^{2+}$ fails to act as a cofactor for DNA pols including RB69pol [36] and BST pol [46]. Similarly, based on the values in Table 2, $Co^{2+}$, $Cd^{2+}$, and $Zn^{2+}$ would be expected to be more effective as cofactors but this prediction is not borne out as is clear from the results obtained with various DNA pols [16–18,20,36,42–44]. In two separate studies, Vashishtha et al. showed that $Zn^{2+}$ was not able to catalyze nucleotidyl transfer reaction with RB69pol [36] and BST pol [46]. Moreover, $Ni^{2+}$ was also not able to support the polymerase activity of BST pol [46] despite having a comparable p$K_a$ value to $Mn^{2+}$ but was a weak activator of RB69pol [36]. Based of its inability to lower the p$K_a$ of the 3′-OH group of the primer, $Ca^{2+}$ is not able to support catalysis with DNA pols and Dpo4 is the only exception in this regard which is able to utilize $Ca^{2+}$ as a cofactor albeit with poor incorporation efficiency compared to $Mg^{2+}$ [11]. This suggests that the ability of divalent cations to lower the p$K_a$ of the 3′-OH group is not the sole factor for the ability of a given divalent cation to support catalysis for DNA pols, therefore other factors must be considered.

Besides the ability to lower the p$K_a$ of the 3′-OH group, the ionic radii of a given divalent cation plays a crucial role in determining its ability to support catalysis. The divalent cation present in the "A site" essentially determines the proximal distance between the 3′-hydroxyl group of the growing primer strand and the α-phosphorous atom of the incoming dNTP as the transition state is being approached leading to phosphodiester bond formation. The ionic radii of various divalent cations are summarized in Table 2. The ionic radii of $Mg^{2+}$ is 0.86 Å, and $Mg^{2+}$ is a universal activator of all DNA pols known to date. Based on this fact it would be expected that other divalent cations whose ionic radii are close to that of $Mg^{2+}$ should also be able to support catalysis with various DNA pols. The ionic radii of $Mn^{2+}$, $Co^{2+}$, $Ni^{2+}$ and $Zn^{2+}$ are comparable to that of $Mg^{2+}$, hence these divalent cations should be able to bring the 3′-hydroxyl group and α-phosphate atom of

the incoming dNTP within the required distance for phosphodiester formation but the results show that $Ni^{2+}$ and $Zn^{2+}$ fail to activate

**Table 2.** Ionic radii, coordination geometry, and p$K_a$ of water molecules coordinated to $Mg^{2+}$, $Mn^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Zn^{2+}$, $Cd^{2+}$, and $Ca^{2+}$.

| Parameter | Metal Ion | | | | | | |
|---|---|---|---|---|---|---|---|
| | $Mg^{2+}$ | $Mn^{2+}$ | $Co^+$ | $Ni^{2+}$ | $Zn^{2+}$ | $Cd^{2+}$ | $Ca^{2+}$ |
| Ionic radius (Å) | 0.86 | 0.81 | 0.89 | 0.83 | 0.88 | 0.95 | 1.1 |
| Coordination | Oct | Oct | Oct | Oct | Oct | Oct | Oct |
| Geometry | Td | Td | Td | Td | Td[b] | Td | PBP |
| | Sq | Sq | Sq | TBP | TBP | HBP | |
| | TBP | TBP | | | | | |
| p$K_a$ of the water molecule | 11.4 | 11.5 | 10.0 | 10.6 | 7.0 | 9.0 | 12.8 |

[a] Td represents Tetrahedral, Sq represents Square planar, TBP represents Trigonal bipyramidal, Oct represents Octahedral, PBP represents pentagonal bipyramid, and HBP represents hexagonal bipyramidal.

[b] Even though $Zn^{2+}$ can form Octahedral complexes, majority of $Zn^{2+}$ complexes are tetrahedral.

BST pol and $Ni^{2+}$ is only able to weakly activate RB69pol [36]. The ionic radii of $Fe^{2+}$ and $Cd^{2+}$ (0.92 and 0.95 Å) are slightly larger than that of $Mg^{2+}$ but surprisingly none of these metal ions support catalysis with RB69pol [36]. In contrast, $Cd^{2+}$ is able to activate BST pol [46]. Moreover, with BST pol, the $k_{pol}$ value obtained in the presence of $Cd^{2+}$ is comparable to that obtained with $Mg^{2+}$ while $Fe^{2+}$ whose ionic radii is very similar to $Cd^{2+}$ fails to support phosphodiester bond formation. $Ca^{2+}$ fails to activate all DNA pols except Dpo4 which is the only pol which can be weakly activated by this divalent cation [42]. The inability of $Ca^{2+}$ to activate DNA pols can be rationalized on the basis of its larger ionic radii as compared to other divalent cations (1.1 Å for $Ca^{2+}$ vs. 0.86 Å for $Mg^{2+}$). This large ionic radii prevents $Ca^{2+}$ occupying the "A" site from allowing the 3′-hydroxyl group and α-phosphate atom of the incoming dNTP to come close to the proximal distance for the phosphodiester bond formation [11].

Another important factor is the preference of different divalent cations for a given coordination geometry. Studies by Xia et al. [11] using metal ion ($Mg^{2+}$ or $Mn^{2+}$) bound complexes of a variant of RB69pol showed that the metal ion occupying the "B" site is present in a perfect octahedral geometry while the metal ion present in the "A" site is present in a distorted octahedral geometry. It is well known that other divalent cations including $Co^{2+}$, $Cd^{2+}$, $Fe^{2+}$, and $Ni^{2+}$ can also form octahedral complexes and hence can theoretically support the phosphoryl transfer reaction with DNA pols. Contrary to this expectation, studies by Vashishtha et al. have shown that only $Co^{2+}$, and $Ni^{2+}$ can support the reaction with RB69pol [36]. In contrast, studies with BST pol showed that $Co^{2+}$, and $Cd^{2+}$ could support catalysis but $Fe^{2+}$, and $Ni^{2+}$ are not able to act as cofactors for BST pol [46]. Interestingly, $Ca^{2+}$ can also form octahedral complexes but since the ionic radii is much larger $Ca^{2+}$ prefers pentagonal bipyramidal and hexagonal bipyramidal geometries over octahedral geometry which helps to explain the inability of $Ca^{2+}$ to support catalysis with various pols except Dpo4 where $Ca^{2+}$ acts as a weak activator [42]. Based on its ability to effectively lower the p$K_a$ of hydroxyl group and its similar ionic radii compared to $Mg^{2+}$, $Zn^{2+}$ is expected to act as a cofactor for DNA pols but $Zn^{2+}$ fails to catalyze the phosphoryl transfer reaction except with pol β [14] and pol α [64]. The inability of $Zn^{2+}$ to act as a cofactor for DNA pols could be explained on the basis of its preference

for tetrahedral geometry as opposed to octahedral geometry required for metal ions bound in the "A" and "B" sites. The ability of only selected divalent cations (including $Mg^{2+}$, $Co^{2+}$, $Mn^{2+}$, and $Cd^{2+}$ for BST pol and $Mg^{2+}$, $Co^{2+}$, $Mn^{2+}$, and $Ni^{2+}$ for RB69pol) to act as cofactors for these pols is consistent with: 1) the ability of these metal ions to form octahedral complexes [34]; 2) similar ionic radii of these metal ions and; 3) their ability to effectively lower the $pK_a$ of the water molecule (and presumably the 3′-hydroxyl group of the primer).

## 13.  Determination of Error Rates for DNA pols

As discussed above, DNA pols are susceptible to making errors during DNA replication resulting in mutations being preserved in the DNA if not corrected by the exonuclease activity. It is important to study these mutations in cases such as tumors and several types of cancers [65]. Frederico et al. have used the M13 reversion assay to determine the rate of cytosine deamination in DNA [66]. The basis of this assay is the reversion of a mutant in the *lacZα* gene coding sequence of bacteriophage M13mp2 [67]. Recent developments in DNA sequencing have led to the evolution of a new era of DNA sequencing known as Next-generation DNA sequencing (NGS) which has revolutionized sequencing both in terms of the cost as well as the amount of data generated from sequencing [65,68]. Schmitt et al. have developed a method called "Duplex Sequencing" which is based on individually tagging and sequencing each of the two DNA strands resulting in a theoretical background error rate of less than one mutation per billion nucleotides sequenced [65]. These authors have used this method to determine the frequency and pattern of random mutagenesis in mitochondrial DNA from human cells underlying the importance of this technique. Interestingly, Yasukawa et al. have also developed a simple and rapid method to determine the fidelity of reverse transcriptase using NGS [68,69].

## 14.  Conclusion

DNA pols from different families are able to catalyze primer extension utilizing different divalent cations. Both $Cd^{2+}$, and $Zn^{2+}$ fail to support catalysis with RB69pol [36], while $Zn^{2+}$ is unable to activate BST pol [46]. Interestingly, crystal soaking experiments with pol β have shown that $Cd^{2+}$, and $Zn^{2+}$ could support catalysis leading to primer extension with blunt-end DNA [14]. $Ni^{2+}$ has been shown to support primer- extension with all DNA pols albeit with greatly reduced activity except for Dpo4 [42], human pol α [50], pol β [14] and BST pol [46]. On the other hand, $Co^{2+}$ is able to activate all DNA pols known till date and detailed pre-steady-state kinetic studies showed that $Co^{2+}$ is in fact better than $Mg^{2+}$ in terms of its ability to support correct nucleotide incorporation. Moreover, $Ca^{2+}$ is unable to support catalysis with all DNA pols known except for Dpo4, although, $Ca^{2+}$ is very poor compared to $Mg^{2+}$ [42]. Thus, it is difficult to generalize as to which DNA pol can be activated by specific divalent cations solely based on their properties.

## Conflict of interest

The authors declare no conflict of interest.

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