Structural insights into the translational infidelity mechanism

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The decoding of mRNA on the ribosome is the least accurate process during genetic information transfer. Here we propose a unified decoding mechanism based on 11 high-resolution X-ray structures of the 70S ribosome that explains the occurrence of missense errors during translation. We determined ribosome structures in rare states where incorrect tRNAs were incorporated into the peptidyl-tRNA-binding site. These structures show that in the codon–anticodon duplex, a G·U mismatch adopts the Watson–Crick geometry, indicating a shift in the tautomeric equilibrium or ionization of the nucleobase. Additional structures with mismatches in the 70S decoding centre show that the binding of any tRNA induces identical rearrangements in the centre, which favours either isosteric or close to the Watson–Crick geometry codon-anticodon pairs. Overall, the results suggest that a mismatch escapes discrimination by preserving the shape of a Watson–Crick pair and indicate that geometric selection via tautomerism or ionization dominates the translational infidelity mechanism.
The misincorporation of amino acids into a polypeptide chain is a common occurrence during translation. A comprehensive estimation of missense errors has been a significant challenge in the field, due to the difficulties in detecting and quantifying these errors. Today, the average efficiency of miscoding is estimated to be as high as 10^-3 - 10^-4 per amino-acid site. Under normal physiological conditions, 18% of the proteins expressed from an average 400-codon-long gene contain at least one misincorporated amino acid. More often, misincorporation is not deleterious and is important for the selective pressure on coding sequence evolution and cell fitness; nevertheless, 10-50% of random substitutions affect protein function. In bacteria and higher organisms, the rate of missense errors is similar, reflecting the universality of the genetic code.

In recent decades, X-ray crystallography has remained indispensable for understanding the molecular mechanisms of biological processes. Here, we present several high-resolution structures of Thermus thermophilus 70S ribosomes programmed by templates carrying missense errors. The collection of our structures provides a decoding mechanism that, for the first time, sets the molecular basis behind the phenomenon of translational fidelity and is in good agreement with in vivo studies of the missense errors that occur during protein synthesis.

Results

Mismatch in the peptidyl-tRNA-binding site. We have successfully solved the structure of the 70S ribosome in two post-incorporation states (Fig. 1). In one case, we modelled the post-incorporation state based on the well-known in vitro misincoding system where polyuridylic acid served as a template and the leucyl-tRNA^Leu served as a substrate for peptidyl-tRNA synthesis (Fig. 1a,b). In this complex, the GAG anticodon of tRNA^Leu formed two simultaneous G-U mismatches with the first and third positions of the phenylalanine UUU codon in the peptidyl-tRNA-binding site (P-site; Fig. 2a,b). Another messenger RNA (mRNA) construct and tRNA^Tyr let us model the second G-U mismatch with the cysteine codon UCG and the anticodon QUA bound in the P-site (Figs 1c and 2c). The structures of both states (Supplementary Table 1) revealed the remarkable finding that a G-U mismatch mimics a canonical Watson–Crick pair at either of the first two positions of the codon–anticodon duplex (Fig. 2b,c). Further analysis showed that the codon–anticodon duplexes containing G-U mismatches have an overall geometry that is identical to that of the corresponding cognate duplexes consisting of standard Watson–Crick pairs (Supplementary Fig. 1a, Fig. 1a). Moreover, we did not find any changes in the ribosomal environment (that is, A790, G926 and C1400 of the 16S ribosomal RNA (rRNA); ref. 12) of the near-cognate duplexes in the P-site. These results are particularly striking because in contrast to the restrictive decoding centre, where a G-U mismatch adopts the Watson–Crick geometry of conserved ribosomal elements, the P-site does not impose any obvious constraints on the codon–anticodon duplex that are discernible at 3 Å resolution. Nevertheless, ribosomal parts tightly hold the P-site transfer RNA with 16S residues forming A-minor groove-like contacts with two base pairs of the anticodon stem and with residue 790 blocking the anticodon stem on the other side (Supplementary Fig. 1b). In addition, C1400 in 16S rRNA stacks over the base pair at the third codon–anticodon position and G966 forms van der Waals contacts with the ribose of the 34th tRNA nucleotide (Supplementary Fig. 1b). At the same time, the mRNA path is also constrained by the ribosome by a bend (the E/P-kINK) at the phosphate between the last (−1) and the first (+1) nucleotides that is stabilized by hydrogen bonds. Moreover, the P-codon is fixed by several interactions of the ribosome with its sugar-phosphate backbone (Supplementary Fig. 1c,d).

Mismatch in the aminoacyl-tRNA-binding site. To expand our previous findings, we investigated non-Watson–Crick pairs other than the G-U mismatch in the 70S ribosomal decoding centre. For this study, we chose a ‘challenging’ pyrimidine–purine mismatch, G-A, and a purine–purine mismatch, A-A, and solved seven high-resolution structures where these mispairs were placed at either of the first two positions of a codon–anticodon duplex in the aminoacyl-tRNA-binding site (A-site). We also solved a control structure to demonstrate that the decoding centre is specific in our system and can only bind cognate tRNA or near-cognate tRNA that resembles cognate substrates. In this control complex, where the tRNA-programmed the ribosome with the AAA codon in the A-site and where tRNA^Phe with the GAG anticodon was given as a substrate, no binding of tRNA^Phe to the A-site was detected (Supplementary Table 1).

The analysis of the models reinforced one of our earlier findings, namely, that the binding of near-cognate and cognate tRNA to the 70S ribosome induces identical rearrangements of (i) the small ribosomal subunit (that is, shift of the shoulder domain by 2–3 Å) and (ii) the decoding centre itself (Figs 3 and 4; see Supplementary Fig. 2c and Supplementary Movie). Independently of which near-cognate tRNA was present in the centre, the first cytosine 1493 and 1492/G530 of the 16S rRNA stabilized the first and second codon–anticodon pairs in a manner identical to that of the cognate models (Figs 3 and 4). In addition, the conserved A1913 of helix 69 (H69) in the 23S rRNA stabilized the first codon–anticodon position through contacts with the 37th nucleotide of the near-cognate and cognate tRNA anticodon loops.

A close-up analysis at every mismatch revealed that despite the stabilization of the sugar-phosphate backbones by A-minor groove interactions with the A1492/1493 and G530 of the 16S rRNA, the nitrogen bases of the A-A and A-A mismatches did not interact stably (Figs 3 and 4; see Supplementary Fig. 3). Thus, the G-A mismatch at the first codon–anticodon position was shifted from the Watson–Crick geometry; however, the shift did not quite reach the wobble position, possibly reflecting a metastable or average state (Fig. 3a). The resulting interatomic distances and putative bond angles of the mismatch suggested that the formation of hydrogen bonds was highly unlikely. When the first G-A mismatch was modelled with tRNA^Tyr, the presence of a queuosine modification, which was not visible in previous structures of tRNA^Tyr, at the first anticodon position led to the displacement of the cytosine from the codon–anticodon helix and distortion of the latter. This change emphasized the lack of stable interactions in the G-A mispair and pointed to an amending role of tRNA modifications in translational accuracy. The base pair geometry of the G-A at the second codon–anticodon position was very similar to that of the first G-A mismatch in the absence of queuosine (Fig. 3c).

No definite density signal was observed for the base of the mRNA adenosine in the structure with the first A-A mispair in the codon–anticodon duplex, demonstrating its mobility (Fig. 4a). One of the possible conformations could be stabilized by the queuosine of tRNA^Tyr (as was the case for the first cytosine; Fig. 3b); however, any interaction with the anticodon adenosine was unlikely. The structure with the A-A mismatch at the second codon–anticodon position provided further evidence of the canonical constraints of the 70S decoding centre. Limited by...
A1492/G530 and stacking interactions with the standard Watson–Crick pairs at the first and third duplex positions, the adenosine of mRNA was found in the syn conformation with its Hoogsteen plane exposed to the Watson–Crick surface of the opposing adenosine in tRNA (Fig. 4b). Nevertheless, interatomic distances of >3.6 Å excluded possibility of strong interactions between the two adenosines, stressing the fact that the 70S decoding centre suppresses the formation of non-Watson–Crick pairs by restrictive steric and geometrical constraints.

We also determined structures with the A•A and G•U mismatches at the first and second positions of the codon–anticodon duplexes, respectively, in the presence of the miscoding aminoglycoside paromomycin. Binding of the antibiotic did not affect the geometry of the mismatches and resulted in the same relaxation of the decoding pocket and shift of H69 towards tRNA that we described previously (Supplementary Fig. 4)\textsuperscript{13}.

**Discussion**

The results obtained for the G•U mismatches presented here as well as those that were previously published\textsuperscript{13} are closely related to the work of Topal and Fresco, who discussed base-pairing schemes and attempted to explain translational errors\textsuperscript{17}. Their work extended the hypothesis of Watson and Crick, who suggested that spontaneous mutagenesis in replication is caused by a base adopting one of its rare tautomeric forms\textsuperscript{18}. Topal and Fresco implied that a non-Watson–Crick pair matching the abundant keto isomers\textsuperscript{17} (Fig. 2d). Most likely the formation of minor tautomers of G or U isomeric state after binding to the ribosome. In some cases, it leads to the rare enol tautomers being favoured over the more abundant keto isomers\textsuperscript{17} (Fig. 2d).

Although the 3 Å resolution of our models is not sufficient to distinguish between the two tautomeric forms, the observed Watson–Crick-like geometries for the G•U pairs can be rationalized by the presence of enol tautomers in the P-site (Fig. 2d). Most likely the formation of minor tautomers of G or U either in mRNA or in tRNA happens before their binding to the ribosome, that is, in solution. While this paper was under review, NMR relaxation dispersion measurements showed that in RNA duplexes a wobble G•U pair exists in dynamic equilibrium with short-lived, low-populated Watson–Crick-like pairs that are stabilized by rare enolic or anionic bases (see page 318 in ref. 19). Our present structure with the P-site G•U mismatches trapped in a Watson–Crick geometry, as well as the previous report\textsuperscript{13} on the A-site with G•U in Watson–Crick-like geometry with its conformation with its syn conformation with its Hoogsteen plane exposed to the Watson–Crick surface of the opposing adenosine in tRNA (Fig. 4b). Nevertheless, interatomic distances of >3.6 Å excluded possibility of strong interactions between the two adenosines, stressing the fact that the 70S decoding centre suppresses the formation of non-Watson–Crick pairs by restrictive steric and geometrical constraints.

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In an analogous fashion to the mRNA P/A-kink13,14,20, the mRNA constraints between the E-codon and P-codon11,14 together with the tight ribosome grip surrounding the tRNA anticodon stem-loop12,14 contribute to the fixing of the P-site codon–anticodon mini-helix in place (Supplementary Fig. 2b–d). Thus, this fixation would restrain the first codon nucleotide from be also forbidden because the tight shape of the codon–anticodon duplex rather than translocation. Recently published studies dealing with the in vivo frequencies of mismatches in translation ranked G•U, U•U or C•U mismatches as the most frequent, and A•A and C•A mispairs as the least probable errors during protein synthesis4,8, fully in accordance with the conclusions derived from our structural data.

Taken together, the present structures along with our earlier models of the 70S ribosome primed by long templates and native geometries, fully support these observations. According to the NMR relaxation dispersion calculations, frequencies of occurrence of minor enolic or anionic bases spans the range of 10–3–10–5 that are not far from the accepted translation error rate of 10–3–10–4 (refs 3,4).

The present results suggest that the extent of molecular adaptability allowing a non-complementary pair to form an isosteric pair with the Watson–Crick-like geometry defines the probability and efficiency of a miscoding event. We can infer that among the described complexes, those ribosomes bearing mRNA and tRNA with G•U mismatches would be by far the most stable24, while those bearing C•A and A•A mismatches would be less homogeneous and less stable. In the context of the kinetic scheme of decoding5, such complexes will be prone to dissociation rather than translocation.
tRNA\textsuperscript{11,13,15} provide an extensive library of various states of the P-site and the decoding centre on the 70S ribosome. Our models suggest an advanced mechanism of decoding that, for the first time, describes how a missense error can skip discrimination, leading to translational infidelity (Fig. 5). Although our structures were obtained in a non-enzymatic system, numerous lines of experimental data support the unified principles that underlie the basic functions of the ribosome and hence allow us to generalize the proposed mechanism.

For the present mechanism of decoding (Fig. 5), we want to emphasize the crucial role of the large ribosomal subunit and, in particular, its helix 69 that forms the intersubunit bridge B2a (ref. 25) and acts as a regulator of nucleotide A1492 of the 16S rRNA in the decoding centre\textsuperscript{15}. The tRNA selection begins with the binding of tRNA to the unoccupied centre (Fig. 5a, i), which is predisposed to accept tRNA\textsuperscript{26}. In this unoccupied centre, A1493 in 16S rRNA prevents strong pairing interactions in the C$\cdot$A mismatch by constraining its sugar-phosphate backbone by hydrogen bonding. In b, the left and middle panels demonstrate a lack of pairing in the C$\cdot$A mismatch reflected by the weak electron density signal corresponding to the cytosine base and misshaping of the mini-helical structure due to displacement of the cytosine by queuosine (right). (c) The C$\cdot$A mismatch at the second position of the codon-anticodon duplex; as in a and b, the left panel depicts conserved A1492 and G530 in 16S rRNA tightening around the mismatch and contorting it. The density maps are contoured at 1.8 $\sigma$ level. In a-c, the right panels depict overall geometry of the mismatches in the codon-anticodon mini-helices.

Figure 3 | The C$\cdot$A mismatch does not form a stable pair in the 70S ribosome-decoding centre. (a,b) The C$\cdot$A mismatch at the first position of the codon-anticodon duplex in the absence (a) or presence (b) of the queuosine modification in the tRNA anticodon. In a, the left panel shows that A1493 in 16S rRNA prevents strong pairing interactions in the C$\cdot$A mismatch by constraining its sugar-phosphate backbone by hydrogen bonding. In b, the left and middle panels demonstrate a lack of pairing in the C$\cdot$A mismatch reflected by the weak electron density signal corresponding to the cytosine base and misshaping of the mini-helical structure due to displacement of the cytosine by queuosine (right). (c) The C$\cdot$A mismatch at the second position of the codon-anticodon duplex; as in a and b, the left panel depicts conserved A1492 and G530 in 16S rRNA tightening around the mismatch and contorting it. The density maps are contoured at 1.8 $\sigma$ level. In a–c, the right panels depict overall geometry of the mismatches in the codon-anticodon mini-helices.

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interactions between A1492 and the second codon position (Fig. 5b, middle). These rearrangements essentially define the decoding pocket from the side of mRNA. The second rearrangement is the slight movement of the shoulder domain of the small subunit towards the anticodon loop that brings together G530 (which switches its conformation from syn to anti) with the second anticodon position and finalizes the formation of the decoding centre (Fig. 5b, middle). Considering early evidence with the second anticodon position and finalizes the formation of the small subunit towards the anticodon loop that brings together G530 (which switches its conformation from syn to anti) with the second anticodon position and finalizes the formation of the decoding centre. In this framework, cognate tRNA will be accommodated in the decoding centre and further translocated to the P-site, resulting in misincorporation of an amino acid into a polypeptide chain (Fig. 5a, iv). This scenario agrees with the studies of the tRNA selection process using the single-molecule fluorescence resonance energy transfer approach and fits well into the contemporary kinetic scheme of the process that suggests that decoding on the ribosome is evolutionarily optimized towards a higher speed of translation at the cost of fidelity.

Our data provide evidence that steric complementarity and shape acceptance but not the number of hydrogen bonds between the decoding centre and a codon–anticodon duplex play the discriminatory role during decoding. Our translational fidelity mechanism finds support in recent studies where multiple 2'-fluoro substitutions in mRNA disrupting the hydrogen bonds between the mRNA codon and the decoding centre only had a modest effect on the tRNA selection efficiency. Our models further reinforce the specific role of tautomerism or base ionization in infidelity mechanisms of other biological processes, such as DNA replication and we propose an original view of the phenomena that may involve non-canonical Watson–Crick pairs, for example, in non-canonical decoding or during the initiation from alternative start codons.

Figure 4 | The A-A mismatch in the decoding centre of the 70S ribosome. The left panels demonstrate the A-A mismatch at the first (a) and second (b) positions of the codon-anticodon duplexes. As for the C-A mismatches, A1493 (a) and A1492 with G530 (b) in 16S rRNA constrain the sugar-phosphate backbones of the first and second mismatches by hydrogen bonding. In a, the exact position of the codon adenosine was not detectable and the figure shows one of the possible positions of this nucleoside; in b, the distances between adenosines exceed 3.6 Å demonstrating absence of strong interactions. In a and b, the right panels depict overall geometry of the mismatches in the codon-anticodon mini-helices.
the ribosomes were dialysed against buffer D (10 mM Mg(CH₃COO)₂, 50 mM KCl, 10 mM NH₄Cl, 1 mM DTT, 10 mM HEPES, pH 7.5), applied on the 5–20% sucrose gradient prepared in buffer D and further centrifuged at 15,400 r.p.m. for 17 h in the SW28 rotor (Beckman). The peaks corresponding to 70S ribosomes were combined and the ribosomes were pelleted by ultracentrifugation at 45,000 r.p.m. for overnight in the type 45 Ti rotor (Beckman). The 70S ribosome pellet was resuspended in buffer D with 5 mM HEPES, pH 7.5, flash frozen in liquid nitrogen and stored in small aliquots at –80°C.

Uncharged native individual tRNAPhe, tRNATyr and tRNAfMet from *Escherichia coli* were purchased from Chemical Block (Russia). All mRNA constructs whose

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**Figure 5 | Proposed mechanism of translational infidelity.** (a) Misincorporation of an amino acid by the ribosome. The main steps of the tRNA selection process are shown including hydrolysis of GTP (black asterisk) by elongation factor Tu on establishing codon–anticodon interactions in the decoding centre; (i–iv) indicate sequential steps of the process (see the text). (b) Conformation of the main nucleotides of the decoding centre without tRNA (left), bound by cognate or near-cognate aa-tRNA at the initial recognition step (right). The crucial nucleotides of 16S and 23S rRNA are shown in cyan and red, respectively. Ribosomal protein S12, which belongs to the shoulder domain of the small ribosomal subunit and additionally restricts the second codon–anticodon pair, is depicted in green. The three nucleotides of the mRNA codon in the A-site are numbered according to the standard system (see legend to Fig. 2). (c) Overall states of the small ribosomal subunit during selection of tRNA. The left panel pictures spontaneous movement of the shoulder domain (black arrows) when the decoding centre is unoccupied; the middle and right panels show that the shoulder is shifted and stabilized on binding of near-cognate tRNA during the initial selection step and further accommodation (see text); sh, h, pl denote the shoulder, head and platform domains of the small ribosomal subunit, respectively. (d) Strengthening of cognate tRNA binding by protein tails from the small (S) and large (L) ribosomal subunits. Fastening of cognate tRNA in the A-site is also represented by formation of the additional intersubunit bridge between protein L31 and proteins S13 and S19 (PDB codes 3I8H and 3I8I). The protein extensions are disordered when near-cognate tRNA is bound in the A-site (present work; the model of the 70S ribosome with tRNA disturbing bound to the UUU codons in the P- and A-sites). The absence of additional stabilization by the proteins can promote dissociation of near-cognate tRNA from the ribosome.
sequences are specified below were from Thermo Scientific (USA) and deprotected following the supplier procedure. Aminoglycoside antibiotic paromomycin was purchased from Sigma-Aldrich.

The ribosomal complexes were formed in 10 mM Tris-acetate, 40 mM KCl, 7.5 mM Mg(CH3COO)2, 0.5 mM DTT at pH 7.0 at 37°C. For all complexes, the 70S ribosomes (3 µm) were incubated with fivefold stoichiometric excess of mRNA and three to fivefold excess of tRNA. For the control complex containing the GTU mismatches in the P-site, the 70S ribosomes (3 µm) were incubated with mRNA-1 and tRNAeo or mRNA-2 and tRNAAT (Fig. 1b,c) for 15 min. For comparison of the near-cognate complexes with GTU at the first codon–anticodon position, we used our previous model of the 70S ribosome with cognate tRNAeo bound to the UUC codon in the P-site. For the second GTU mismatch, we made a separate control complex by incubating 70S ribosomes with mRNA-3 and tRNAAT (Fig. 1c).

For the near-cognate complexes with mismatches in the decoding centre, mRNA sequences were 30 nucleotides long and contained 5′-GGG-GAG-GAG-AAA-5′ (Z at the 5′-end). The exact sequences were as follows: mRNA-4= ZAUAGCUCA; mRNA-5= ZAUAGCAAC; mRNA-6= ZUAGGUC; mRNA-7= ZUAAGACG; and mRNA-8= ZUAUGACG (the start codon and the Shine–Dalgarno sequence are underlined).

The 70S ribosomes (3 µm) were pre-incubated with mRNA-4, mRNA-5, mRNA-6, mRNA-7 or mRNA-8 and tRNATyr for 15 min to fill the P-site. The complexes modelling the GTU mismatch at the first and second codon–anticodon positions were obtained by incubating tRNAe with the 70S/tRNAfMet/mRNA-5 mixture, with the complexes with the GTU mismatch at the first position was also prepared by incubation of the 70S/tRNAfMet/mRNA-5 mixture with tRNATyr. The complexes with the GTU mismatch at the first and second codon–anticodon positions were made by addition of mRNAAT and tRNAAT to the 70S/tRNAAT/mRNA-5 and 70S/tRNAAT/mRNA-8 mixtures, respectively, and incubated as described above.

Complexes with paromomycin obtained by incubating the antibiotic (60 µM) into the incubation mixture containing 70S/tRNAAT/mRNA-6/7/tRNAAT. Crystals were grown at 24°C via vapour diffusion in sitting drop plates (CrysChem, Hampton Research). The ribosomal complex (2 µl) containing 2.8 mM Decoy Big Chaps (CalBioChem) was mixed with the equal volume of the crystallization solution (3.9–4.2% (v/v) PEG 20K, 3.9–4.2% (v/v) PEG500monine, 100 mM Tris-acetate, pH 7.0, 100 mM KSCN). The crystals grew for 2–3 weeks and were then dehydrated by exchanging the reservoir for 60% (v/v) 2-methyl-2,4-pentanediol. Before freezing in the nitrogen stream, crystals were then cryo-protected by the addition of 30% (v/v) 2-methyl-2,4-pentanediol and 14 mM Mg(CH3COO)2.

Data collection, processing and structure determination. Data for all complexes were collected at the PX1 beamline of Swiss Light Source, Switzerland, at 100 K. A very low dose mode was used and huge redundancy data were collected. For the 70S ribosome with paromomycin, with the 70S complex with mRNA-6/7/tRNAAT. The crystals were grown at 24°C using the vapour diffusion method. The data were processed and scaled using XDS65. All crystals belong to space group P21,2,1 and contain two ribosomes per asymmetric unit. One of the previously published structures with tRNA, mRNA, tRNA and metal ions removed, was used for refinement with Phenix65. The initial model was placed within each data set by refinement with Phenix. The refinement with Phenix43. The initial model was placed within each data set by refinement with Phenix. The refinement with Phenix43. The initial model was placed within each data set by refinement with Phenix. The refinement with Phenix43. The initial model was placed within each data set by refinement with Phenix. The refinement with Phenix43. The initial model was placed within each data set by refinement with Phenix. The refinement with Phenix43. The initial model was placed within each data set by refinement with Phenix. The refinement with Phenix43. The initial model was placed within each data set by refinement with Phenix. The refinement with Phenix43. The initial model was placed within each data set by refinement with Phenix. The refinement with Phenix43. The initial model was placed within each data set by refinement with Phenix. The refinement with Phenix43. The initial model was placed within each data set by refinement with Phenix. The refinement with Phenix43.

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Author contributions

A.R. and N.D. conducted the experiments. A.R., N.D., E.W., M.Y. and G.Y. interpreted the structures. All authors contributed to the final version of the paper.

Additional information

Accession codes: The atomic coordinates and structure factors for the reported crystal structures have been deposited in the Protein Data Bank under accession codes 4WQ1 (A-site: 1st C\textsuperscript{15}A with tRNATyr), 4WQR (A-site: 1st C\textsuperscript{15}A with tRNAPhe), 4WR6 (A-site: 1st A\textsuperscript{15}A), 4WRA (A-site: 1st A\textsuperscript{15}A with paromomycin), 4WRO (A-site: 2nd C\textsuperscript{15}A), 4WSD (A-site: 2nd C\textsuperscript{15}A with paromomycin), 4WT1 (A-site: 2nd A\textsuperscript{15}A), 4WSM (P-site: 1st G\textsuperscript{15}U), 4WU1 (P-site: 2nd G\textsuperscript{15}U), 4WZD (P-site: 2nd G\textsuperscript{15}G) and 4WZO (A-site: control).

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