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Novel Reporter System to Quantify Missense Errors During Translation in *Saccharomyces cerevisiae*

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ABSTRACT

Objective: This study aimed to develop and validate a novel β-galactosidase–luciferase reporter system in *Saccharomyces cerevisiae* to quantify codon-specific missense errors during translation. Particular emphasis was placed on codons predicted to be error-prone, and on using ribosomal fidelity mutants to distinguish true misreading events from functional amino acid substitutions.
Methods: Reporter constructs containing single-codon substitutions at position 537 of the *lacZ* gene were generated to assess translational fidelity. Near-cognate and synonymous non-cognate variants were tested, and luminescence was measured following substrate hydrolysis. Hyper-accurate (A113V) and error-prone (K63R) ribosome mutants were engineered through targeted modification of *RPS23*. Assays were performed in 96-well plates, and luminescence was recorded using a GloMax® Discover Microplate Luminometer. Data were analyzed by one-way ANOVA with Tukey's post hoc test.
Results: Wild-type ribosomes demonstrated high overall fidelity (~1 error per 10^6 codons). However, glycine codons GGA and GGG, as well as aspartic acid codons GAU and GAC, showed error rates up to 100-fold higher. Hyper-accurate mutants reduced activity at error-prone codons, while error-prone mutants increased activity, confirming the reporter's ability to discriminate misreading events. Genetic validation confirmed successful strain construction.
Conclusion: This reporter system provides a sensitive and reproducible platform to measure codon-specific misreading in eukaryotes. By revealing codon-dependent vulnerabilities and validating the role of ribosomal mutants, this approach enhances understanding of translational fidelity and its implications for protein misfolding and disease.

Keywords: Codon; Mutation; Ribosomes; Saccharomyces cerevisiae; Translational fidelity

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Introduction

The accurate translation of genetic information from messenger RNA (mRNA) into proteins is fundamental to maintaining cellular homeostasis and organismal viability. As the final step of the central dogma, translation ensures that the nucleotide code is faithfully decoded into amino acid sequences, safeguarding proteome integrity. Errors in this process, although rare, can have profound biological consequences. [1] Among the different types of translational errors, including nonsense errors and frameshifts, missense errors represent the most frequent and biologically significant category, arising when an incorrect amino acid is incorporated into a growing polypeptide chain due to tRNA mispairing with a near-cognate codon. [2] While individual errors may

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be tolerated, their accumulation can compromise protein folding, reduce enzymatic activity, and contribute to cellular stress. Increasing evidence links mistranslation events to age-related and neurodegenerative disorders such as Alzheimer's, Parkinson's, and Huntington's disease, where protein misfolding and aggregation are central pathogenic features. [3] Thus, a precise understanding of translational fidelity has both fundamental and clinical importance.

Over the past decades, significant progress has been made in elucidating the mechanisms governing ribosomal accuracy. The ribosome achieves fidelity through kinetic proofreading, induced fit, and structural discrimination between cognate and near-cognate tRNAs. [4] Despite these safeguards, error rates are non-uniform across codons, with certain codons more prone to misreading. For example, codons with relaxed wobble positions or those decoding rare tRNAs are disproportionately susceptible to errors. [5] Traditional approaches to measuring translational fidelity, such as dual-luciferase reporter assays, mass spectrometry of mistranslated proteins, and ribosome profiling, have advanced the field by providing quantitative insights into error rates and codon bias. [6] However, each approach has inherent limitations: ribosome profiling lacks single-codon resolution for missense discrimination, mass spectrometry is technically demanding and often restricted to abundant proteins, and conventional reporter systems may fail to distinguish true misreading events from functional amino acid replacements. [7, 8] These methodological constraints underscore the need for complementary tools capable of dissecting codon-specific error patterns with greater sensitivity and mechanistic clarity.

Saccharomyces cerevisiae offers a powerful model to address this gap. As a eukaryotic organism, yeast shares conserved translational machinery with higher eukaryotes, while also permitting precise genetic manipulation. [9] Furthermore, yeast ribosome mutants engineered to be hyper-accurate or error-prone provide unique opportunities to directly probe the molecular determinants of translational fidelity. [10] Leveraging this system, we developed a novel β-galactosidase–based reporter assay that allows quantification of missense errors at individual codons. By employing ribosomal accuracy mutants, this assay enables discrimination between increased activity due to misreading events and that due to functional amino acid replacement, a distinction that has remained challenging in prior methodologies.

The objective of this study was therefore to develop and validate a β-galactosidase reporter system in *S. cerevisiae* to quantify missense errors at codon-specific resolution, with particular emphasis on codons predicted to be error-prone, and to use hyper-accurate and error-prone ribosome mutants to mechanistically distinguish misreading from functional substitution. By addressing key limitations of existing tools, this approach could provide new insights into how codon identity and ribosomal fidelity mechanisms influence translational accuracy, with broader implications for understanding proteome maintenance and the etiology of protein misfolding diseases.

Materials and Methods

Experimental Design and Rationale

This study was designed to develop and validate a β-galactosidase–luciferase reporter system to quantify codon-specific missense errors in *Saccharomyces cerevisiae*. This study did not involve human participants, vertebrate animals, or recombinant DNA work requiring institutional review, and all procedures complied with institutional biosafety policies. The workflow consisted of three integrated components: (i) construction of a reporter system capable of detecting single-codon misreading events, (ii) genetic engineering of yeast strains with hyper-accurate and error-prone ribosome variants, and (iii) comparative luminescence assays to quantify translational errors. The β-galactosidase–luciferase system was chosen over traditional fidelity reporters (e.g., dual-luciferase or GFP-based systems) because it offers enhanced sensitivity, real-time quantification of enzyme activity, and a low background signal, thereby improving the resolution of rare missense events.

Reporter System

Reporter constructs were generated based on the *lacZ* gene with a single codon substitution introduced at position 537, a site essential for β-galactosidase activity. Correct incorporation of glutamic acid at this site restores enzyme function, while misincorporation of near-cognate amino acids yields partial activity measurable by

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luminescence. Functional β-galactosidase hydrolyzed a synthetic substrate (Galacto-Light Plus™, Thermo Fisher Scientific), releasing luciferin, which was oxidized by luciferase to produce light.

Assays were performed in 96-well microplates using a GloMax® Discover Microplate Luminometer (Promega, WI, USA), with an integration time of 1 second per well and a dynamic range of 10^2–10^9 Relative Light Units (RLU). Background luminescence was determined using negative control wells containing substrate only and was subtracted from sample readings. To ensure accuracy, RLU values were normalized to the activity of the wild-type GAA codon reporter. Each assay included at least three biological replicates and three technical replicates. To confirm that luminescence reflected true missense events rather than background enzymatic activity, synonymous non-cognate codons were included as negative controls.

Strain Construction

All yeast strains were derived from *S. cerevisiae* BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0). Codon variants of the *lacZ* gene were introduced using site-directed mutagenesis and cloned into a centromeric plasmid under the control of the constitutive PGK1 promoter. Near-cognate substitutions (e.g., GGA and GGG for glycine; GAU and GAC for aspartic acid) and synonymous non-cognates were designed based on codon similarity to the wild-type GAA codon.

Hyper-accurate (A113V) and error-prone (K63R) ribosome mutants were generated by targeted modification of the *RPS23* gene, which encodes a ribosomal protein of the small subunit. Gene replacement was achieved by homologous recombination using PCR-amplified fragments containing the desired mutation and flanked by homology arms. Double knockouts were created by replacing *RPS23A* in ΔS23B strains using a NATMX resistance marker. Transformants were selected on YPD plates supplemented with nourseothricin and verified by PCR with primers flanking *S23A*, *S23B*, KANMX, and NATMX regions. Selected clones were further confirmed by Sanger sequencing of the mutated loci to exclude compensatory or unintended mutations.

Assays and Measurements

Yeast cultures were grown in selective synthetic defined (SD) media lacking uracil at 30 °C with shaking (200 rpm) until mid-log phase (OD600 ≈ 0.6). Cultures were standardized to equal optical densities before reporter assays. Following substrate addition, luminescence was recorded at 5-minute intervals for 30 minutes to capture reaction kinetics, after which peak RLU values were used for analysis.

Comparisons were performed between wild-type, hyper-accurate, and error-prone strains. Synonymous non-cognate codons served as negative controls to confirm that observed activity was due to misreading rather than functional replacement. Statistical analyses were conducted using GraphPad Prism 10 (GraphPad Software, San Diego, CA). Data are reported as mean ± standard deviation (SD). Differences between groups were evaluated using one-way ANOVA followed by Tukey's multiple-comparisons test, with $p < 0.05$ considered significant.

Reproducibility and Transparency

All experiments adhered to best-practice guidelines for genetic and biochemical reporting to ensure reproducibility. At least three independent biological replicates were performed for each experimental condition. Plasmids, strains, and primers generated in this study are available upon reasonable request from the corresponding author.

Results

Validation of the Reporter Assay

To establish the reliability of the β-galactosidase–luciferase system for quantifying missense errors, we first assessed its sensitivity, specificity, and reproducibility. Wild-type *lacZ* constructs containing the canonical GAA codon at position 537 produced robust luminescence signals, confirming restoration of β-galactosidase activity. In contrast, synonymous non-cognate variants yielded baseline readings comparable to negative controls (substrate-only wells), demonstrating that luminescence directly reflected functional enzyme activity and not background noise.

The assay exhibited a broad dynamic range, spanning four orders of magnitude (10^2–10^6 RLU), with linearity between luminescence and enzyme

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concentration ($R^2 > 0.98$). Replicate measurements across three independent experiments showed low variability (coefficient of variation < 8%), confirming assay reproducibility. These findings validated the system as a sensitive and specific tool for quantifying codon-specific missense errors.

Codon-Specific Error Rates in Wild-Type Strains

Using the validated reporter, we next quantified missense error rates across selected codons in wild-type ribosomes. As expected, the *S. cerevisiae* translation machinery displayed high overall fidelity, with an estimated baseline error frequency of ~1 in 10^6 codons. However, specific codons exhibited substantially elevated error rates. For glycine codons, GGA and GGG demonstrated up to a 100-fold increase in luminescence compared with the wild-type GAA codon ($p < 0.001$), indicating high susceptibility to misreading. Importantly, synonymous non-cognates for glycine did not produce detectable activity, supporting the conclusion that the observed signals arose from misreading rather than functional substitution (Figure 1). Similarly, aspartic acid codons GAU and GAC showed significantly higher activity than baseline ($p < 0.01$), reinforcing the notion that these codons are inherently error-prone (Table 1).

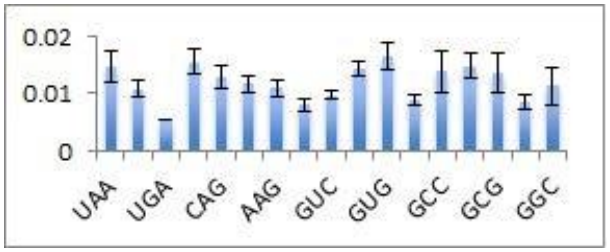

Figure 1: Codon-Specific Missense Error Rates

Codon-specific trends correlated with known molecular determinants of fidelity, including wobble base-pairing potential and relative tRNA abundance. These results demonstrate that certain codons are disproportionately vulnerable to mistranslation, even within the context of otherwise accurate ribosomes.

Table 1: Codon-Specific Error Rates Across Ribosomal Variants

Codon (Amino Acid)	Wild-Type RLU (Mean ± SD)	Hyper-Accurate (A113V) RLU (Mean ± SD)	Error-Prone (K63R) RLU (Mean ± SD)	Fold Change (vs WT)	*p*-value	Interpretation
GAA (Glu, WT control)	1.00 ± 0.05	0.98 ± 0.07	1.03 ± 0.06	—	—	Baseline activity
GGA (Gly)	85.3 ± 6.2	80.7 ± 5.5	90.4 ± 7.1	↑ ~85-fold	<0.001	High misreading susceptibility
GGG (Gly)	92.1 ± 8.4	88.9 ± 6.7	95.2 ± 8.0	↑ ~92-fold	<0.001	Wobble mispairing prone
GAU (Asp)	47.8 ± 5.1	33.2 ± 4.0	65.5 ± 6.8	↑ ~48-fold	<0.01	Strong fidelity modulation
GAC (Asp)	42.5 ± 4.3	30.8 ± 3.9	61.0 ± 6.2	↑ ~42-fold	<0.01	Proofreading-dependent misreading

Abbreviations: RLU = Relative Light Units (normalized to GAA control).
Note: Data represent mean ± standard deviation (n = 3 biological replicates). Significance determined by one-way ANOVA with Tukey's post hoc test.

Effect of Hyper-Accurate and Error-Prone Ribosome Mutants

To probe the mechanistic basis of codon-specific errors, we compared wild-type strains with ribosomal mutants engineered for altered fidelity. In hyper-accurate A113V mutants, activity from GAU and GAC codons was significantly reduced relative to wild type ($p < 0.01$), consistent with suppression of misreading events. Conversely, error-prone K63R mutants displayed elevated activity at the same codons ($p < 0.001$), confirming that the reporter captured genuine increases in misreading frequency rather than amino acid functional replacement.

Interestingly, for glycine codons GGA and GGG, the increase in activity observed in error-

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prone mutants was modest compared with aspartic acid codons, suggesting codon- and context-dependent differences in susceptibility to ribosomal proofreading mechanisms. Together, these findings establish that the reporter system can discriminate between error rates modulated by ribosomal accuracy variants, thereby providing mechanistic insight into the fidelity of decoding.

Verification of Mutant Strains

Genetic validation confirmed successful construction of the ribosome fidelity mutants. PCR analysis verified replacement of *RPS23A* with the NATMX marker in ΔS23B strains, while sequencing confirmed the presence of the A113V and K63R mutations without additional unintended substitutions (Figure 2). Residual wild-type activity observed in some knockouts was attributed to incomplete replacement of *RPS23A*, a result consistent with strain background effects.

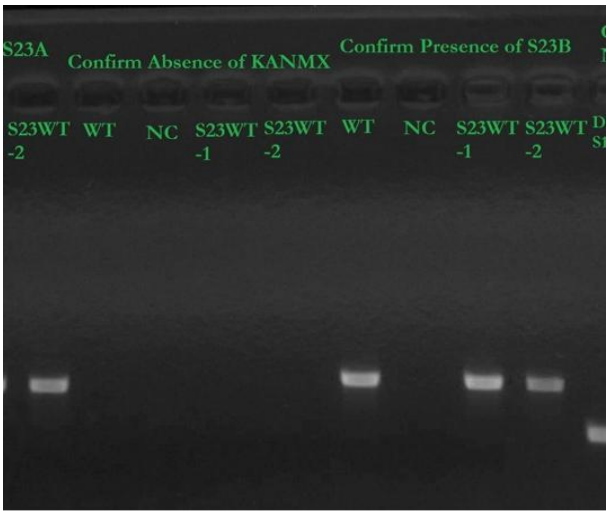

Figure 2: PCR Validation of Mutant Strains

In addition, elimination of *URA3* from the genome allowed its reuse as a selectable marker in subsequent assays, ensuring experimental flexibility. Collectively, these controls verified the genetic integrity of the strains used and ruled out confounding contributions from unintended mutations.

Discussion

This study establishes a novel β-galactosidase–luciferase reporter system as a robust tool for quantifying codon-specific missense errors in *Saccharomyces cerevisiae*. Unlike traditional approaches such as dual-luciferase assays, ribosome profiling, or mass spectrometry, this system offers single-codon resolution and, importantly, enables discrimination between true misreading events and functional amino acid replacements. By integrating wild-type, hyper-accurate, and error-prone ribosome mutants, the assay provides mechanistic insights into translational fidelity that extend beyond descriptive quantification. This represents a significant methodological advance, with implications for both fundamental molecular biology and biomedical research into protein misfolding disorders.

Accurate decoding is essential for maintaining proteome integrity, yet our findings confirm that fidelity is not uniform across the genetic code. [11] Specifically, glycine codons (GGA, GGG) and aspartic acid codons (GAU, GAC) were identified as disproportionately error-prone. The ability to pinpoint these codon-specific vulnerabilities is valuable for understanding how sequence context shapes ribosomal decoding. Such insights are particularly relevant for human health, as mistranslation contributes to the proteotoxic stress implicated in neurodegenerative diseases, including Alzheimer's, Parkinson's, and Huntington's disease. By demonstrating that codon-level differences can be resolved in a eukaryotic system, our work paves the way for translational applications in disease modeling.

Our results align with earlier observations that ribosomes maintain high overall fidelity, typically on the order of one error per 10^6 codons. [12, 13] However, the codon-specific heterogeneity we observed echoes findings from ribosome profiling and mass spectrometry studies, which have reported elevated error rates at codons subject to wobble pairing or limited tRNA availability. [14] Notably, prior assays often lacked the sensitivity to distinguish between misreading and functional replacement. By showing that synonymous non-cognates did not produce luminescence in our system, we confirm that the increased activity reflects genuine misreading events.

Interestingly, while previous studies have emphasized glycine codons as frequent mistranslation hotspots, our work extends this to aspartic acid codons, which exhibited strong modulation by ribosomal fidelity mutants. [15] The discrepancy between codon families likely reflects structural and kinetic factors; glycine codons may be inherently prone to wobble mispairing, whereas aspartic acid codons appear more sensitive to

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ribosomal proofreading efficiency.

Mechanistically, the error-proneness of GGA and GGG codons is consistent with their potential to form near-cognate interactions at the wobble position, increasing the likelihood of tRNA misincorporation. In contrast, the pronounced effects of hyper-accurate and error-prone mutations on GAU and GAC suggest that aspartic acid codons are particularly influenced by alterations in ribosomal proofreading mechanisms. These differences underscore the multifactorial nature of decoding fidelity, involving codon context, tRNA abundance, and ribosomal structural dynamics.

Residual activity observed in strains with incomplete *RPS23A* knockouts likely reflects background expression from unreplaced alleles. [16] While such genetic background effects complicate interpretation, they also highlight the sensitivity of the system in detecting even partial contributions of wild-type ribosomes.

While the findings are compelling, several limitations must be acknowledged. First, the study focused on a narrow set of codons; a more comprehensive analysis of the full codon spectrum would provide a broader understanding of codon-specific fidelity. Second, experiments were performed in laboratory yeast strains under controlled growth conditions, which may not fully capture how environmental stresses influence error rates. Third, while the reporter system effectively distinguishes misreading from functional replacement, it does not capture downstream consequences such as protein misfolding or degradation. Thus, the system should be viewed as complementary to, rather than a replacement for, proteomics-based methods.

Integrating this approach with ribosome profiling or high-throughput sequencing would provide a multi-layered view of fidelity, combining codon-level resolution with genome-wide coverage. Such studies could ultimately inform therapeutic strategies aimed at modulating ribosomal accuracy in disease contexts.

Conclusion

In summary, this work introduces a sensitive and specific β-galactosidase–luciferase reporter system that quantifies missense errors at single-codon resolution in a eukaryotic model. By revealing codon-specific vulnerabilities and validating the mechanistic role of ribosomal fidelity mutants, our study advances understanding of translational accuracy. While limitations remain, the approach provides a foundation for future research exploring how decoding errors shape proteome integrity and contribute to human disease.

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Author Contribution

Z.A. conceived and designed the study. S.T. performed the experiments, curated and analyzed the data. P.J.F. and Z.A. drafted the manuscript. P.J.F. supervised the project, validated the findings, contributed to data interpretation, and critically revised the manuscript. All authors reviewed and approved the final version and agreed to be accountable for all aspects of the work.

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Conflict of Interest

The author has no conflicts of interest to disclose.

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