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Inducing nonsense suppression by targeted pseudouridylation

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Abstract

Isomerization from uridine to pseudouridine (pseudouridylation) is largely catalyzed by a family of small ribonucleoproteins called box H/ACA RNPs, each of which contains one unique small RNA—the box H/ACA RNA. The specificity of the pseudouridylation reaction is determined by the base-pairing interactions between the guide sequence of the box H/ACA RNA and the target sequence within an RNA substrate. Thus, by creating a new box H/ACA RNA harboring an artificial guide sequence that base-pairs with the substrate sequence, one can site-specifically introduce pseudouridines into virtually any RNA (e.g., mRNA, ribosomal RNA, small nuclear RNA, telomerase RNA and so on). Pseudouridylation changes the properties of a uridine residue and is likely to alter the role of its corresponding RNA in certain cellular processes, thereby enabling basic research into the effects of RNA modifications. Here we take a *TRM4* reporter gene (also known as *NCL1*) as an example, and we present a protocol for designing a box H/ACA RNA to site-specifically pseudouridylate TRM4 mRNA. Disease-related mutation can result in early termination of translation by creating a premature termination codon (PTC); however, pseudouridylation at the PTC can suppress this translation termination (nonsense suppression). Thus, the experimental procedures described in this protocol may provide a novel way to treat PTC-related diseases. This protocol takes 10–13 d to complete.

INTRODUCTION

Pseudouridine (ψ), the 5'-ribosyl isomer of uridine¹, was once considered as the fifth ribonucleoside in addition to the four other canonical ribonucleosides, namely adenosine, guanosine, cytidine and uridine². Compared with uridine, the most distinct feature of ψ is the presence of an extra hydrogen-bond donor at its N1 position (Fig. 1a), which allows ψ to be involved in inter- or intramolecular hydrogen bonding through a water bridge. The water-bridged intramolecular hydrogen-bonding network is formed between the water molecule and the N1 imino-hydrogen and the 5' phosphate oxygen of ψ as well as the phosphate oxygen of the 5' preceding nucleotide³. In addition, ψ has been shown to improve base-stacking interactions within RNAs⁴ and be capable of altering RNA structure⁵. Furthermore, ψ is more polar than uridine⁶. In short, the presence of ψ in RNA could profoundly influence the properties of the RNA molecule and the cellular process in which it participates.

Pseudouridylation is a naturally occurring process that occurs post-transcriptionally. In eukaryotic nuclei, a group of noncoding RNAs, termed box H/ACA guide RNAs, are

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responsible for directing site-specific pseudouridylation of ribosomal RNA (rRNA) and spliceosomal small nuclear RNA^{7,8}. Structurally, box H/ACA RNAs assume a functional hairpin-hinge-hairpin-tail conformation^{9,10} (Fig. 1b). The conserved box 'H' (5'-ANANNA-3', where *N* is any nucleotide) resides in the hinge region, whereas the box 'ACA' (5'-ACA-3') is located in the tail region just three nucleotides upstream of the 3' end¹¹. Within each hairpin, there is a single-stranded internal loop termed the pseudouridylation pocket, consisting of two discontinuous tracts of guide sequences (g1 and g1', and g2 and g2') that provide pseudouridylation-site specificity (Fig. 1b). Indeed, the guide sequences recognize the substrate RNA by base-pairing interactions^{12,13}. Notably, the uridine residue to be pseudouridylated is always positioned between the two tracts of guide sequences and remains unpaired. The pseudouridylation site is usually 14–16 nt upstream of either box H or box ACA.

By constructing artificial guide RNAs, we recently demonstrated that site-specific modification can be introduced post-transcriptionally into a variety of RNA molecules, including mRNAs^{14–16}, spliceosomal small nuclear RNAs¹⁷, rRNAs and telomerase RNA¹⁸. The ability to manipulate RNA modification allows us to probe RNA structure, identify important nucleotides and study RNA function.

Translation of an mRNA terminates when the ribosome encounters a nonsense/termination codon (UAA, UGA or UAG) in an mRNA^{19–21}. Termination at one of these three nonsense codons yields a functional full-length protein. However, genetic mutations have been shown to generate a PTC within genes implicated in a large number of human diseases, including muscular dystrophy, cystic fibrosis and various types of cancer. When translation terminates at a PTC, a nonfunctional C-terminally truncated protein is produced, potentially leading to deleterious effects on cell survival and organ function²². Approximately one-third of human genetic diseases may be associated with PTCs²³. Therefore, developing a protocol to specifically attenuate undesirable translation termination is attractive to both the academic and clinical communities.

The fact that a uridine residue appears in the first position of all three nonsense codons prompted us to investigate the possibility of whether introducing pseudouridylation into a PTC would result in nonsense suppression. To test this idea, we took advantage of the yeast *Saccharomyces cerevisiae* system and the well-established yeast box H/ACA guide RNAs²⁴. Specifically, we generated a plasmid-borne PTC-containing TRM4 mRNA as a substrate and an artificial box H/ACA RNA (Fig. 1b) to target the PTC for pseudouridylation. Our data have shown that the uridine of this PTC is indeed pseudouridylated and that the pseudouridylated PTC triggers translational read-through (nonsense suppression) to produce a full-length Trm4 protein in yeast. Mass spectrometry indicated that the amino acids inserted at pseudouridylated PTCs are either serine/threonine (for ψ AA and ψ AG) or tyrosine/phenylalanine (for ψ GA)²⁵. Specifically, although ψ AA codes for serine and threonine almost equally well, ψ AG codes mostly for serine (90%) and far less efficiently for threonine (10%). On the contrary, ψ GA codes mostly for tyrosine (80%) and less efficiently for phenylalanine (20%). Currently, it is not clear as to why specific amino acids are used to decode a particular pseudouridylated PTC. Nonetheless, our data suggest that RNA-guided pseudouridylation of RNA may represent a novel approach for inducing nonsense suppression *in vivo*. Although insertion of the amino acids (serine/threonine or tyrosine/phenylalanine) at the pseudouridylated PTCs could change the property of the protein, the benefit of producing a full-length protein, although not exactly a wild type (WT), often outweighs the harm that a single amino acid mutation brings. Indeed, a successful rescue of protein function through nonsense suppression was observed (judged by growth phenotype)²⁵.

With respect to codon alterations, this approach also offers the possibility to study the potential expansion of the genetic code. As specific amino acids (serine/threonine or tyrosine/phenylalanine) can be incorporated at pseudouridylated PTCs, it is likely that pseudouridylation of sense codons will result in changes of coding specificity as well. In the canonical genetic code, over 50% of codons contain uridines. In theory, any of these codons can be targeted for pseudouridylation and codon alteration.

Our artificial box H/ACA guide RNAs were all derived from a naturally occurring box H/ACA RNA (snR81) with only guide sequences being altered, thus affording a simple and straightforward method. Notably, given the large number of patients who have PTC-related genetic diseases, such as cystic fibrosis, Duchenne muscular dystrophy, β -thalassemia and Hurler syndrome, this strategy holds promise for clinical applications as well.

One relatively major limitation of this protocol is the low efficiency of nonsense suppression in yeast cells. On the basis of western blot analysis, the efficiency of nonsense suppression is currently about 5–10% (read-through product/WT product). In our experience, the efficiency of nonsense suppression directly correlates with the efficiency of PTC pseudouridylation. Thus, our future goal is to improve the efficiency of mRNA pseudouridylation. This can probably be achieved through manipulating the box H/ACA RNAs used to guide ψ formation.

Experimental design

Construction of a TRM4-PTC expression cassette—To evaluate the potential power of pseudouridylation in nonsense suppression, we first must generate a PTC-containing mRNA that is stably expressed in yeast. To this end, we chose a yeast 2 μ plasmid, pTRM4-WT (Fig. 2), which encodes a C-terminally tagged Trm4p. The endogenous *TRM4* gene encodes an *S*-adenosyl-L-methionine-dependent tRNA methyltransferase²⁶. pTRM4-WT was a generous gift from E. Phizicky/E. Grayhack's laboratory. This system is ideal because it is capable of producing a large amount of C-terminally tagged Trm4p in yeast cells²⁷, which will greatly facilitate the subsequent detection and purification of the nonsense suppression product.

Although all three nonsense codons (UAA, UAG, UGA) are available for targeting, here we take UAA as an example. For two reasons, we chose the phenylalanine at position 602 (F602) in Trm4p as the site for a PTC. First, the proximity of the F602 codon to the 3' end of the gene maximized the stability of PTC-containing TRM4 mRNA, which would otherwise be degraded by nonsense-mediated mRNA decay²². Second, after tryptic digestion, F602 is located in the middle of a peptide fragment consisting of 12 aa residues (596–607; Fig. 3) that can be quantified by mass spectrometry, thus facilitating the identification of any amino acid encoded by the pseudouridylated PTC.

The introduction of a mutation in TRM4 mRNA at F602 was accomplished by using the site-directed mutagenesis kit from Stratagene together with pTRM4-WT and appropriate oligodeoxynucleotide primers (TRM4-F602X(TAA)-F1 and TRM4-F602X(TAA)-R1). As a result, the codon of F602 was changed from TTT to TAA, thus generating pTRM4-F602X(TAA). To target the PTC, an artificial TRM4-F602X(TAA)-specific box H/ACA guide RNA (based on the yeast naturally occurring snR81 box H/ACA RNA)²⁴ was also constructed.

Construction of artificial TRM4-F602X(TAA)-specific snR81 box H/ACA RNAs

—Construction of an artificial snR81 is facilitated by a four-primer overlapping PCR strategy. Note that as an alternative, a two-primer overlapping PCR or single oligonucleotide strategy, which require two long primers (~100 nt) or one overly long oligonucleotide (> 82

nt), respectively, could also be used to construct the artificial guide RNA. However, from a cost-savings perspective, it is desirable to use four-primer PCR (as four short oligonucleotides are much less expensive than one or two long oligonucleotides). Given the length of snR81 (182 nt) and the discontinuous guide sequences in each pseudouridylation pocket, it is convenient to design four overlapping pieces of oligodeoxynucleotide primers, each containing one guide sequence. Of the four pieces, there is one sense strand, snR81-TRM4-F602X(TAA)-F1, and three antisense strands, snR81-TRM4-F602X(TAA)-R1, snR81-TRM4-F602X(TAA)-R2 and snR81-TRM4-F602X(TAA)-R3. Within each primer, the guide sequences ($g1$, $g1'$, $g2$, $g2'$; see Figs. 1b and 4) were altered according to the target sequence (TRM4 mRNA) both at and flanking the PTC. Except for the guide sequences, all the other nucleotides of snR81 remain unchanged. Notably, the first (U) and second (A) residues of the PTC codon should be left unpaired by guide sequences to allow for pseudouridylation. In addition, using both pseudouridylation pockets of snR81 to target the PTC will enhance the modification efficiency.

In the four-primer overlapping PCR reaction, the four primers serve as templates for each other and are used consecutively to create an artificial snR81 (Fig. 4). The PCR reaction requires a different molar concentration for each of the four primers to efficiently generate the full-length snR81 (a 210-nt-long PCR product). Specifically, the overall relative ratio (in terms of $\text{pmol } \mu\text{l}^{-1}$) is 20:1:1:20 for the primers snR81-TRM4-F602X(TAA)-F1:snR81-TRM4-F602X(TAA)-R1:snR81-TRM4-F602X(TAA)-R2:snR81-TRM4-F602X(TAA)-R3. If the PCR products are heterogeneous, one can purify the band of correct size and then perform a second round of PCR using the purified band as template and snR81-TRM4-F602X(TAA)-F1 and snR81-TRM4-F602X(TAA)-R3 as primers. The PCR products are then digested with restriction enzymes and inserted into a high-copy *Escherichia coli* yeast shuttle plasmid, pSEC (snoRNA expression cassette, available on request)²⁸, between BamHI and HindIII sites. The transcription of artificial snR81 is under the control of the Gal promoter, which turns on in the presence of galactose.

Similarly, in a control experiment, we designed four different oligodeoxynucleotide primers (snR81-Control-F1, snR81-Control-R1, snR81-Control-R2, snR81-Control-R3) to generate psnR81-Control, whose guide sequences were altered such that they have no targets within the yeast genome. Thus, psnR81-Control was used to serve as a guide-specificity control (Fig. 5 and Table 1).

Transformation of yeast cells—The *S. cerevisiae* parental strain, BY4741, was used. Given the genotype of BY4741 (MATa his3 Δ 1 leu2 Δ 0 met15 Δ 0 ura3 Δ 0), *URA3* and *LEU2* genes were chosen as markers for selecting pTRM4 and psnR81 variants, respectively. Two alternate yeast transformation strategies can be used: co-transformation and sequential transformation. For co-transformation, both the pTRM4 and psnR81 variants are transformed into competent yeast cells in a single experiment, and transformants are screened on a –Leu/–Ura double-dropout solid medium. Sequential transformation, however, involves two independent consecutive transformations of the pTRM4 and psnR81 variants. Practically, co-transformation yields relatively lower (but still acceptable) transformation efficiency but is much less laborious compared with sequential transformation. Thus, only the co-transformation procedure is described in this protocol.

Detection of TRM4 expression—To prepare the yeast crude extract, cells are grown to mid-log phase in SGal–Leu–Ura double-dropout liquid medium. Because we experienced loss of expression of the plasmid-borne gene owing to the relaxation of auxotrophic pressure, YPGal medium should not be used to grow transformed cells to prepare extract. The presence of 2% (wt/wt) galactose in the culture medium is essential for activating the

Gal promoter in the pSEC plasmid, which drives expression of both the TRM4 and snR81 variants.

For small-scale preparation of yeast crude extract (1 ml of settled wet cells), the sterile acid-washed glass beads method is ideal (see PROCEDURE). For large-scale preparation, we recommend using a homogenizer to process 10–20 ml of settled wet cells and using a mortar and pestle or French press to process more than 20 ml wet cells.

To evaluate the expression level of Trm4p, monoclonal anti-protein A (Sigma-Aldrich) and anti-Eno1p (gift from M. Dumont) are used to visualize both Trm4p and Eno1p (as a loading control). Eno1p is the phosphopyruvate hydratase that catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate during glycolysis²⁹. Although the two primary antibodies are compatible with each other, we recommend that the membrane be probed sequentially to greatly reduce the nonspecific bands and background. Other than *Eno1p*, many housekeeping genes in *S. cerevisiae* could also serve as loading controls. If hybridization is carried out at 4 °C, the primary antibody solution can be reused several times.

MATERIALS

REAGENTS

- Oligodeoxyribonucleotides (Table 1)
- Taq DNA polymerase (5 U μl^{-1} ; Fermentas, cat. no. EP0401)
- Taq DNA polymerase buffer (10 $\times$; Fermentas, cat. no. B34)
- dNTPs (10 mM; Fermentas, cat. no. R0181)
- DNA loading dye (6 $\times$; Fermentas, cat. no. R0611)
- Pfu DNA polymerase (5 U μl^{-1} and 10 $\times$ buffer; Stratagene, cat. no. 600250)
- BamHI restriction endonuclease (Fermentas, cat. no. ER0051) and 10 $\times$ buffer (cat. no. B57)
- HindIII restriction endonuclease (Fermentas, cat. no. ER0501) and 10 $\times$ buffer (cat. no. BR5)
- T4 DNA ligase (1 U μl^{-1} and 5 $\times$ buffer; Fermentas, cat. nos. EL0011 and B69)
- DH5 α -competent cells (Invitrogen, cat. no. 18265-017)
- XL1-blue-competent cells (Stratagene, cat. no. 200521)
- Phenol (Alfa Aesar, cat. no. A15760-0E) ! **CAUTION** It is a corrosive solution; wear gloves while handling.
- Chloroform (J.T. Baker, cat. no. 9182-01) ! **CAUTION** It is a corrosive solution; wear gloves while handling.
- Isoamyl alcohol (Sigma-Aldrich, cat. no. I9392) ! **CAUTION** It is a highly flammable solution; handle in a fume hood.
- Glycogen (Sigma-Aldrich, cat. no. G0885)
- Sodium acetate (J.T. Baker, cat. no. 127093)
- Ethanol (EMD Millipore, cat. no. EM-4450S) ! **CAUTION** It is a highly flammable solution; handle in a fume hood.

- Agarose (VWR international, cat. no. VW1468-07)
- Ethidium bromide (VWR International, cat. no. VW1475-01) ! **CAUTION** It is a carcinogen; wear gloves while handling.
- Ampicillin (IBI Scientific, cat. no. IB02040)
- *S. cerevisiae* strain BY4741 (Open Biosystems, cat. no. YSC1048)
- Yeast extract (BD Diagnostics, cat. no. 90000-444)
- Peptone (BD Diagnostics, cat. no. 90000-264)
- Sodium chloride (NaCl; BDH, cat. no. BDH4534-5KGP)
- Agar (EMD Millipore, cat. no. EMD-10283.0201)
- Yeast nitrogen base (AMRESCO, cat. no. 97064-322)
- Ammonium sulfate (EMD Millipore, cat. no. EM-AX1385-1)
- L-Isoleucine (Sigma-Aldrich, cat. no. I2752)
- L-Valine (Sigma-Aldrich, cat. no. V0500)
- Adenine hemisulfate salt (Sigma-Aldrich, cat. no. A9126)
- L-Arginine monohydrochloride (Sigma-Aldrich, cat. no. A5131)
- L-Histidine monohydrochloride (Sigma-Aldrich, cat. no. H8125)
- L-Lysine monohydrochloride (Sigma-Aldrich, cat. no. L5626)
- L-Methionine (Sigma-Aldrich, cat. no. M9625)
- L-Phenylalanine (Sigma-Aldrich, cat. no. P2126)
- L-Tryptophan (Sigma-Aldrich, cat. no. T0254)
- L-Tyrosine (Sigma-Aldrich, cat. no. T3754)
- Galactose (J.T. Baker, cat. no. M672-07)
- Lithium acetate (Alfa Aesar, cat. no. AA13417-30)
- Polyethylene glycol (PEG-3350; J.T. Baker, cat. no. JTU221-8)
- Tris base (J.T. Baker, cat. no. JTX171-3)
- HCl (J.T. Baker, cat. no. JT9535-1) ! **CAUTION** It is a corrosive solution, handle in fume hood with protective gear.
- HEPES (EMD Millipore, cat. no. EM-5320)
- Potassium chloride (KCl; EMD Millipore, cat. no. EM-PX1405-1)
- Magnesium chloride (MgCl₂; EMD Millipore, cat. no. EM-MX0045-1)
- Boric acid (EMD Millipore, cat. no. EM-BX0865-3)
- EDTA (EMD Millipore, cat. no. EM-EX0539-5) ! **CAUTION** It is an irritant.
- DTT (EMD Millipore, cat. no. EM-3860)
- PMSF (Sigma-Aldrich, cat. no. P7626) ! **CAUTION** It is an irritant.
- Glycerol (J.T. Baker, cat. no. JT2136-3)
- Acid-washed glass beads (0.5 mm; BioSpec Products, cat. no. 11079105)

- Tris-HCl ready gels (4–15% (wt/vol); Bio-Rad, cat. no. 161-1104)
- Tris-glycine-SDS buffer (10×; Bio-Rad, cat. no. 161-0732)
- Tris-glycine buffer (10×; Bio-Rad, cat. no. 161-0734)
- Methanol (Pharmaco-AAPER, cat. no. 339000000) ! **CAUTION** It is a highly flammable solution; handle it in a fume hood.
- BSA (Rockland Immunochemical, cat. no. RLBSA50)
- Tween-20 (J.T. Baker, cat. no. X251-07)
- Monoclonal anti-protein A, mouse (Sigma-Aldrich, cat. no. P2921)
- Antibody against Eno1p (a generous gift from M. Dumont, University of Rochester Medical Center)
- Goat anti-mouse IgG (H + L)-AP conjugate (Bio-Rad, cat. no. 170-6520)
- One-step NBT/BCIP (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate; Pierce, cat. no. 34042)
- ddH₂O

EQUIPMENT

- Petri dishes (VWR International, cat. no. 25384-088)
- Miniprep kit (Qiagen, cat. no. 27104)
- Gel purification kit (Qiagen, cat. no. 28704)
- NanoDrop spectrophotometer (Thermo Scientific)
- UV spectrophotometer (Agilent Technologies, Cary 50 Bio)
- Screw-cap tubes (2 ml; VWR International, cat. no. 16466-040)
- Screw-cap with O-ring (VWR International, cat. no. 16466-080)
- Filter paper (Whatman, cat. no. 21427-411)
- Nitrocellulose membranes (0.1 µm; Whatman, cat. no. 10402096)
- PCR machine (PCR Thermal Cycler MP, Takara Bio)
- Sorvall RC-5C Plus centrifuge
- Incubators (30 °C and 37 °C; Thermo Scientific Precision)
- Orbital heated shaker (200 r.p.m.; Phoenix Equipment)
- Mini gel electrophoretic cell (Bio-Rad, cat. no. 165-3301)
- Mini blot transfer cell (Bio-Rad, cat. no. 170-3930)
- Fume hood (Kewaunee Scientific)
- Autoclave (BetaStar)
- Rocker platform (VWR International)
- Parafilm
- Benchtop centrifuge

REAGENT SETUP

Glycogen (10 mg ml⁻¹) Dissolve 100 mg of glycogen in 10 ml of ddH₂O. It can be stored at – 20 °C for a year.

TBE buffer (5×) To prepare TBE buffer, mix 445 mM Tris, 445 mM boric acid and 16 mM EDTA. This buffer can be stored at room temperature (about 23–25 °C) for 1 month.

TBE buffer (0.5×) Mix 100 ml of 5× TBE buffer with 900 ml of ddH₂O. This buffer should be freshly prepared.

LB liquid medium To prepare LB medium, dissolve 10 g of NaCl, 10 g of peptone and 5 g of yeast extract; fill to 1 liter with ddH₂O and autoclave. This medium should be freshly prepared.

LB-ampicillin solid medium To prepare LB-ampicillin solid medium, mix 20 g of agar, 10 g of sodium chloride, 10 g of peptone, 5 g of yeast extract; fill to 1 liter with ddH₂O and autoclave. Allow the mixture to cool and add 1 ml of 100 mg ml⁻¹ ampicillin and mix well. Pour 20–25 ml of medium in Petri dishes to achieve a bed height of ~0.5 cm. This solid medium should be freshly prepared.

YPD liquid medium To prepare YPD liquid medium, mix 10 g of yeast extract, 20 g of peptone and 20 g of dextrose; fill with ddH₂O to 1 liter and autoclave. This medium should be freshly prepared.

One-step transformation buffer This buffer is prepared by combining 100 mM lithium acetate (pH does not need to be adjusted) and 50% (wt/vol) PEG-3350 solution. It can be stored at – 20 °C for 1 year.

SGal–Leu–Ura double-dropout liquid medium Liquid medium is prepared by mixing 7.5 g of synthetic leucine/uracil double-dropout powder from Table 2 and 20 g of galactose; fill to 1 liter with ddH₂O and autoclave. The liquid medium should be freshly prepared.

SGal–Leu–Ura double-dropout solid medium Solid medium is prepared by mixing 7.5 g of synthetic leucine/uracil double-dropout powder from Table 2, 20 g of agar and 20 g of galactose; fill to 1 liter with ddH₂O and autoclave. Pour 20–25 ml medium in Petri dishes to achieve a bed height of ~0.5 cm. The solid medium should be freshly prepared.

Phenol/chloroform/isoamyl alcohol (PCA) PCA (25:24:1 (vol/vol/vol)) saturated (1:1 (vol/vol)) with 20 mM Tris-HCl (pH 8.0). Tris-HCl–saturated PCA can be aliquotted to 50-ml tubes and stored at 4 °C for several months. Discard if the phenol phase turns pink.

G50 buffer To prepare G50 buffer, mix 20 mM Tris-HCl at pH 7.5, 300 mM sodium acetate, 2 mM EDTA and 0.2% (wt/vol) SDS. G50 buffer can be stored at room temperature for more than 2 months.

Yeast extract preparation buffer To prepare yeast extract preparation buffer, mix 20 mM HEPES at pH 7.9, 200 mM KCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF (add before use) and 20% (vol/vol) glycerol. It can be stored at 4 °C for 1 month.

Western blot running buffer Mix 100 ml of 10× Tris-glycine-SDS buffer with 900 ml ddH₂O. Freshly prepare and precool it to 4 °C before use.

Western blot transferring buffer Mix 100 ml of 10× Tris-glycine buffer with 200 ml of methanol and 700 ml of ddH₂O. Freshly prepare and precool it to 4 °C before use.

Western blot washing buffer Mix 100 ml of 10× Tris-glycine buffer with 100 µl of Tween-20 and 899.9 ml of ddH₂O. Freshly prepare and use it at room temperature.

Western blot blocking buffer Dissolve 5 g of BSA in 100 ml of ddH₂O, and then pass it through a 0.2-µm filter. Freshly prepare and use it at room temperature.

Ethanol (70%) Mix 30 ml of ethanol with 70 ml of ddH₂O. It can be stored at room temperature for 1 year.

EQUIPMENT SETUP

PCR thermal cycler program 1 One cycle of 95 °C for 30 s; 18 cycles of 95 °C for 30 s, 55 °C for 1 min and 68 °C for 9 min; and 1 cycle of 72 °C for 2 min.

PCR thermal cycler program 2 One cycle of 95 °C for 2 min; 35 cycles of 95 °C for 30 s, 42 °C for 30 s, 72 °C for 30 s; 1 cycle of 72 °C for 2 min.

PROCEDURE

Construction of pTRM4-F602X(TAA) expression plasmid • TIMING 3 d

- 1| Prepare freshly purified pTRM4-WT plasmid at 1 µg µl⁻¹ using the Qiagen miniprep kit according to the manufacturer's instructions. Standard microbiology sterile practice is required to avoid bacterial contamination.
- 2| In a 0.2-ml PCR tube, mix 5 µl of 10× Pfu DNA polymerase buffer, 2 µl of 10 mM dNTPs, 2 µl of 10 ng µl⁻¹ pTRM4-WT, 2 µl of 10 µM TRM4-F602X(TAA)-F1 primer, 2 µl of 10 µM TRM4-F602X(TAA)-R1 primer, 1 µl (5 U µl⁻¹) of Pfu DNA polymerase and 38 µl of ddH₂O.
- 3| Set the sample(s) in a PCR thermal cycler, and run program 1 (see EQUIPMENT SETUP).
- 4| Mix 10 µl of PCR product from Step 3 with 2 µl of 6× DNA loading dye and load the sample onto a 1% (wt/vol) agarose gel containing 0.05% (vol/vol) ethidium bromide. Perform electrophoresis at constant voltage (10 V cm⁻¹ gel length) in 0.5× TBE buffer for 30 min, and then visualize the bands with UV shadowing.

▲ CRITICAL STEP The band intensity of the amplified plasmid should be readily visible using UV shadowing; otherwise, it indicates an inefficient amplification and may substantially compromise subsequent steps. Always include a negative control PCR in which ddH₂O is added instead of primers.

? TROUBLESHOOTING

- 5| Add 2 µl (10 U µl⁻¹) of DpnI to each PCR tube, and then incubate at 37 °C for 2 h.
- 6| Add 200 µl of ethanol to the tube and centrifuge it at 14,000g at 4 °C for 10 min to precipitate the nucleic acids. Discard the supernatant and wash the pellet with 70% (vol/vol) ethanol, air-dry the pellet for 5 min and dissolve the pellet in 10 µl of ddH₂O.
- 7| In a 1.5-ml tube, mix 2 µl of dissolved PCR product with 100 µl of competent cells (XL1-blue).

- 8| Put the tube on ice for 20 min, heat-shock it at 42 °C for 90 s, and then place it on ice again for 2 min.
- 9| Add 900 µl of LB liquid medium, seal the tube with Parafilm, shake it at 200 r.p.m. at 37 °C for 1 h, and then plate the sample on LB-ampicillin solid medium.
- 10| You should observe 20–30 well-separated colonies (1 mm diameter) after 16 h of incubation at 37 °C.

? TROUBLESHOOTING

- 11| Prepare plasmid DNA from several colonies using Qiagen miniprep kit according to the manufacturer's instructions, and then confirm the nucleotide sequence of the plasmid by sequencing. Once confirmed, the plasmid is then named pTRM4-F602X(TAA).

construction of TRM4-F602X(TAA)-specific snR81 expression plasmid • TIMING 3 d

- 12| In a 200-µl PCR tube, mix 5 µl of 10× Taq DNA polymerase buffer, 2 µl of 10 mM dNTPs, 2 µl of 10 µM snR81-TRM4-F602X(TAA)-F1 primer, 1 µl of 1 µM snR81-TRM4-F602X(TAA)-R1 primer, 1 µl of 1 µM snR81-TRM4-F602X(TAA)-R2 primer, 2 µl of 10 µM snR81-TRM4-F602X(TAA)-R3 primer, 1 µl (5 U µl⁻¹) of Taq DNA polymerase and 36 µl of ddH₂O.

▲ CRITICAL STEP The ratio of these four oligodeoxynucleotide primers is crucial and optimized to generate the maximum amount of full-length PCR product.

- 13| Set the sample(s) in a PCR thermal cycler and run program 2 (see EQUIPMENT SETUP).
- 14| Mix 10 µl of PCR product from Step 13 with 2 µl of 6× DNA loading dye and load the sample onto a 1% (wt/vol) agarose gel containing 0.05% (vol/vol) ethidium bromide. Perform electrophoresis at constant voltage (10 V cm⁻¹ gel length) in 0.5× TBE buffer for 30 min, and then visualize the bands with UV shadowing.

▲ CRITICAL STEP Usually, a band of correct size and one of a higher molecular weight are observed. It is highly recommended to gel-purify the band of correct size using Qiagen's gel purification kit (see Step 16).

? TROUBLESHOOTING

- 15| Mix the remaining 40 µl of PCR product from Step 13 with 460 µl of G50 buffer and 500 µl of PCA and vortex for 30 s. Centrifuge at 14,000g at 4 °C for 5 min in a benchtop centrifuge. Transfer the aqueous phase to a new 1.5-ml tube, and add 1 µl of 10 mg ml⁻¹ glycogen and 1 ml of 100% ethanol; centrifuge at 14,000g at 4 °C for 10 min in a benchtop centrifuge. Discard the supernatant and wash the pellet with 70% (vol/vol) ethanol, air-dry the pellet for 5 min and dissolve the pellet in 10 µl of ddH₂O.
- 16| Mix 10 µl of the precipitated PCR product from Step 15 with 2 µl of 6× DNA loading dye and load the sample onto a 1% (wt/vol) agarose gel containing 0.05% (vol/vol) ethidium bromide. Perform electrophoresis at constant voltage (10 V cm⁻¹ gel length) in 0.5× TBE buffer for 30 min. Gel-purify the band of the expected size using the Qiagen gel purification kit according to the manufacturer's instructions. Elute the PCR product from the excised gel slice

with 40 μl of ddH₂O. Quantify 1 μl of the PCR product with a NanoDrop or UV spectrophotometer.

■ **PAUSE POINT** PCR products can be stored at -20°C for up to 1 year.

- 17| In a 1.5-ml tube, add 5 μl of 1 $\mu\text{g } \mu\text{l}^{-1}$ PCR products from Step 16, 5 μl of 10 $\times$ BamHI digestion buffer and 2.5 μl of BamHI (10 U μl^{-1}); bring the total volume to 50 μl with ddH₂O. Incubate in a 37 $^{\circ}\text{C}$ water bath for 2 h.
- 18| In a second 1.5-ml tube, add 1 μl of 1 $\mu\text{g } \mu\text{l}^{-1}$ of pSEC, 5 μl of 10 $\times$ BamHI digestion buffer, 2.5 μl of BamHI (10 U μl^{-1}) and bring the total volume to 50 μl with ddH₂O. Incubate in a 37 $^{\circ}\text{C}$ water bath for 2 h.
- 19| To both tubes, add 450 μl of G50 buffer and 500 μl of PCA and vortex for 30 s. Centrifuge at 14,000 g at 4 $^{\circ}\text{C}$ for 5 min in a benchtop centrifuge.
- 20| Transfer each aqueous phase to a new 1.5-ml tube, and add 1 μl of 10 mg ml^{-1} glycogen and 1 ml of 100% ethanol; centrifuge at 14,000 g at 4 $^{\circ}\text{C}$ for 10 min in a benchtop centrifuge.
- 21| Discard the supernatant and add 1 ml of 70% (vol/vol) ethanol, and then centrifuge at 14,000 g at 4 $^{\circ}\text{C}$ for 5 min in a benchtop centrifuge.
- 22| Remove the supernatant and allow the pellet to air-dry for 5 min. Dissolve each pellet in 10 μl of ddH₂O.

■ **PAUSE POINT** PCR products can be stored at -20°C for up to 1 year.

- 23| To both tubes from Step 22, add 5 μl of 10 $\times$ HindIII digestion buffer, 2.5 μl of HindIII (10 U μl^{-1}) and bring the total volume to 50 μl with ddH₂O. Incubate in a 37 $^{\circ}\text{C}$ water bath for 2 h.
- 24| Repeat Step 19, transfer the aqueous phases to new 1.5-ml tubes, add 1 ml of 100% ethanol and centrifuge the tubes at 14,000 g at 4 $^{\circ}\text{C}$ for 10 min in a benchtop centrifuge.
- 25| Repeat Steps 21 and 22.

■ **PAUSE POINT** PCR products can be stored at -20°C for up to 1 year.

- 26| In a new 1.5-ml tube, add 5 μl of digested PCR product from Step 25, 1 μl of digested pSEC plasmid from Step 25, 2 μl of 5 $\times$ T4 DNA ligase buffer, 2 μl of T4 DNA ligase (1 U μl^{-1}) and 10 μl of ddH₂O.
- 27| Incubate the ligation mix in a 16 $^{\circ}\text{C}$ water bath overnight.
- 28| Mix 5 μl of ligation mix with 100 μl of DH5 α -competent cells and place them on ice for 10 min. Heat-shock the mixture at 42 $^{\circ}\text{C}$ for 45 s and place it on ice for 2 min.
- 29| Add 900 μl of LB liquid medium (without ampicillin) and shake the mixture at 200 r.p.m. at 37 $^{\circ}\text{C}$ for 45 min.
- 30| Centrifuge the mix at 4,000 g for 5 min in a benchtop centrifuge.
- 31| Remove the supernatant, and resuspend the cell pellet in 100 μl of LB-ampicillin liquid medium. Spread the cells on an LB-ampicillin solid medium plate.
- 32| Incubate the plate in a 37 $^{\circ}\text{C}$ incubator overnight to observe 20–30 well-separated colonies (1 mm in diameter).

- 33| Prepare plasmid DNA from several colonies using the Qiagen miniprep kit according to the manufacturer's instructions and confirm the nucleotide sequence of the plasmid by sequencing. Once confirmed, the plasmid is then named psnR81-TRM4-F602X(TAA).

■ **PAUSE POINT** Plasmids can be stored at -20°C for up to 1 year.

Co-transformation of yeast with pTRM4-F602X(TAA) and psnR81-TRM4-F602X(TAA) • TIMING 3 d

- 34| Inoculate a single yeast colony into 5 ml of YPD liquid medium and shake it at 200 r.p.m. at 30°C for 16 h to achieve maximum cell growth. Measure the optical density at 600 nm (OD_{600}) using fresh YPD liquid medium as a blank. Use standard microbiology sterile practice to avoid bacterial contamination and cross-contamination.
- 35| Dilute the cells in 5 ml of fresh YPD medium to obtain an OD_{600} of 0.5.
- 36| Closely monitor the OD, and when it reaches an OD_{600} of 2, collect the cells at $2,500g$ using an SH3000 rotor in a Sorvall RC-5C Plus centrifuge at 4°C for 5 min. Remove the YPD medium completely.

? TROUBLESHOOTING

- 37| Resuspend the cell pellet in 200 μl of one-step transformation buffer to obtain an OD_{600} of 50.
- 38| Aliquot 50 μl of the mixed cell solution to a new sterile 1.5-ml tube, add 1 μl of $1\text{ }\mu\text{g }\mu\text{l}^{-1}$ of pTRM4-F602X(TAA) from Step 11 and 1 μl of $1\text{ }\mu\text{g }\mu\text{l}^{-1}$ psnR81-TRM4-F602X(TAA) from Step 33, and then mix thoroughly with a pipette.
- 39| Incubate the mixture in a 42°C water bath for 30 min. Add 300 μl of YPD medium and rotate the tubes in a shaker at 200 r.p.m. at 30°C for 1 h.
- 40| Centrifuge the cells at $2,500g$ at room temperature for 5 min in a benchtop centrifuge and remove the supernatant.
- 41| Resuspend cells in 100 μl of autoclaved ddH_2O , and spread them on the surface of a SGal-Leu-Ura solid medium plate.
- 42| Incubate the cells at 30°C for 2–3 d to observe colonies (usually 1 mm in diameter).

■ **PAUSE POINT** The transformed yeast cells can be stored at 4°C for up to 2 weeks.

? TROUBLESHOOTING

Preparation of crude cell extract from *S. cerevisiae* • TIMING 2 d

- 43| Inoculate a single yeast colony (1–2 mm in diameter) into 100 ml of SGal-Leu-Ura liquid medium, and shake it at 200 r.p.m. at 30°C until $\text{OD}_{600} = 2$.
- 44| Centrifuge the cells at $2,300g$ at 4°C for 5 min, discard the supernatant and transfer the cell pellet (a total $\sim 200\text{ }\mu\text{l}$) to a 2-ml screw-cap tube.
- 45| Add 200 μl of $2\times$ yeast extract preparation buffer and 200 μl of glass beads to the cell pellet. Screw the cap on firmly and seal it with Parafilm.

▲ CRITICAL STEP For best results, the volume ratio of glass beads and precipitated wet cells should be approximately 1:1. If the ratio is >1:1, the recovery of supernatant will decrease accordingly; if the ratio is <1:1, the efficiency of cell lyses will decrease accordingly. The 2-ml tubes with screw caps are highly recommended because common 1.5-ml tubes may leak during vortexing.

- 46| Vortex the tube vigorously for 1 min, place it on ice for 30 s and then repeat this step five more times.
- 47| Centrifuge the tube at 14,000g at 4 °C for 5 min, and transfer the supernatant to a new 1.5-ml tube. Repeat this step three more times (each time, transfer the supernatant to a new tube and discard the pellet) until the supernatant is free of cell debris.
- 48| Aliquot the supernatant at 10 µl per 1.5-ml tube. Use one tube at a time for western blotting.

■ PAUSE POINT The rest of cell extract is stable for at least 1 year at – 80 °C.

? TROUBLESHOOTING

Detection of Trm4 protein expression by western blotting • TIMING 2 d

- 49| Chill the Bio-Rad mini gel box, the Bio-Rad transfer box, 1 liter of western blot running buffer and 1 liter of western blot transfer buffer at 4 °C overnight.
- 50| Mix 10 µl of 2× loading dye with 1 µl (about 10 µg protein) of cell crude extract from Step 48 and 9 µl of ddH₂O, mix by pipetting, and heat the mixture at 95 °C for 5 min. Briefly centrifuge the mixture in a benchtop centrifuge to spin down the sample.
- 51| Load 8 µl into each lane of a 4–14% (wt/vol) Tris-HCl ready gel, run it at a constant 150 V at 4 °C for 1 h (or until the bromophenol blue dye runs to the bottom of the gel).
- 52| Separate the gel plates and transfer the gel onto a sheet of prewetted (with transfer buffer) Whatman nitrocellulose membrane (pore size, 0.1 µm; cut the membrane such that it is identical to the gel size).

▲ CRITICAL STEP Mark the membrane at a corner with a pencil to indicate the orientation and side of the membrane that bears the transferred protein. Avoid touching the membrane at any part that bears transferred protein. Always keep the membrane wet.

- 53| Make a sandwich in the transfer cassette in the following order: the pad (prewetted with transfer buffer), one piece of prewetted Whatman filter paper (slightly larger than the gel size), gel, nitrocellulose membrane, a second piece of prewetted Whatman filter paper, and a second prewetted pad.
- ▲ CRITICAL STEP** During sandwich assembly, use a glass rod to roll out air bubbles between layers. Recheck the order of the gel and membrane so that proteins migrate toward the membrane. For best results, the entire sandwich should be assembled in a tray containing the transfer buffer.
- 54| Insert the sandwich into the transfer box with gel facing the anode and the membrane facing the cathode.
- 55| Pour 1,000 ml of cold western blot transfer buffer in the transfer box until the buffer covers the entire transfer cassette. Run at constant 100 V at 4 °C for 2 h.

- 56| After the transfer is completed, disassemble the sandwich and immerse the membrane in 20 ml of 5% (wt/vol) BSA solution with the protein side facing up. Gently shake it at room temperature for 1 h.
- 57| Wash the membrane with 20 ml of washing buffer and gently shake it at room temperature for 10 min.
- 58| Hybridize the membrane with 10 ml of freshly made primary hybridization buffer containing 5 μ l of monoclonal anti-protein A or anti-Eno1p. Gently shake at 4 °C overnight (> 16 h).
- 59| Collect the primary hybridization buffer and store it at – 20 °C for possible reuse.
- 60| Wash the membrane with 20 ml of washing buffer and gently shake it at room temperature for 10 min. Repeat three more times.
- ▲ **CRITICAL STEP** Extensive washing of the membrane is important to achieve a low background in the subsequent signal-generation step.
- 61| Hybridize the membrane with 10 ml of freshly made secondary hybridization buffer containing 1 μ l of goat anti-mouse (H + L)-AP conjugated IgG. Gently shake it at room temperature for 1 h.
- 62| Wash the membrane with 20 ml washing buffer and gently shake it at room temperature for 10 min. Repeat three more times.
- 63| Immerse the membrane in 10 ml of 1-step NBT/BCIP solution. Keep the membrane/container in the dark, and gently shake it at room temperature for 5–15 min to visualize protein bands.

? TROUBLESHOOTING

- 64| Stop the reaction by removing the 1-step NBT/BCIP solution and washing the membrane with 20 ml of ddH₂O.
- ▲ **CRITICAL STEP** Close monitoring is required to control the extent of the color-forming reaction. Stop the reaction when the desired band intensity is achieved.
- 65| Keep the membrane in the dark and air-dry it at room temperature for 20 min before scanning.

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 3.

● TIMING

Steps 1–11, construction of pTRM4-F602X(TAA) expression plasmid: 3 d

Steps 12–33, construction of TRM4-F602X(TAA)-specific snR81 expression plasmid: 3 d

Steps 34–42, co-transformation of yeast with pTRM4-F602X(TAA) and psnR81-TRM4-F602X(TAA): 3 d

Steps 43–48, preparation of crude cell extract from *S. cerevisiae*: 2 d

Steps 49–65, detection of Trm4 protein expression by western blotting: 2 d

ANTICIPATED RESULTS

As mentioned in the INTRODUCTION, pTRM4-F602X(TAA) is the construct that allows the use of the C-terminal tag to visualize nonsense suppression (translational read-through) products. It is then expected that when the PTC is not pseudouridylated, the C-terminally truncated Trm4p (without the tag) will be produced as a result of premature translation termination. When the PTC is pseudouridylated, however, nonsense suppression is induced, thus leading to the production of full-length Trm4p and its C-terminal tag, which allows for the detection of the nonsense suppression product by western blotting.

Figure 5 shows the western blot analysis of extracts prepared from cells expressing TRM4-WT (nonsense-free) or TRM4-F602X(TAA). When cells were transformed with pTRM4-WT, a strong protein A-tag signal was detected (Fig. 5, lane 1). However, when cells were co-transformed with TRM4-F602X(TAA) and a guide RNA containing a control guide sequence, the protein A-tag signal was undetectable (Fig. 5, lane 3). In contrast, when cells were co-transformed with pTRM4-F602X(TAA) and psnR81-TRM4-F602X(TAA), a fairly strong protein A-tag signal was detected, indicating nonsense suppression (Fig. 5, lane 2).

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Figure 1.

Schematic representation of Ψ formation and an artificial box H/ACA RNA. **(a)**

Pseudouridylation reaction. a, hydrogen-bond acceptor; d, hydrogen-bond donor. **(b)** The artificial snR81 (based on WT snR81), whose guide sequences are altered to target TRM4-F602X(TAA) for pseudouridylation. snR81 adopts a hairpin-hinge-hairpin-tail structure. The H box and ACA box are shaded. Two pseudouridylation pockets exist as internal loops/guide sequences (g1 and g1', g2 and g2', indicated by the blue lines) within each hairpin, which are capable of base-pairing with complementary sequences within TRM4-PTC mRNA (shown in red). The uridine residue targeted for pseudouridylation (indicated by a black arrow) as well as the adjacent downstream nucleotide are left unpaired so as to remain accessible for modification.

Figure 2.

Schematic representation of the construction of the PTC-containing TRM4 expression cassette. pTRM4-F602X(TAA) is derived from pTRM4-WT, which contains a C-terminally tagged wild-type TRM4 construct. In pTRM4-F602X(TAA), the codon for amino acid residue 602 was changed from UUU (for phenylalanine) to UAA (PTC, indicated by a hatched box) by site-directed mutagenesis. The sizes of the elements are not drawn to scale. pTRM4-F602X(TAA) is an *E. coli*-yeast shuttle plasmid. 2μ allows the plasmid to exist in high copy number, and URA3 is the auxotroph selective marker in yeast. pUC ori is the replication origin, and ampR is an ampicillin resistance marker in *E. coli*.

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1  MARRKNFKKG NKKTFGARDD SRAQKNWSEL VKENEKWEKY YKTLALFPED
51  QWEEFKKTCQ APLPLTFRIT GSRKHAGEVL NLFKERHLPN LTNVEFECEK
101 IKAPVELPWY PDHLAWQLDV PKTVIRKNEQ FAKTQRFLVV ENAVGNISRQ
151 EAVSMIPPIV LEVKPHHTVL DMCAAPGSKT AQLIEALHKD TDEPSGFVVA
201 NDADARRSHM LVHQLKRLNS ANLMVVNHDA QFFPRIRLHG NSNNKNDVLK
251 FDRILCDVPC SGDGTMRKNV NVWKDWNTQA GLGLHAVQLN ILNRGLHLLK
301 NNGRLVYSTC SLNPIENEAV VAEALRWGD KIRLVNCDDK LPGLIRSKGV
351 SKWPVYDRNL TEKTKGDEGT LDSFFSPSEE EASKFNLQNC MRVYPHQNT
401 GGFFITVFEK VEDSTEAAATE KLSSETPALE SEGPQTKKIK VEEVQKKERL
451 PRDANEFPFV FVDPQHEALK VCWDFYGIDN IFDRNTCLVR NATGEPTRVV
501 YTVCPALKDV IQANDRLKI IYSGVKLFVS QRSIECSWR IQSESLPIMK
551 HHMKSNRIVE ANLEMLKHLI IESFPNFDDI RSKNIDNDFV EKMTKLSSGC
601 AFDVSRNDP AKENLFLPVW KGNKCINLMV CKEDTHELLY RIFGIDANAK
651 ATPSAEEKEK EKETTESPAP TTTGTSTEAP SAAN*

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Figure 3.

Amino acid sequence of Trm4p. The sequence of the C-terminal tag is not depicted. The shaded residues represent the 12-residue tryptic fragment of interest. The codon for phenylalanine at position 602, shaded in black, was changed to a premature termination codon. The natural termination site/codon of wild-type *TRM4* is indicated by an asterisk (*).

Figure 4.

PCR strategy for generating artificial snR81. Four oligodeoxynucleotide primers were designed: one for the sense-strand sequence of snR81 and the other three for the antisense-strand sequence of snR81 (as indicated by red arrows). The guide sequences are depicted in blue (g1, g1', g2, g2'). The four primers are designed in a way that allows them to combine to generate an artificial snR81 gene with BamHI and HindIII restriction sites at each end. The artificial snR81 gene is subsequently cloned into plasmid pSEC under the control of the Gal promoter. pSEC is an *E. coli*-yeast shuttle plasmid. 2μ allows the plasmid to exist in high copy numbers, and LEU2 is the auxotroph selective marker in yeast. pUC ori is the replication origin, and ampR is an ampicillin resistance marker in *E. coli*.

Figure 5.

Western blot analysis of TRM4-PTC nonsense suppression. The Trm4 proteins, expressed in BY4741 cells that were transformed with either pTRM4-WT and a psnR81-Control (lane 1), pTRM4-F602X(TAA) and psnR81-TRM4-F602X(TAA) (lane 2), or pTRM4-F602X(TAA) and psnR81-Control (lane 3), were visualized by anti-protein A immunoblotting. Eno1p was probed as a loading control. The level of Trm4p generated in lane 2 (the nonsense suppression or read-through product) is about 5% of that of lane 1 (the WT product).

TABLE 1

Oligodeoxynucleotides.

Name	Sequence	Convenient stock concentration (μM)
snR81-TRM4-F602X(TAA)-F1	5'-GCGGGATCCGGGACTGCATCAATTGCGGCGAGGCAGCCACATCAAGTGGAACAC-3'	10
snR81-TRM4-F602X(TAA)-R1	5'-TGTTAGGATTGCTCTTGGGACCGTTGCGCCGCGACAAGGAAGTCTGTGTAGTTCCACTTG-3'	1
snR81-TRM4-F602X(TAA)-R2	5'-CCATCCGTGGACTGTACAGGTTACGCGGGGAATTGATGTTTGCTTGTAGGATTGCTCT-3'	1
snR81-TRM4-F602X(TAA)-R3	5'-GGCGAAGCTTAGATGTGAAAAAGCGCGCCCCCGAATCATATAACTTCTGCACCATCCGTGGACTGT-3'	10
snR81-Control-F1	5'-GCGGGATCCGGGACTGCTCGATTAGCGGCGAGGCAGCCACATCAAGTGGAACAC-3'	10
snR81-Control-R1	5'-TGTTAGGATTGCTCTTGGGACCGATTACTCGCGACAAGGAAGTCTGTGTAGTTCCACTTG-3'	1
snR81-Control-R2	5'-CCATCCGTGGACTGTACAGGTTACGCGGGGTAATCGATTTTGCTTGTAGGATTGCTCT-3'	1
snR81-Control-R3	5'-GCGAAGCTTAGATGTGAAAAAGCGACTCCCCCGAATCATATAACTTCTGCACCATCCGTGGACTGT-3'	10
TRM4-F602X(TAA)-F1	5'-AAATTAAGCTCTGGTTGCGCCTAAATTGATGTGTCAAGA-3'	10
TRM4-F602X(TAA)-R1	5'-TCTTGACACATCAATTTAGGCGCAACCAGAGCTTAATTT-3'	10

TABLE 2

Synthetic leucine/uracil double-dropout powder.

Ingredients	Amount per 15 L
Yeast nitrogen base	25.1 g
Ammonium sulfate	75.4 g
Isoleucine	450 mg
Valine	2.25 g
Adenine	300 mg
Arginine	300 mg
Histidine	300 mg
Leudne	–
Lysine	450 mg
Methionine	300 mg
Phenylalanine	750 mg
Tryptophan	300 mg
Tyrosine	450 mg
Uracil	–

TABLE 3

Troubleshooting table.

Step	Problem	Possible reason	Solution
4	No band	Too little template	Check the amount of parental plasmid and its purity
		PCR extension time is too short	Increase PCR extension time in program 1
10	Too many colonies	Too much template in Step 4	Decrease the parental plasmid amount in Step 4
		DpnI activity is low	Use newly purchased DpnI restriction enzyme
14	No band	Too little primer	Increase the primer concentration in the PCR
	Heterogeneous bands	Annealing temperature is not optimal	Adjust the annealing temperature in program 2
	Single band at the wrong size	Annealing temperature is not optimal	If the band is smaller than expected, try to gel-purify the band and perform a new round of PCR with only extending primers and purified band as template
		Molar ratio of primers is not optimal	Try a ratio of F1:R1:R2:R3=3:1:1:3, if F1:R1:R2:R3 = 20:1:1:20 is not working
36	No transformation	Cell OD ₆₀₀ is too high	Usually one can have successful transformation when cell OD ₆₀₀ is 1.0–2.0
		Residual YPD medium interference	Wash the cell pellet with sterile ddH ₂ O before adding transformation buffer
42	No colonies	Solid medium plate has dried out	Always use freshly made SGal-Leu-Ura solid medium
	Too many colonies	Selective pressure is insufficient	Ensure that both leucine and uracil are absent from the synthetic medium preparation
	Satellite colonies	Starting cell amount is too high	Reduce the cell amount by diluting cells with transformation buffer
48	Extract is too clear	Insufficient volume of glass beads	Increase the volume of glass beads
		Vortex speed is not fast enough	Increase the vortex speed or add lysozyme
	Extract is too opaque	Too many cells used	Carry out an additional centrifugation step(s) to get rid of cell debris
			Dilute extract with 10× yeast extract preparation buffer
63	Insufficient/no transfer	Air bubbles are present in the sandwich	Immerse the cassette in transfer buffer during assembly
			Remove air bubbles using a glass rod
		Transfer time is too short or too long	Transferring at constant 15 V at 4 °C overnight usually works well
			Transfer efficiency can be evaluated by monitoring the transfer of prestained marker between the gel and membrane
		Sandwich components are in the wrong order	The correct order is anode-pad-filter-gel-membrane-filter-pad-cathode