

Supplementary Information

***Saccharomyces cerevisiae* ASN1 and ASN2 are asparagine synthetase paralogs that have diverged in their ability to polymerize in response to nutrient stress**

Chalongrat Noree^{1,*}, Naraporn Sirinonthanawech¹, and James E. Wilhelm^{2,*}

¹Institute of Molecular Biosciences, Mahidol University, 25/25 Phuttamonthon 4 Road, Salaya, Phuttamonthon, Nakhon Pathom 73170 Thailand

²Section of Cell and Developmental Biology, Division of Biological Sciences, University of California, San Diego, 9500 Gilman Drive (MC 0347), La Jolla, CA 92093-0347 USA

***Correspondence:**

Chalongrat Noree

Tel: +66-2-4419003

Fax: +66-2-4411013

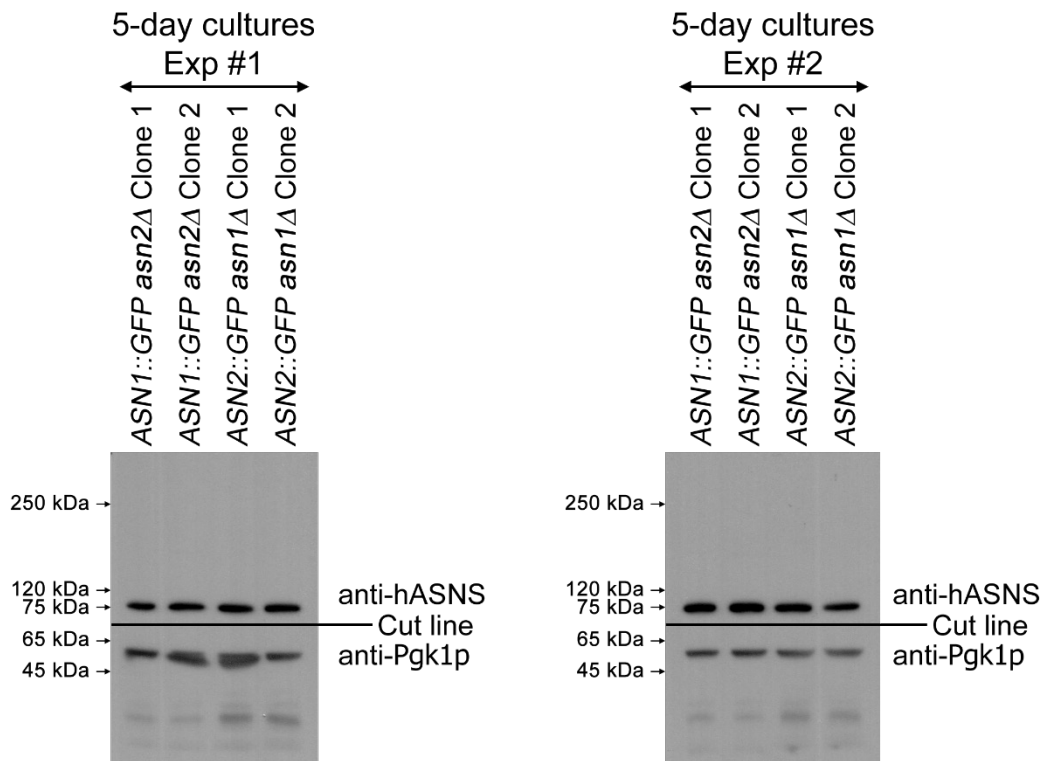
Email: chalongrat.nor@mahidol.edu

James E. Wilhelm

Tel: 1-858-534-9541

Fax: 1-858-534-7214

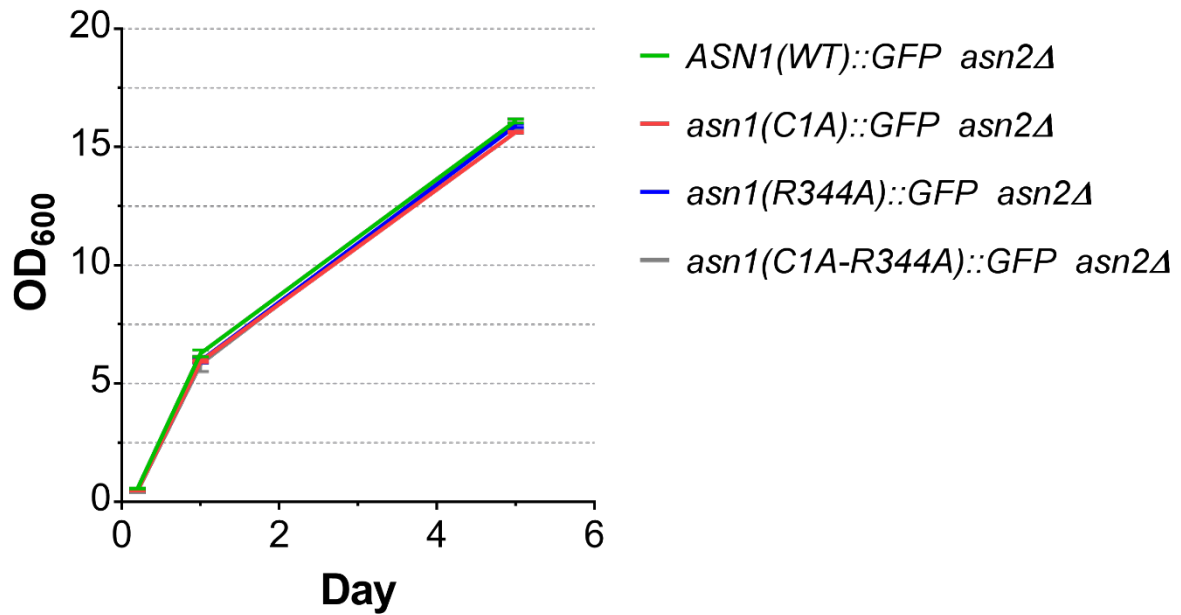
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Supplementary Figure S1. Expression levels of Asn1p-GFP (in *asn2Δ* background) vs. Asn2p-GFP (in *asn1Δ* background). Two different clones of yeast *ASN1::GFP asn2Δ* and *ASN2::GFP asn1Δ* were grown in liquid YPD at 30°C for 5 days with shaking. Then, 10 OD₆₀₀ cells were taken from each culture to prepare whole cell extract for SDS-PAGE and western blot analysis. Each membrane was cut into 2 pieces between 75 and 65 kDa of the pre-stained protein ladder. The upper cuts were used to detect GFP-tagged Asn1/2p-GFP with anti-hASNS (91.7 kDa and 91.9 kDa, respectively). The lower cuts were used to detect Pgk1p (as internal loading control) (44.7 kDa). Both were later assembled and developed the chemiluminescent signal together. Two independent experiments performed to confirm the results.



Supplementary Figure S2. Amino acid sequence alignment of *E. coli*, yeast, and human asparagine synthetases. Amino acid sequences of *E. coli* K12 asparagine synthetase B (P22106), yeast Asn1p (YPR145W, SGDID:S000006349), and human ASNS (P08243) were aligned. Yeast Asn1p shows: 37% identity and 53% similarity with human ASNS, and 48% identity and 64% similarity with *E. coli* asparagine synthetase B, respectively. The multiple alignment was performed with BioEdit version 7.1.9. The red arrow indicates arginine residue at position 325 of *E. coli*, 344 of yeast, and 340 of human asparagine synthetase, respectively.



Yeast strain	Clone #	OD ₆₀₀ (Average ± SEM)		
		Log-phase	Day 1	Day 5
<i>ASN1(WT)::GFP asn2</i> Δ	1	0.57 ± 0.03	6.29 ± 0.10	16.44 ± 0.27
	2	0.57 ± 0.02	6.27 ± 0.14	16.11 ± 0.09
<i>asn1(C1A)::GFP asn2</i> Δ	1	0.47 ± 0.02	5.94 ± 0.05	15.63 ± 0.04
	2	0.46 ± 0.03	5.90 ± 0.04	15.60 ± 0.12
<i>asn1(R344A)::GFP asn2</i> Δ	1	0.49 ± 0.03	5.96 ± 0.10	15.90 ± 0.06
	2	0.54 ± 0.02	6.23 ± 0.20	16.11 ± 0.22
<i>asn1(C1A-R344A)::GFP asn2</i> Δ	1	0.46 ± 0.07	5.84 ± 0.33	15.99 ± 0.21
	2	0.43 ± 0.07	5.97 ± 0.05	16.05 ± 0.13

Supplementary Figure S3. Growth curve comparison of yeast *ASN1(WT)::GFP asn2*Δ, *asn1(C1A)::GFP asn2*Δ, *asn1(R344A)::GFP asn2*Δ, and *asn1(C1A-R344A)::GFP asn2*Δ. These yeast strains were cultured in YPD at 30°C, and measured their optical density (OD₆₀₀) at exponential phase of growth, day 1, and day 5, respectively. Three independent experiments were performed and reported as average ± SEM. Clone#1 of each strain was used to make a growth curve. Summary table is shown below the growth curve.

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      10      20      30      40      50      60      70      80      90     100
Asn1p  MCGIFAAFRHEDVHRYKPKALQLSKRIRHRGPDWSGNAIKNSTIFVHERLAIVGVESGAQPITSSDGEYMLCVNGEITYNHIQLREECADYEFGLSDCEP
Asn2p  MCGIFAAFRHEDIHNFKPKALQLSKRIRHRGPDWSGNAVNMNSTIFVHERLAIVGLDLSGAQPITSSADGEYMLGVNGEITYNHIQLREMCSDYKQTFSDCEP

      110     120     130     140     150     160     170     180     190     200
Asn1p  IIPMYLKHDIIDAPKYLDGMFAWILYDAKQDRIVAARDPIGITTILYMGRSSASPKIVYFASLKLCLTDDCDTITAFPPGHVYDSKTDKITRYFTPDWLDEK
Asn2p  IIPLYLEHDIIDAPKYLDGMFAFCLYDCKDRIVAARDPIGVVTLYMGRSSQSPETVYFASLKLCLTDVCDIISFPFPHVYDSETDKITRYFTPDWLDEK

      210     220     230     240     250     260     270     280     290     300
Asn1p  RIPSTPIDYMAIRHSLKAVRKRRLMAEVPYGVLLSGGLDSSLIASIAARETAKATNDV-EPSTYDSKARHLAGIDDDGKLHTAGWTSLSHFAIGLNPAD
Asn2p  RIPSTPVDYHAIRHSLKAVRKRRLMAEVPYGVLLSGGLDSSLIAAIAARETEKANADANEDNNVDEK--QLAGIDDDQGHHTSGWSRLHFAIGLNPAD

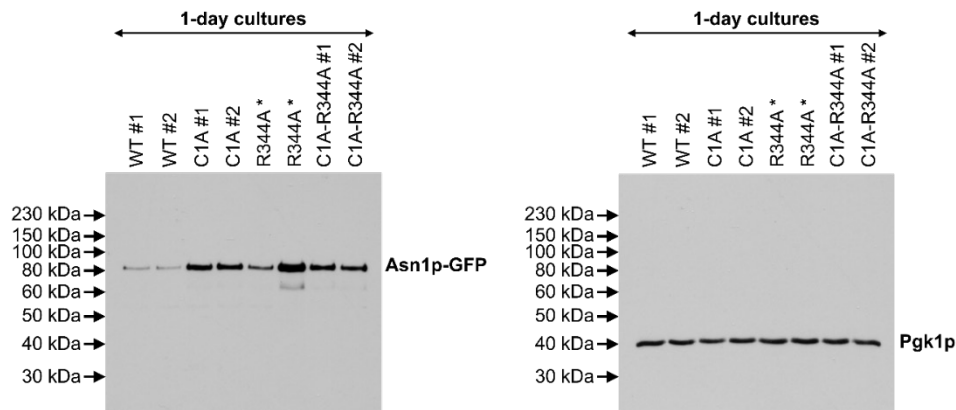
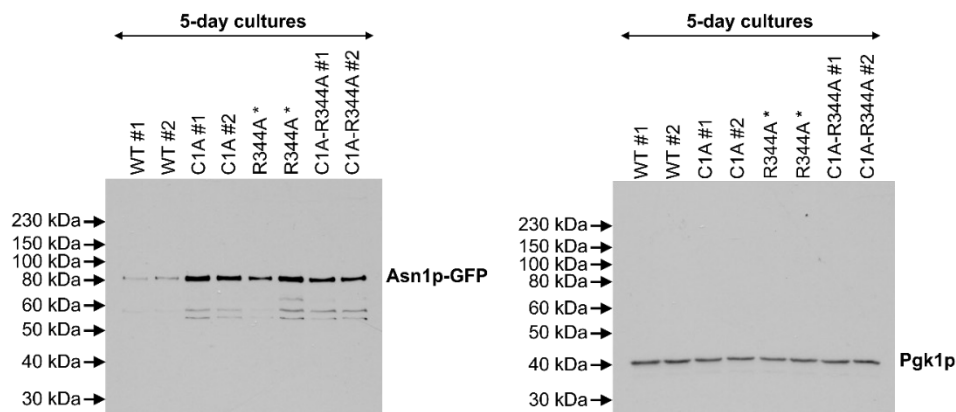
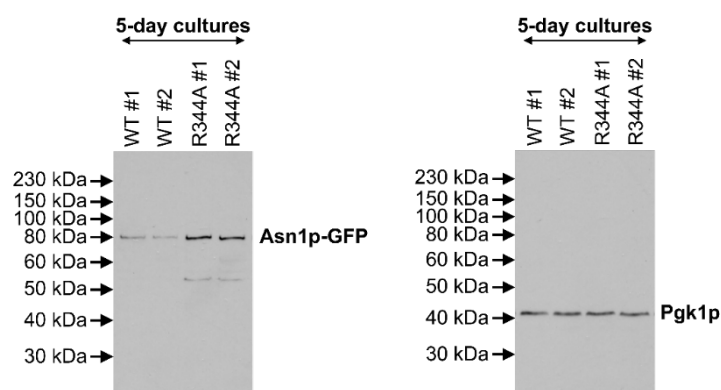
      310     320     330     340     350     360     370     380     390     400
Asn1p  LQAARKVAKFIGSIHHEHTFTLQEGLDALDDVIYHLETYDVTTIRASTPMFLLSRKIKAQGVKMLVLSGEGSDEIFGGYLYFAQAPSAAEFHTESVQRVKN
Asn2p  LQAARKVAKFIGSIHHEHTFTLQEGLDALDDVIYHLETYDVTTIRASTPMFLLSRKIKAQGVKMLVLSGEGSDEIFGGYLYFAQAPSAAEFHTESVQRVKN

      410     420     430     440     450     460     470     480     490     500
Asn1p  LHLADCLRANKSTMAWGLEARVPFLDREFLQLCMNIDPNEKMIKPKKEGRIEKYILRKAFTDTEGPDVAKPYLPEEILWRQKEQFSDGVGYSWIDGLRDTAE
Asn2p  LHLADCLRANKSTMAWGLEARVPFLDKDFLQLCMNIDPNEKMIKPKKEGRIEKYILRKAFTDTEGPDVAKPYLPEEILWRQKEQFSDGVGYSWIDGLRDTAE

      510     520     530     540     550     560     570
Asn1p  AVISDEMFAFPKAEWGSDIPTTKEAFWYRLKFDALFPQKIVADTVMRWIPKADWGCIEDPSGRYAQIHEKHIE-*
Asn2p  RAISDAMFANPKADWGDIDPTTKEAYWYRLKFDALFPQKTAADTVMRWIPKADWGCIEDPSGRYAKIHEKHVSA*

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Supplementary Figure S4. Pairwise amino acid sequence alignment of yeast asparagine synthetase paralogs. The alignment of Asn1p (YPR145W, SGDID:S000006349) and Asn2p (YGR124W, SGDID:S000003356) shows 88% identity and 94% similarity of their amino acid sequences. The pairwise alignment (optimal GLOBAL) was performed with BioEdit version 7.1.9.

A**B****C**

Supplementary Figure S5. Defective structure formation of R344A and C1A-R344A Asn1p-GFP was not caused by their protein expression levels. Yeast *ASN1(WT)::GFP asn2Δ*, *asn1(C1A)::GFP asn2Δ*, *asn1(R344A)::GFP asn2Δ*, and *asn1(C1A-R344A)::GFP asn2Δ* were grown in YPD for 1 day (**A**) and 5 days (**B-C**) at 30°C with shaking. Five OD₆₀₀ cells (1-day cultures) or 10 OD₆₀₀ cells (5-day cultures) were collected for preparing 200-μl whole cell extracts. Twenty μl of each protein sample were resolved on 8% acrylamide gel. Anti-hASNS antibody was used to detect Asn1p-GFP (91.75 kDa). Anti-PGK1 monoclonal antibody was used to detect yeast 3-phosphoglycerate kinase (44.74 kDa) as an internal loading control. Each experiment used the same blot for probing with anti-hASNS and anti-PGK1, one at a time. Stripping buffer [62.5 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 0.7% (w/v) BME] was used

to remove previous antibody from the blot prior to addition of the other antibody. The clones of *asn1(R344A)::GFP asn2Δ*, marked with asterisk in **(A)** and **(B)**, were not used in this study as they showed unequal expression levels (although they both showed the Asn1p-GFP polymerization defect). The clones of *asn1(R344A)::GFP asn2Δ* used for analysis in this study were shown in **(C)**.

Supplementary Table S1. List of primers used for recombinant DNA cloning, making DNA cassettes for yeast transformation, verifying yeast transformants, and DNA sequencing.

Primer Code	Sequence (5' to 3')	Description	Used with	PCR Product Size
For creating yeast <i>ASN1::GFP ASN2::mCherry</i> (colocalization assay) DNA cassette carrying (5' to 3'): 50 nt upstream of the <i>ASN2</i> stop codon, <i>mCherry</i> coding sequence, kanamycin resistance gene, and 50 nt downstream of the <i>ASN2</i> stop codon DNA template: pBS34				
CN0033	5'- AAGATCCTTCAGGTAGATACGCCAAAATACA CGAAAAGCACGTCAAGTCTGGTTCGACGGAT CCCCGGG -3'	Forward, with 50 nt upstream of <i>ASN2</i> stop codon, sequence homology to pBS34 (underlined)	JW1995	2,533 bp
JW1995	5'- CCGCATTTCTTGGTTCACTCGTCAATTATAA GAATACGATTGCGCTCGTAATCGATGAATTC GAGCTCG -3'	Reverse, with 50 nt downstream of <i>ASN2</i> stop codon, sequence homology to pBS34 (underlined)	CN0033	
For verifying the success of introducing mCherry to <i>ASN2</i> in yeast genome (colocalization assay) DNA template: genomic DNA isolated from selected yeast transformants				
JW2041	5'- TTGATCCAAATGAAAAGATGATCAAG -3'	Forward, located at nt1301-1326 of <i>ASN2</i> coding sequence	JW1775	1,154 bp
JW1775	5'- CTAATTGTACAGCTCGTCCATGCC -3'	Reverse, located at nt688-711 of <i>mCherry</i> coding sequence	JW2041	
For creating yeast <i>asn1Δ</i> (assembly dependence assay) DNA cassette carrying (5' to 3'): 50 nt upstream of the <i>ASN1</i> start codon, hygromycin resistance gene, and 50 nt downstream of the <i>ASN1</i> stop codon DNA template: pFA6a-hphMX6				
JW2201	5'- AAAAGTATAACTTGCTTTACGCTAAGGATAT AAATCGGACGTAACCTAAGGGTCGACGGAT CCCCGGG -3'	Forward, with 50 nt upstream of <i>ASN1</i> start codon, sequence homology to pFA6a-hphMX6 (underlined)	JW2202	1,811 bp
JW2202	5'- AAATATCTATAAGATTAATCCATAATCTTTT TCTATTTTTTAATGTTATATCGATGAATTCGA GCTCG -3'	Reverse, with 50 nt downstream of <i>ASN1</i> stop codon, sequence homology to pFA6a-hphMX6 (underlined)	JW2201	
For creating yeast <i>asn2Δ</i> (assembly dependence assay) DNA cassette carrying (5' to 3'): 50 nt upstream of the <i>ASN2</i> start codon, hygromycin resistance gene, and 50 nt downstream of the <i>ASN2</i> stop codon DNA template: pFA6a-hphMX6				
JW2193	5'- TTGCTTCCCAGTTAAGCTCACCAAAACACAA ATACTACTACAATAACAATGGTTCGACGGATC CCCCGGG -3'	Forward, with 50 nt upstream of <i>ASN2</i> start codon, sequence homology to pFA6a-hphMX6 (underlined)	JW2194	1,811 bp
JW2194	5'- CCGCATTTCTTGGTTCACTCGTCAATTATAA GAATACGATTGCGCTCGTAATCGATGAATTC GAGCTCG -3'	Reverse, with 50 nt downstream of <i>ASN2</i> stop codon, sequence homology to pFA6a-hphMX6 (underlined)	JW2193	
For verifying the success of deleting <i>ASN1</i> and <i>ASN2</i> from yeast genome (assembly dependence assay) DNA template: genomic DNA isolated from selected yeast transformants				
JW2010	5'- CTGCCCACTCGAGATGACAAATA -3'	Forward, located 200 nt upstream of <i>ASN1</i> start codon	JW1986	1,355 bp
JW1986	5'- CTCCATACAAGCCAACCACG -3'	Reverse, located at nt713-732 of hygromycin	JW2010	

Primer Code	Sequence (5' to 3')	Description	Used with	PCR Product Size
		resistance coding sequence		
JW2009	5'- CATTGACTCATGGCAAGATTTCTCC -3'	Forward, located 200 nt upstream of ASN2 start codon	JW1986	1,355 bp
JW1986	5'- CTCCATACAAGCCAACCACG -3'	Reverse, located at nt713-732 of hygromycin resistance coding sequence	JW2009	
For cloning ASN1 into pFA6a-GFP-kanMX6 DNA template: genomic DNA isolated from yeast BY4741				
JW2022	5'- CTT <u>GTCGAC</u> ATGTGTGGTATTTTCGCCGCTTC -3'	Forward, for cloning ASN1 , <i>Sall</i> site underlined	JW2023	1,734 bp
JW2023	5'- CTTCCCGGGTTCGATATGTTTTTCATGAATTGGGCATATC -3'	Reverse, for cloning ASN1 , <i>SmaI</i> site underlined	JW2022	
For site-directed mutagenesis of pFA6a-ASN1-GFP-kanMX6 (to introduce C1A or R344A mutation into ASN1 coding sequence) DNA template: pFA6a-ASN1-GFP-kanMX6				
JW2259	5'- GACGTTACCACTATCG CAG CTTCCA CT CCAATG -3'	Forward, with R344A mutation (in bold)	JW2260	6,593 bp
JW2260	5'- GTAAGTTTCCAAATGGTAGATCAC - 3'	Reverse	JW2259	
JW2261	5'- CTGCAGGTCGACATG GCT GGTATTTTCGCCGCT -3'	Forward, with C1A mutation (in bold)	JW2262	6,593 bp
JW2262	5'- CGTACGAAGCTTCAGCTGG -3'	Reverse	JW2261	
For making yeast ASN1(R344A)::GFP DNA cassette carrying (5' to 3'): ASN1 coding sequence (with R344A mutation), kanamycin resistance gene, and 50 nt downstream of the ASN1 stop codon DNA template: pFA6a-ASN1(R344A)-GFP-kanMX6				
JW2038	5'- CGCATTCTTCCACCCCAATAG -3'	Forward, located at nt601-622 of ASN1 coding sequence	JW2160	3,590 bp
JW2160	5'- AAATATCTATAAGATTAATCCATAATTCTTTTCTATTTTTTAATGTTATATCGATGAATTCCGA <u>GCTCG</u> -3'	Reverse, with 50 nt downstream of ASN1 stop codon, sequence homology to pFA6a-ASN1-GFP-kanMX6 (underlined)	JW2038	
For making yeast ASN1(C1A)::GFP and yeast ASN1(C1A-R344A)::GFP DNA cassette carrying (5' to 3'): 50 nt upstream of the ASN1 start codon, ASN1 coding sequence (with desired mutation), kanamycin resistance gene, and 50 nt downstream of the ASN1 stop codon DNA template: pFA6a-ASN1(C1A)-GFP-kanMX6 or pFA6a-ASN1(C1A-R344A)-GFP-kanMX6				
JW2276	5'- AAAAGTATAACTTGCTTTACGCTAAGGATATAAATCGGACGTAAGTAAAGATGGCTGGTATTTCGCCGCT -3'	Forward, with 50 nt upstream of ASN1 start codon, start codon (italicized), C1A mutation (in bold)	JW2160	4,240 bp
JW2160	5'- AAATATCTATAAGATTAATCCATAATTCTTTTCTATTTTTTAATGTTATATCGATGAATTCCGA <u>GCTCG</u> -3'	Reverse, with 50 nt downstream of ASN1 stop codon, sequence homology to pFA6a-ASN1-GFP-kanMX6 (underlined)	JW2276	
For preparing PCR products to send out for DNA sequencing DNA template: genomic DNA isolated from yeast ASN1(C1A)::GFP , yeast ASN1(R344A)::GFP , and yeast ASN1(C1A-R344A)::GFP				
JW2010	5'- CTGCCCACTCGAGATGACAAATA -3'	Forward, located 200 nt upstream of ASN1 start codon	JW1623	2,829 bp

Primer Code	Sequence (5' to 3')	Description	Used with	PCR Product Size
JW1623	5'- GCGACCTCATACTATACCTG -3'	Reverse, located 184 nt downstream of GFP coding sequence	JW2010	
For DNA sequencing				
JW2010	5'- CTGCCCACTCGAGATGACAAATA -3'	Forward, located 200 nt upstream of ASN1 start codon		
JW2038	5'- CGCATTCTTCCACCCCAATAG -3'	Forward, located at nt601-622 of ASN1 coding sequence		
JW2039	5'- ACATCGATCCAAATGAAAAGATG -3'	Forward, located at nt1301-1323 of ASN1 coding sequence		
JW1623	5'- GCGACCTCATACTATACCTG -3'	Reverse, located 184 nt downstream of GFP coding sequence (in pFA6a-ASN1-GFP-kanMX6)		