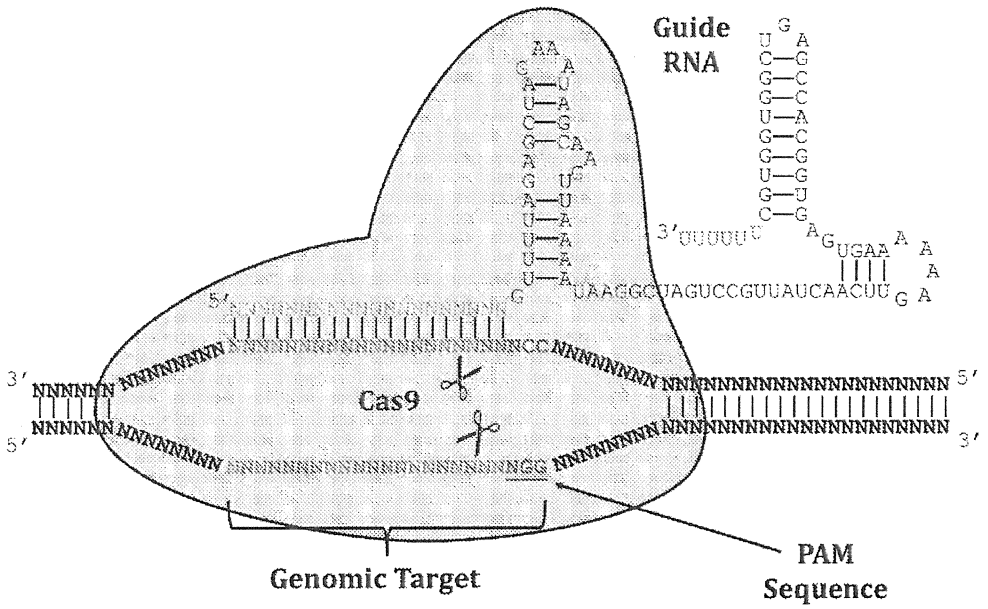
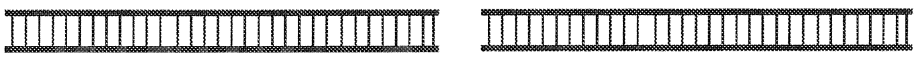
Figure 1

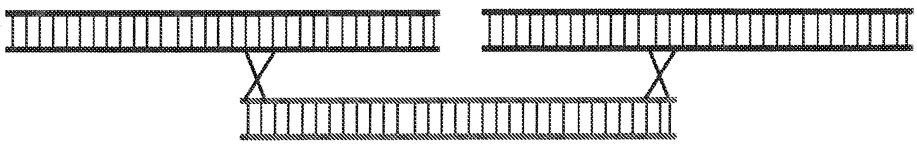
RNA guided Genome cleavage via Cas9



Double-strand break by Cas9 cleavage



Homologous recombination with Donor DNA that removes Cas9 cleavage site



Genomically integrated donor DNA



Figure 2

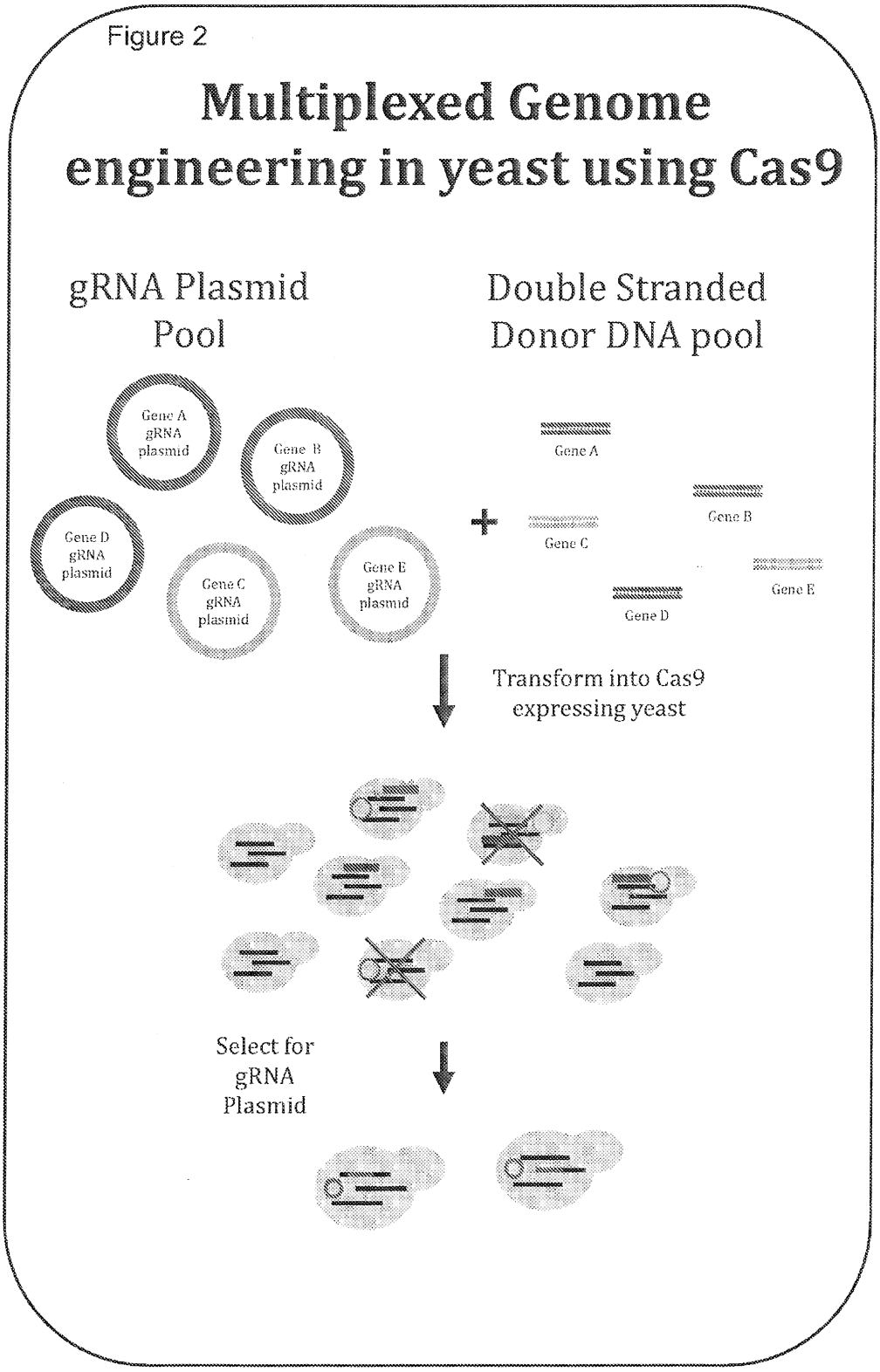

Multiplexed Genome engineering in yeast using Cas9
gRNA Plasmid Pool
Double Stranded Donor DNA pool
Gene A gRNA plasmid
Gene B gRNA plasmid
Gene C gRNA plasmid
Gene D gRNA plasmid
Gene E gRNA plasmid
Gene A
Gene B
Gene C
Gene D
Gene E
Transform into Cas9 expressing yeast
Select for gRNA Plasmid

FIG. 3

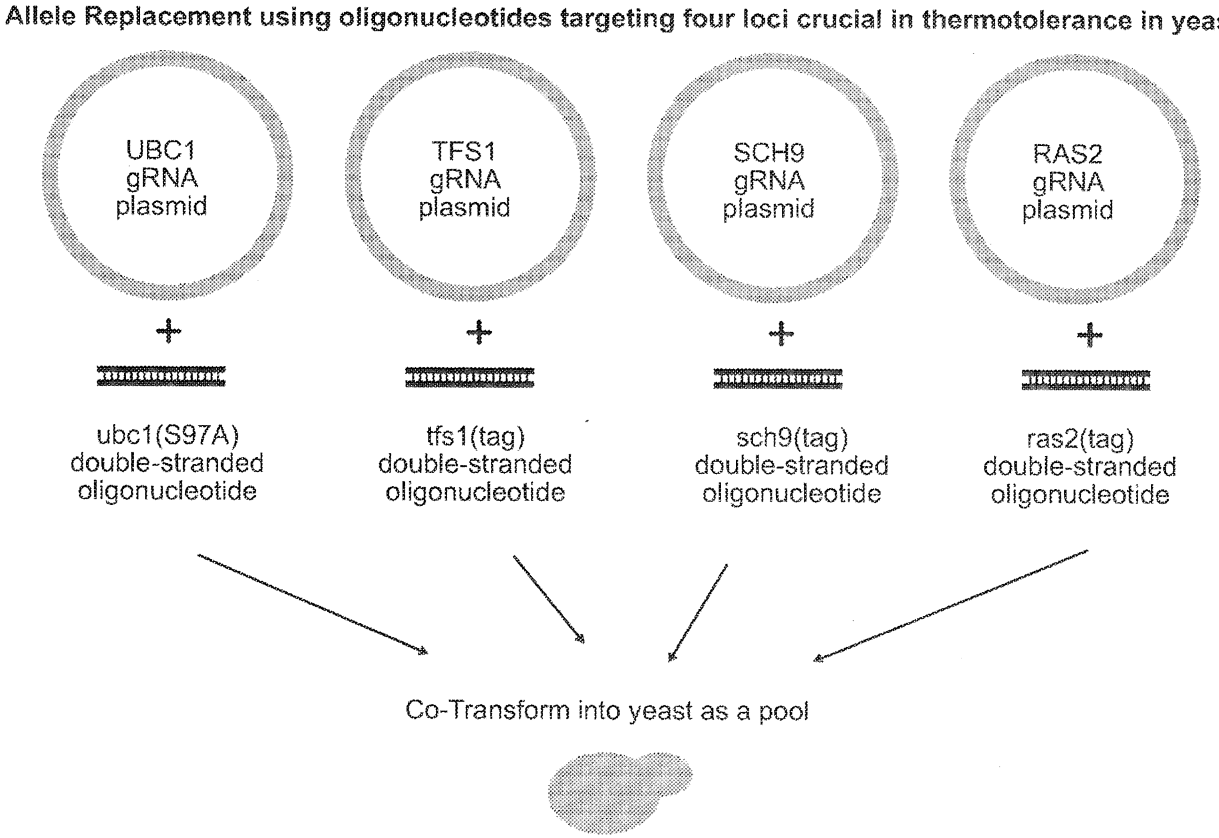
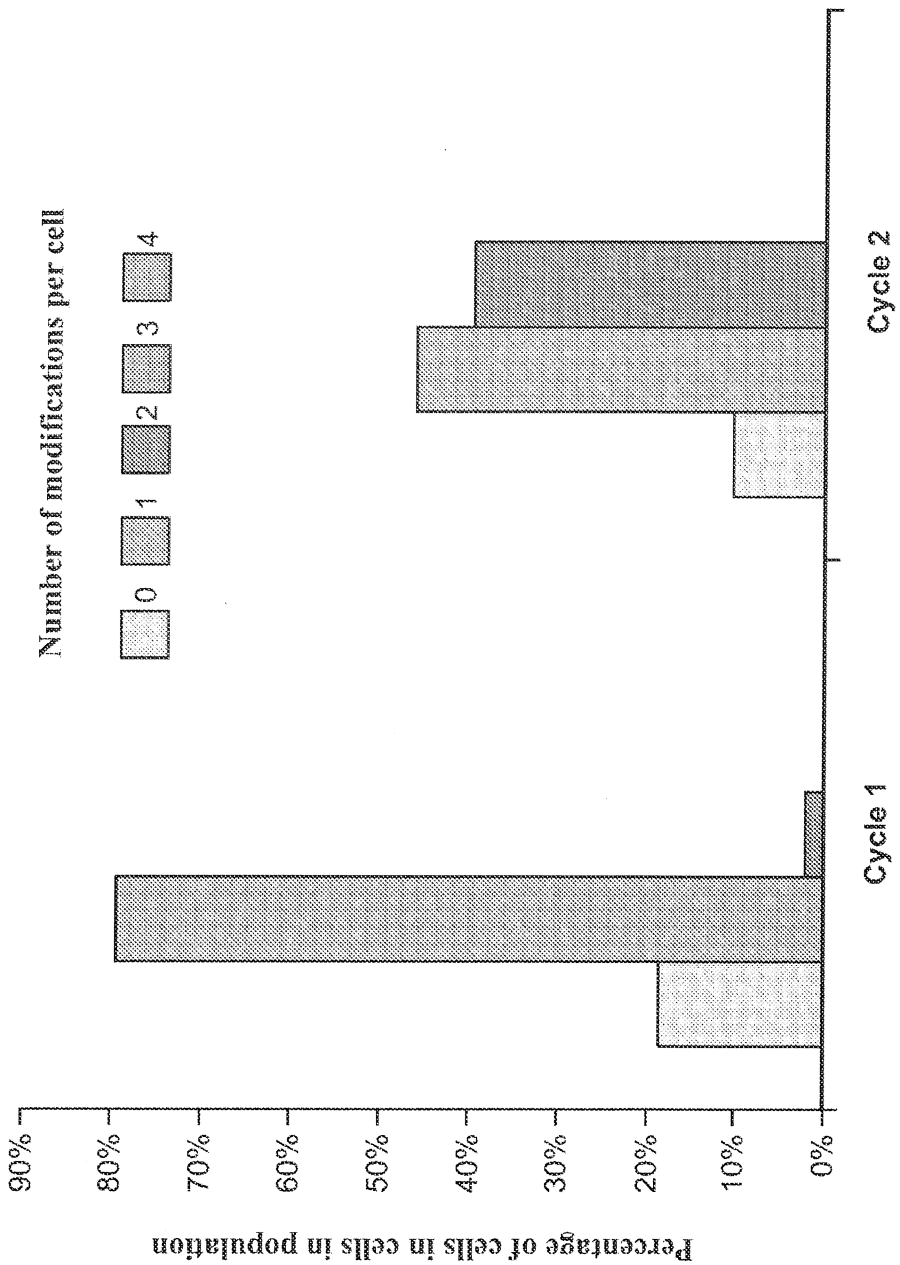

Allele Replacement using oligonucleotides targeting four loci crucial in thermotolerance in yeast
UBC1 gRNA plasmid
TFS1 gRNA plasmid
SCH9 gRNA plasmid
RAS2 gRNA plasmid
ubc1(S97A) double-stranded oligonucleotide
tfs1(tag) double-stranded oligonucleotide
sch9(tag) double-stranded oligonucleotide
ras2(tag) double-stranded oligonucleotide
Co-Transform into yeast as a pool

FIG. 4

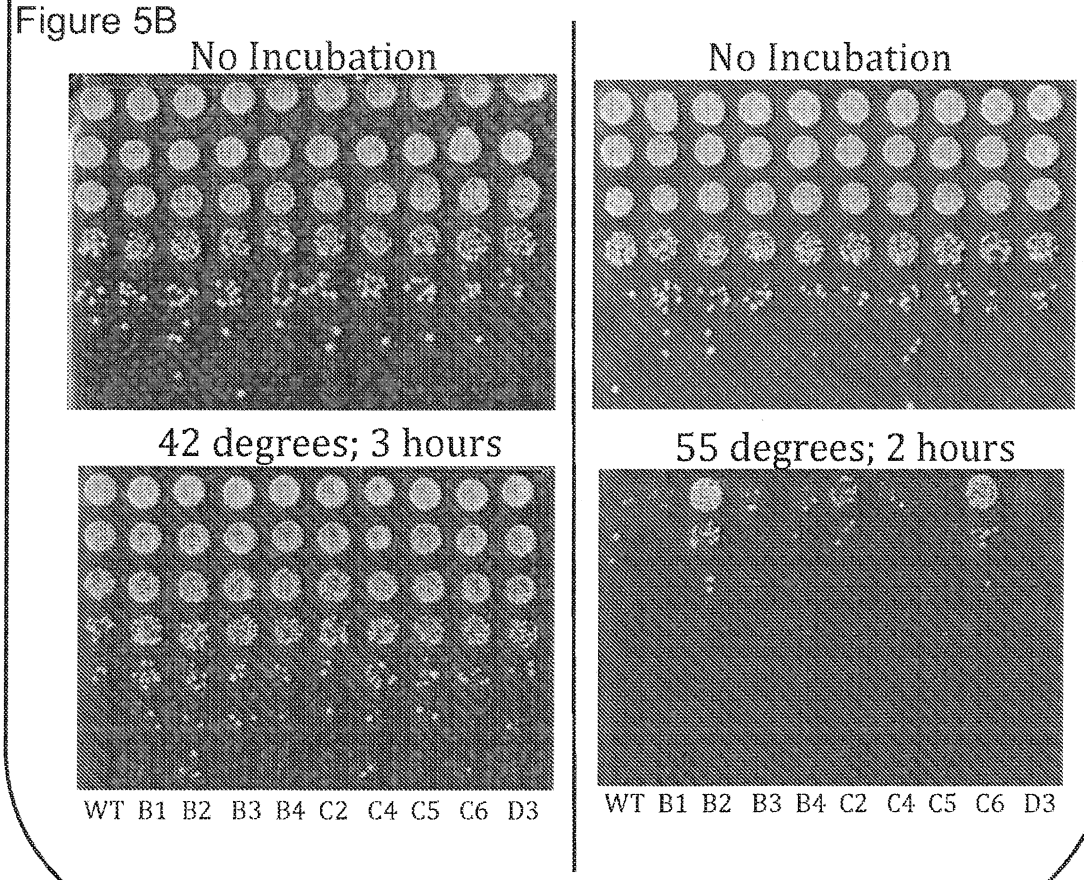

Cycling Data
Number of modifications per cell
0
1
2
3
4
Percentage of cells in cells in population
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Cycle 1
Cycle 2

Thermotolerance to heat shock in select mutants

Figure 5A

Strain	Code
Wild-Type	WT
ras2	B1
sch9	B2
ubc1(S97A)	B3
tfs1	B4
sch9 tfs1	C2
ras2 tfs1	C4
sch9 ubc1(S97A)	C5
tfs1 ubc1(S97A)	C6
ras2 tfs1 ubc1(S97A)	D3

Figure 5B



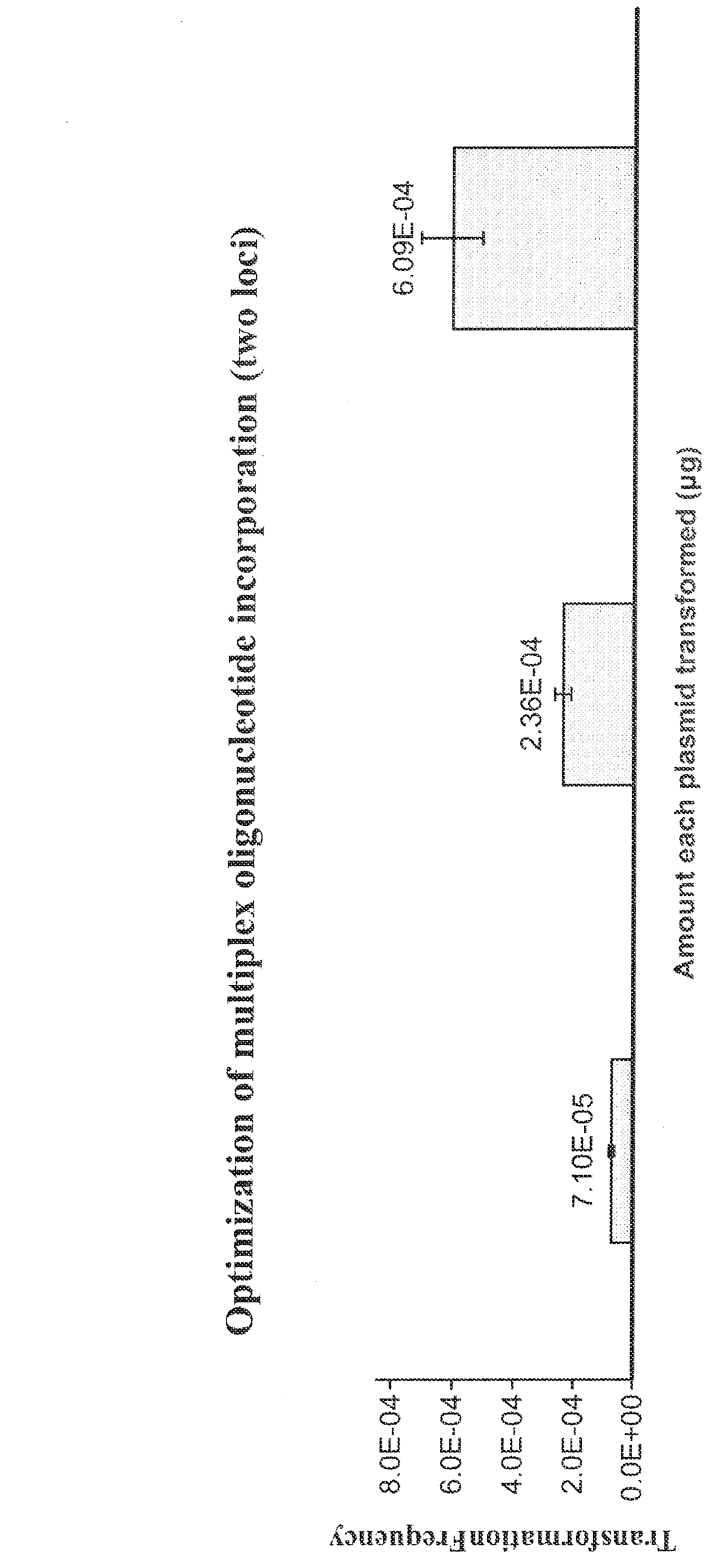

Optimization of multiplex oligonucleotide incorporation (two loci)
TransformationFrequency
8.0E-04
6.0E-04
4.0E-04
2.0E-04
0.0E+00
7.10E-05
2.36E-04
6.09E-04
Amount each plasmid transformed (µg)

FIG. 6A

FIG. 6B

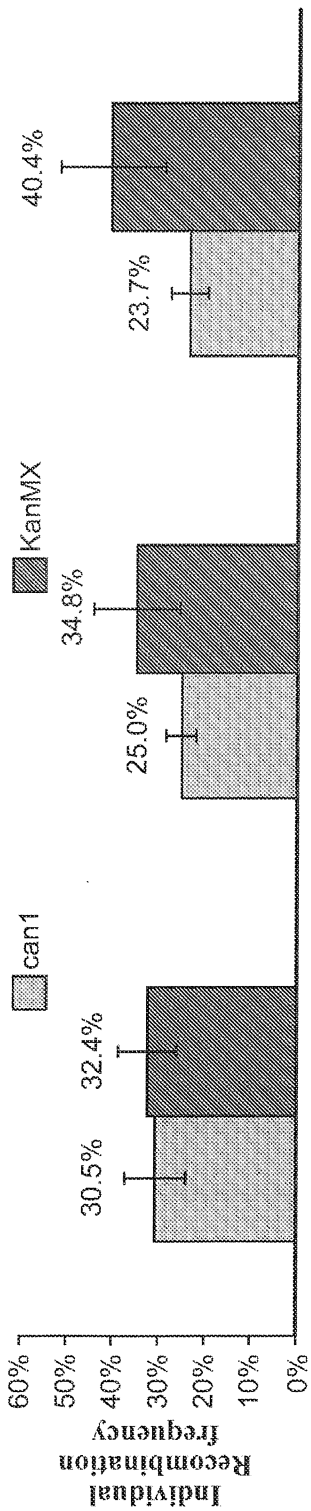
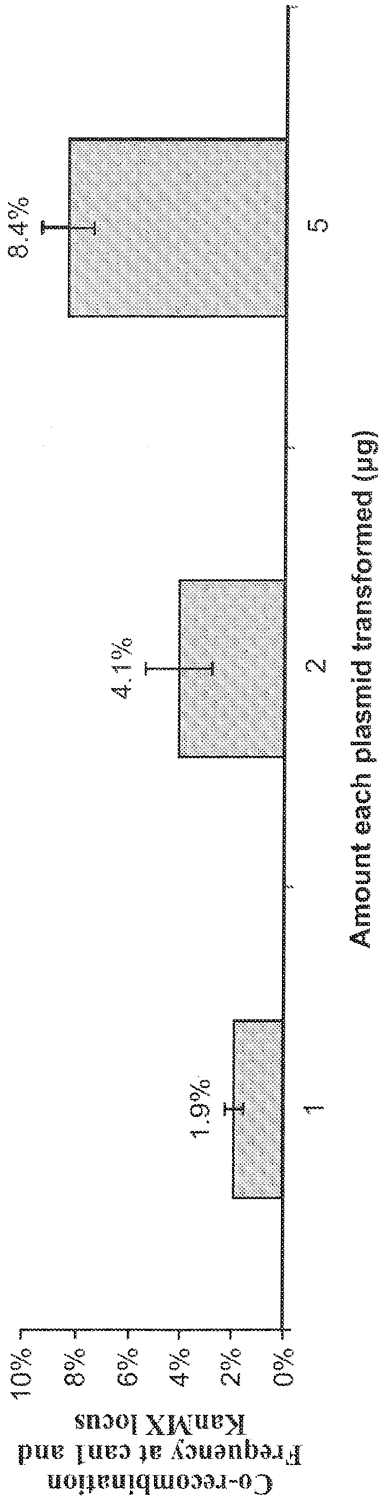

Individual Recombination frequency
60%
50%
40%
30%
20%
10%
0%
can1
KanMX
30.5%
32.4%
25.0%
34.8%
23.7%
40.4%

FIG. 6C


Co-recombination Frequency at can1 and KanMX locus
10%
8%
6%
4%
2%
0%
1.9%
4.1%
8.4%
1
2
5
Amount each plasmid transformed (µg)

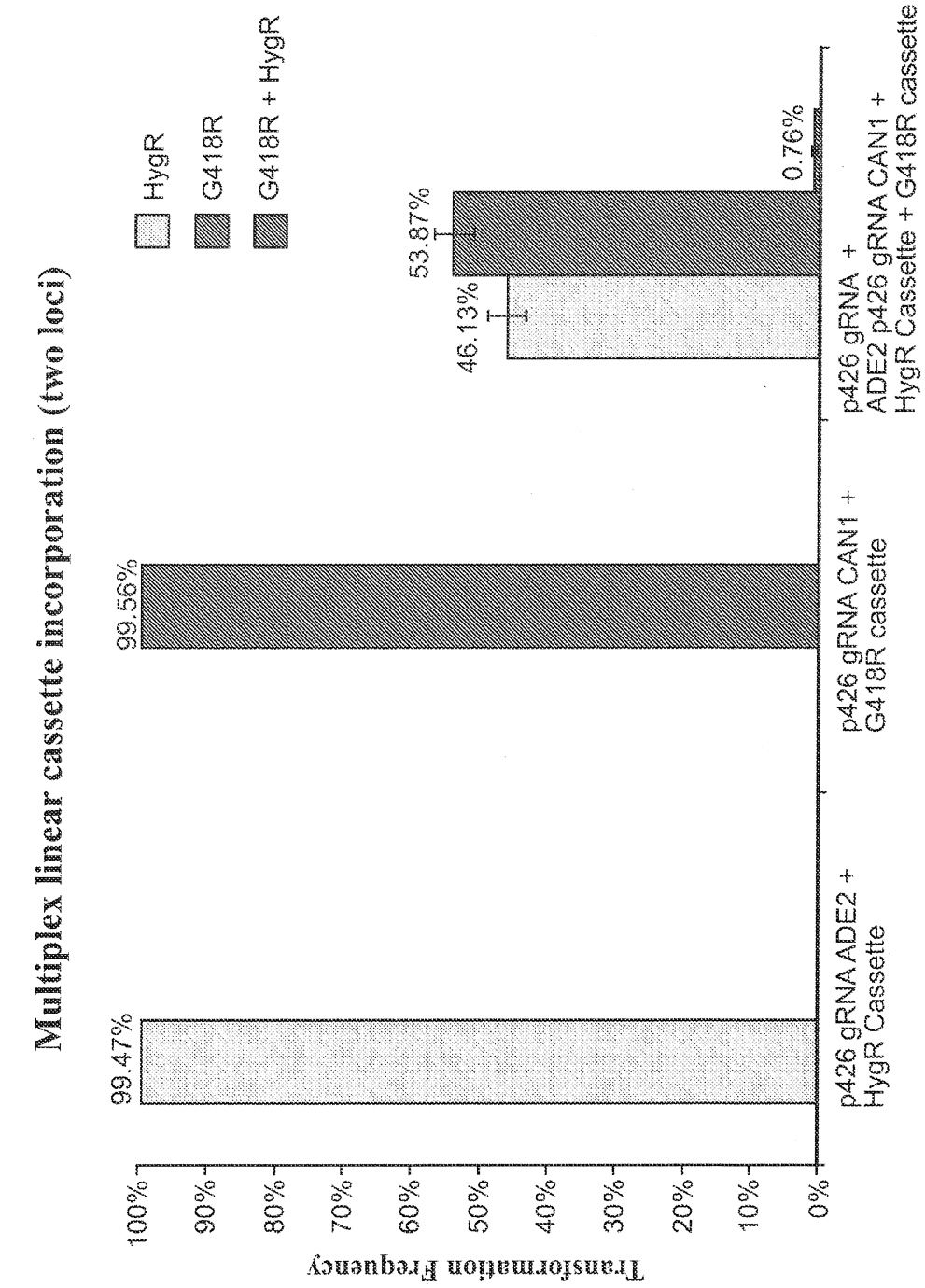

Multiplex linear cassette incorporation (two loci)
Transformation Frequency
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
HygR
G418R
G418R + HygR
99.47%
99.56%
46.13%
53.87%
0.76%
p426 gRNA ADE2 + HygR Cassette
p426 gRNA CAN1 + G418R cassette
p426 gRNA + ADE2 p426 gRNA CAN1 + HygR Cassette + G418R cassette

FIG. 7

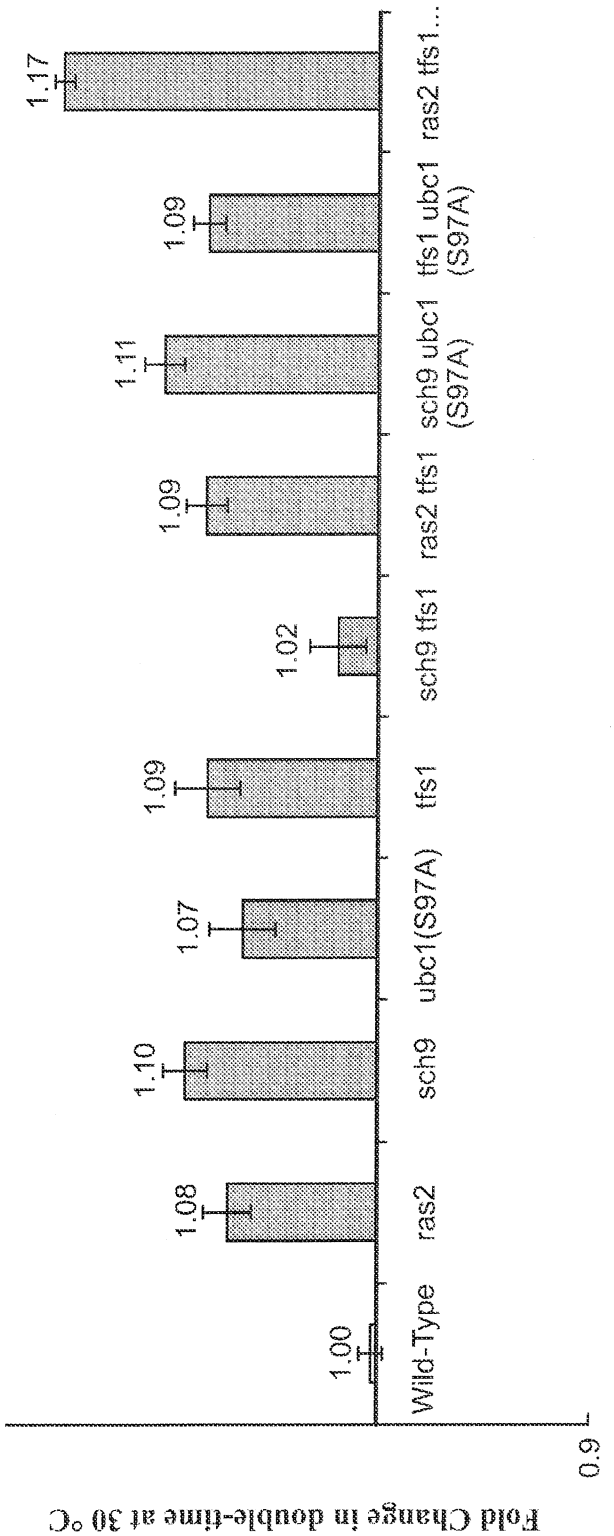

Doubling time in exponential growth in elevated temperatures for select mutants
Fold Change in double-time at 30 °C
0.9
1.00
1.08
1.10
1.07
1.09
1.02
1.09
1.11
1.09
1.17
Wild-Type
ras2
sch9
ubc1(S97A)
tfs1
sch9 tfs1
ras2 tfs1
sch9 ubc1 (S97A)
tfs1 ubc1 (S97A)
ras2 tfs1...

FIG. 8A

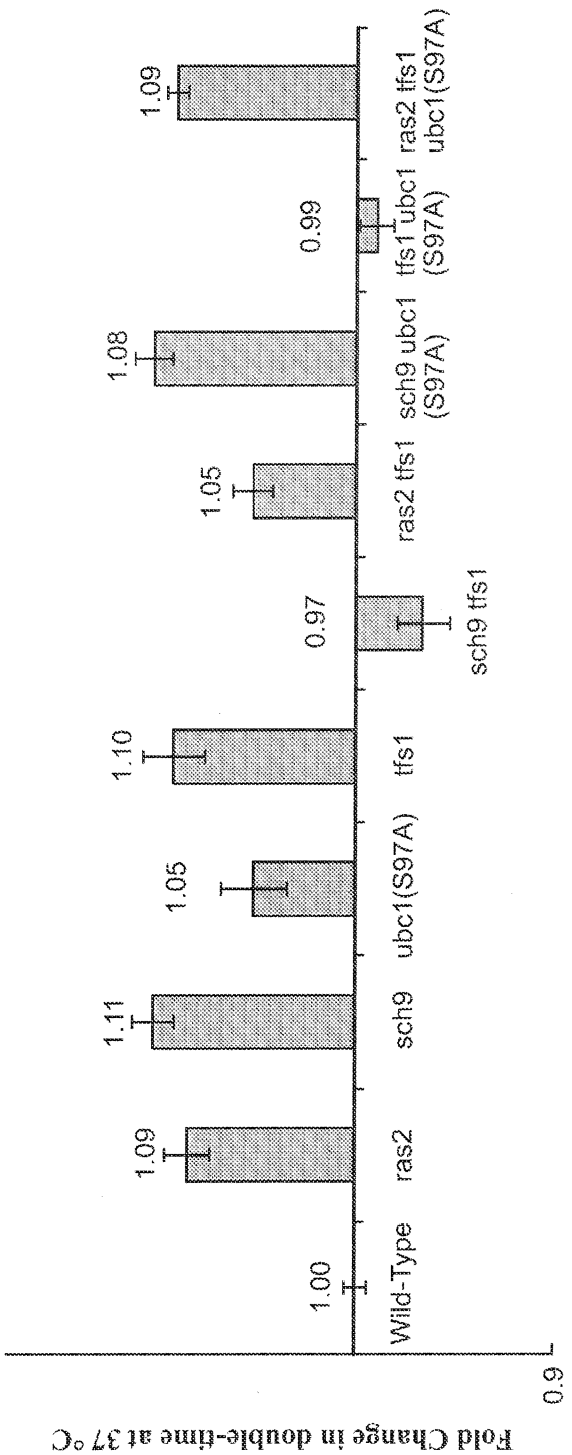

Fold Change in double-time at 37 °C
0.9
1.00
1.09
1.11
1.05
1.10
0.97
1.05
1.08
0.99
1.09
Wild-Type
ras2
sch9
ubc1(S97A)
tfs1
sch9 tfs1
ras2 tfs1
sch9 ubc1 (S97A)
tfs1 ubc1 (S97A)
ras2 tfs1 ubc1(S97A)

FIG. 8B

FIG. 8C

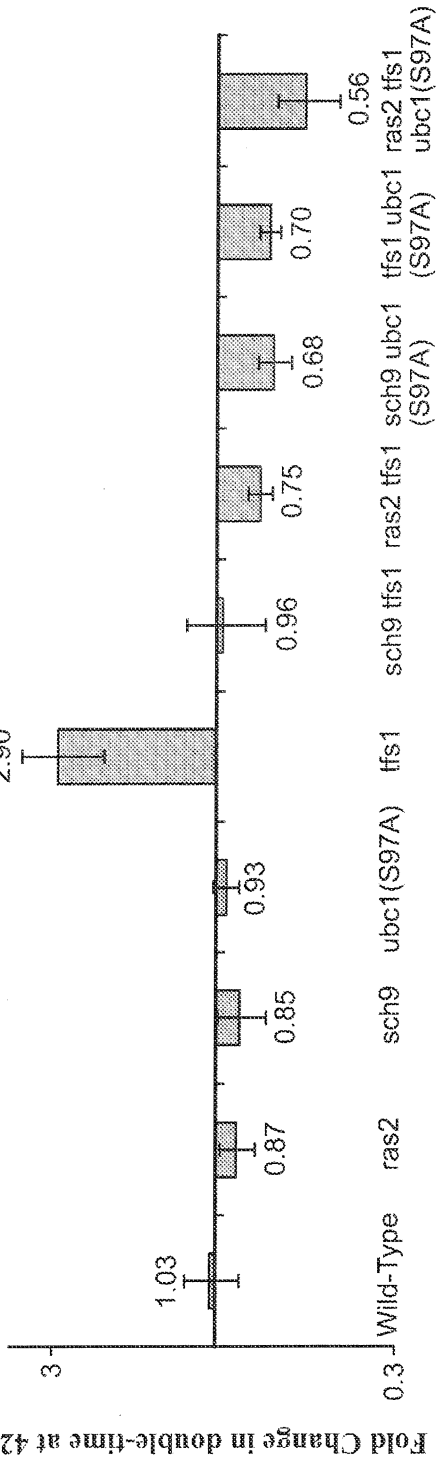
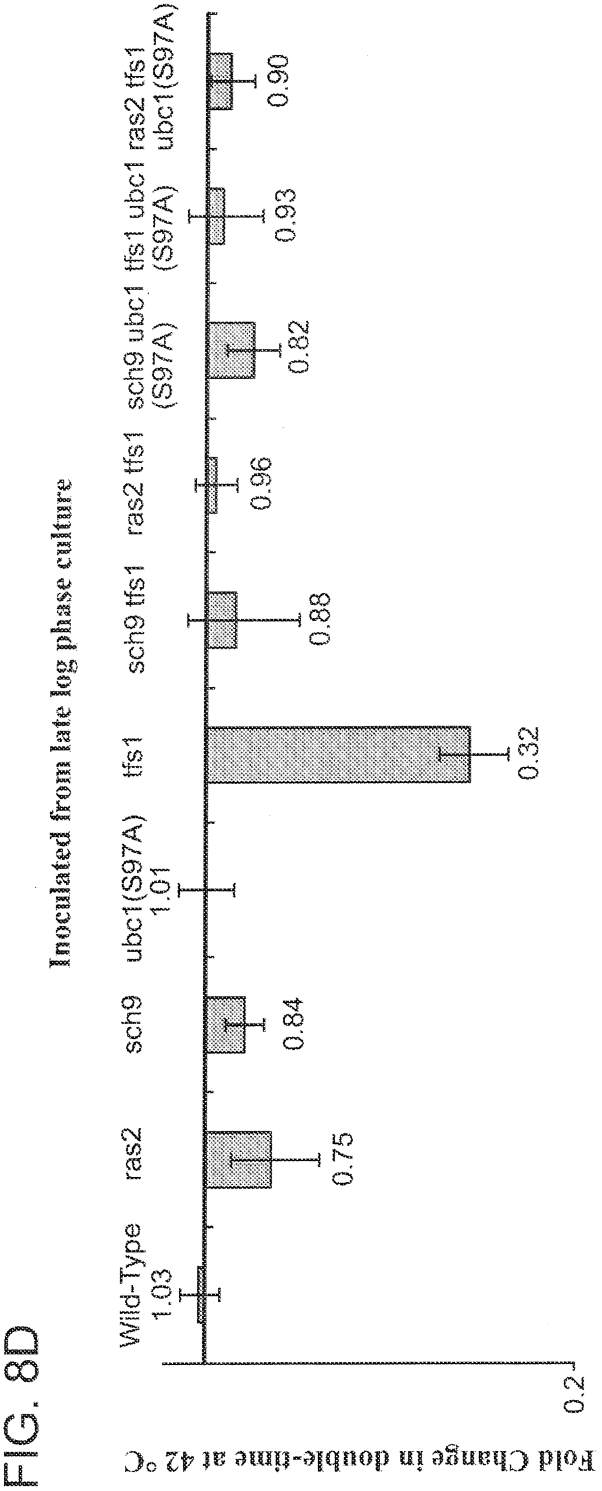

Inoculated from late stationary phase culture
Fold Change in double-time at 42°C
3
0.3
1.03
0.87
0.85
0.93
2.90
0.96
0.75
0.68
0.70
0.56
Wild-Type
ras2
sch9
ubc1(S97A)
tfs1
sch9 tfs1
ras2 tfs1
sch9 ubc1 (S97A)
tfs1 ubc1 (S97A)
ras2 tfs1 ubc1(S97A)

FIG. 8D


Inoculated from late log phase culture
Fold Change in double-time at 42 °C
0.2
Wild-Type
1.03
ras2
0.75
sch9
0.84
ubc1(S97A)
1.01
tfs1
0.32
sch9 tfs1
0.88
ras2 tfs1
0.96
sch9 ubc1 (S97A)
0.82
tfs1 ubc1 (S97A)
0.93
ras2 tfs1 ubc1(S97A)
0.90