Supplementary information for

Modular, synthetic chromosomes as new tools for large scale engineering of metabolism

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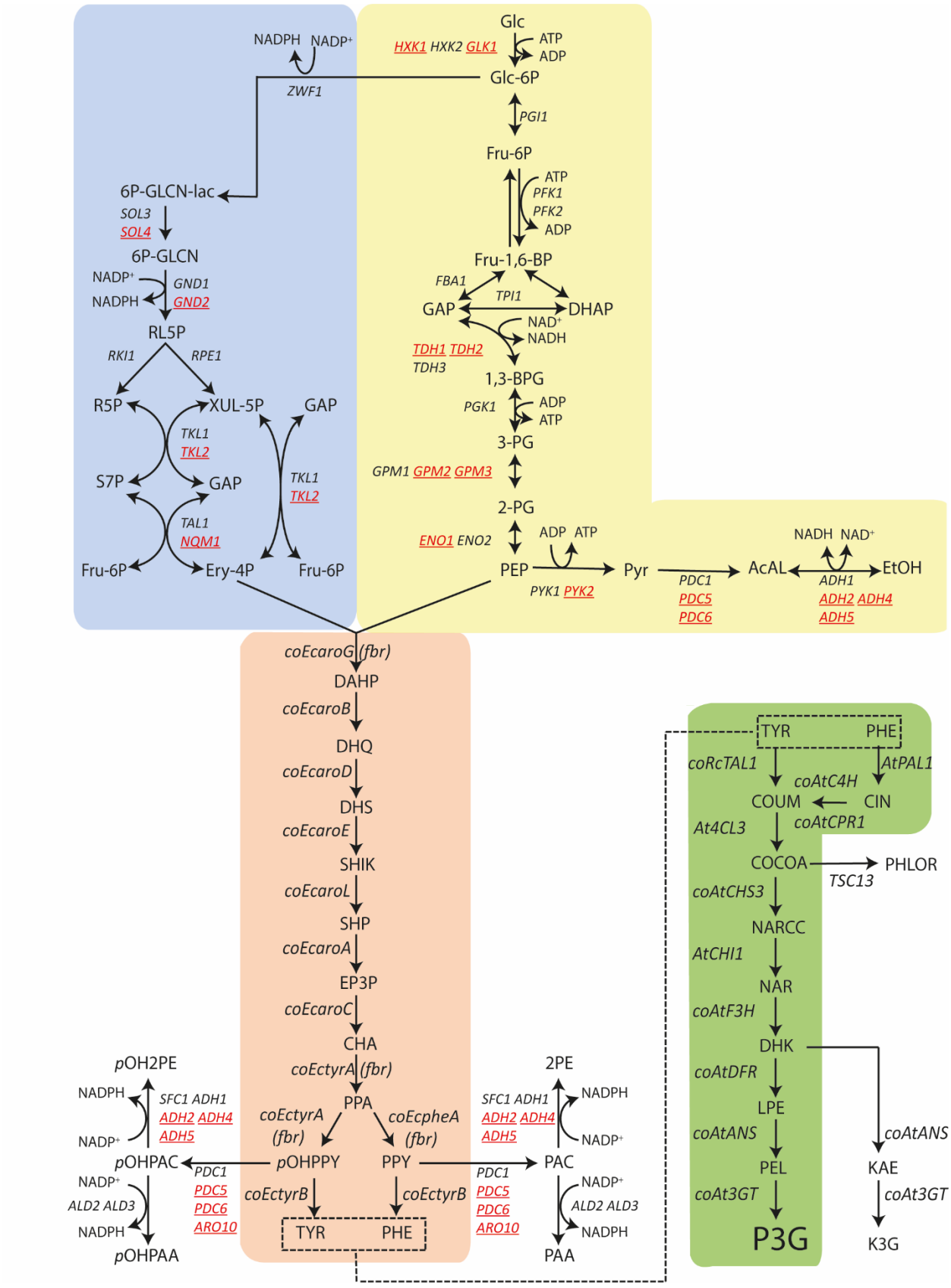
Supplementary Figure 1 - Reactions from glucose to pelargonidin 3-O-glucoside

Yellow: glycolysis an ethanolic fermentation. Blue: pentose phosphate pathway. Brown: *E. coli* shikimate pathway. Green: plant anthocyanin pathway. The gene names encoding the enzymes involved in the indicated reactions are indicated in italics. Genes deleted in the present study are indicated in red and underlined. *Ec E. coli, At Arabidopsis thaliana, Rc Rhodobacter capsulatus, co* codon optimized. *fbr* feedback resistant, Glc glucose, Glc-6P glucose 6-phosphate, Fru-6p fructose-6-phosphate, Fru-1,6-BP fructose 1,6-biphosphate, GAP glyceraldehyde 3-phosphate, DHAP dihydroxyacetone, 1,3-BPG 1,3-biphosphoglycerate, 3-PG 3-phosphoglycerate,2-PG 2-phosphoglycerate, PEP phosphoenolpyruvate, Pyr pyruvate, AcAL acetaldehyde, EtOH ethanol, 6p-GLCN-lac 6-phosphogluconolactone, 6p-GLCN 6-phosphoglucono, RL5P ribulose 5-phosphate, R5P ribose 5-phosphate, S7P sedoheptulose 7-phosphate, XUL-5P xylulose 5-phosphate, GAP glyceraldehyde 3-phosphate, Ery-4P erythrose 4-phosphate, DAHP 3-deoxy-D-arabino-heptulosonate-7-P, DHQ 3-dehydroquinate, DHS 3-dehydroshikimate, SHIK shikimate, SHP shikimate 3-phosphate, EP3P 5-enolpyruvoyl-shikimate 3-phosphate, CHA chorismate, PPA prephenate, PPY phenylpyruvate, PAC phenylacetaldehyde 2PE 2-phenylethanol, PAA phenylacetic acid, PHE L-phenylalanine, *p*OHPPY *p*-hydroxyphenylpyruvate, *p*OHPAC *p*-hydroxyphenylacetaldehyde, *p*OH2PE, *p*-hydroxyphenylethanol, *p*OHPAA, *p*-hydroxyphenylacetic acid, TYR tyrosine, COUM coumaric acid, CIN cinnamic acid, COCOA coumaroyl-CoA, NARCC naringenin chalcone, PHLOR phloretic acid, NAR naringenin, DHK dihydrokaempferol, KAE kaempferol, K3G kaempferol 3-O-glucoside, LPE, leucopelargonidin, PEL pelargonidin, P3G pelargonidin 3-O-glucoside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ADP
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PFK1
PFK2
ADP
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FBA1
TPI1
GAP
DHAP
NAD+
NADH
TDH1 TDH2
TDH3
1,3-BPG
PGK1
ADP
ATP
3-PG
GPM1 GPM2 GPM3
2-PG
ENO1 ENO2
ADP ATP
PEP
Pyr
PYK1 PYK2
PDC1
PDC5
PDC6
AcAL
NADH NAD+
EtOH
ADH1
ADH2 ADH4
ADH5
NADPH NADP+
ZWF1
6P-GLCN-lac
SOL3
SOL4
6P-GLCN
NADP+
NADPH
GND1
GND2
RL5P
RKI1
RPE1
R5P
XUL-5P
GAP
TKL1
TKL2
S7P
GAP
TAL1
NQM1
Fru-6P
Ery-4P
Fru-6P
TKL1
TKL2
coEcaroG (fbr)
DAHP
coEcaroB
DHQ
coEcaroD
DHS
coEcaroE
SHIK
coEcaroL
SHP
coEcaroA
EP3P
coEcaroC
CHA
coEctyrA (fbr)
PPA
coEctyrA (fbr)
coEcpheA (fbr)
pOHPPY
PPY
coEctyrB
coEctyrB
TYR
PHE
pOH2PE
NADPH
NADP+
SFC1 ADH1
ADH2 ADH4
ADH5
pOHPAC
NADP+
ALD2 ALD3
NADPH
pOHPAA
PDC1
PDC5
PDC6
ARO10
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ADH5
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NADP+
PAC
NADP+
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PAA
PDC1
PDC5
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ARO10
TYR
PHE
coRcTAL1
AtPAL1
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COUM
CIN
At4CL3
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COCOA
PHLOR
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NAR
coAtF3H
DHK
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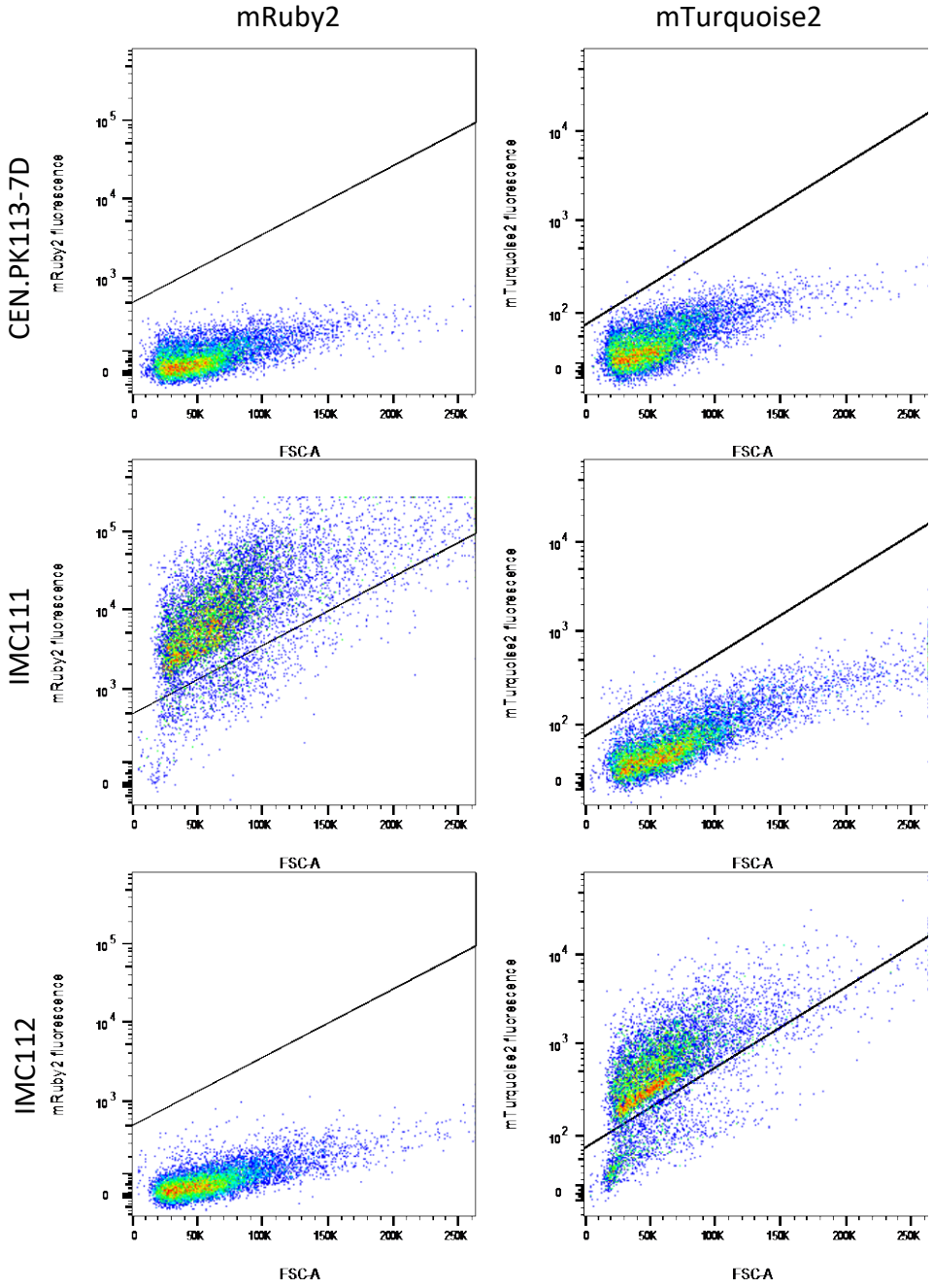
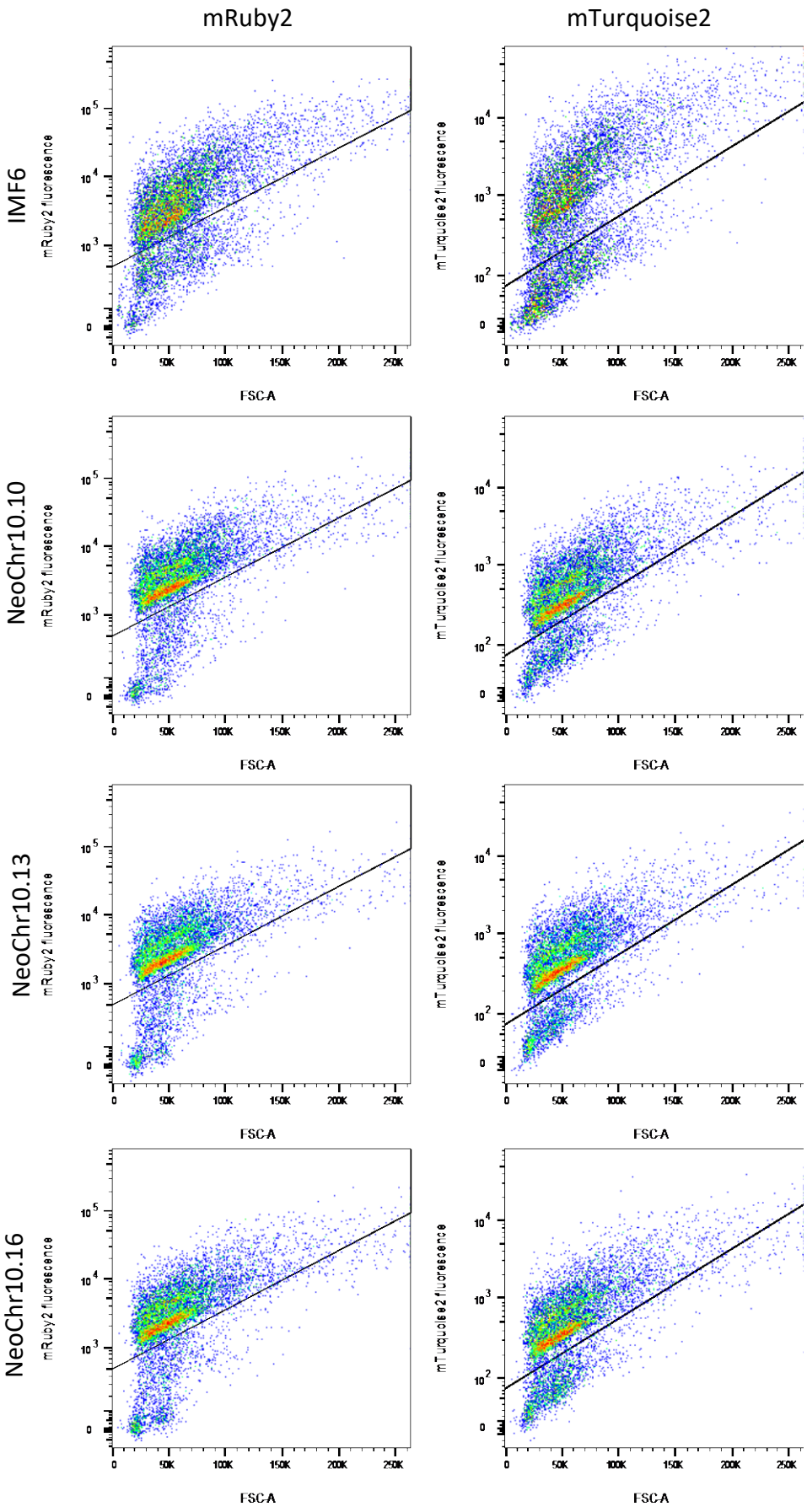
LPE
coAtANS
PEL
coAt3GT
P3G
coAtANS
KAE
coAt3GT
K3G

Supplementary Figure 2 – Flow cytometric analysis of the test linear neochromosomes

Cells from shake flask cultures were analysed by FACS. The fluorescence is plotted on the y-axis and the forward scatter (FSC-A) on the x-axis. Negative control: CEN.PK113-7D. Positive controls: IMC111 (mRuby2), IMC112 (mTurquoise2) and IMF6 (mRuby2 and mTurquoise2). Gates for fluorescence of the two different fluorescent proteins were drawn based on the IMC111 and IMC112 controls. Approximately 10000 events are shown for each plot.

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IMF6
NeoChr10.10
NeoChr10.13
NeoChr10.16
mRuby2 fluorescence
mTurquoise2 fluorescence
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150K
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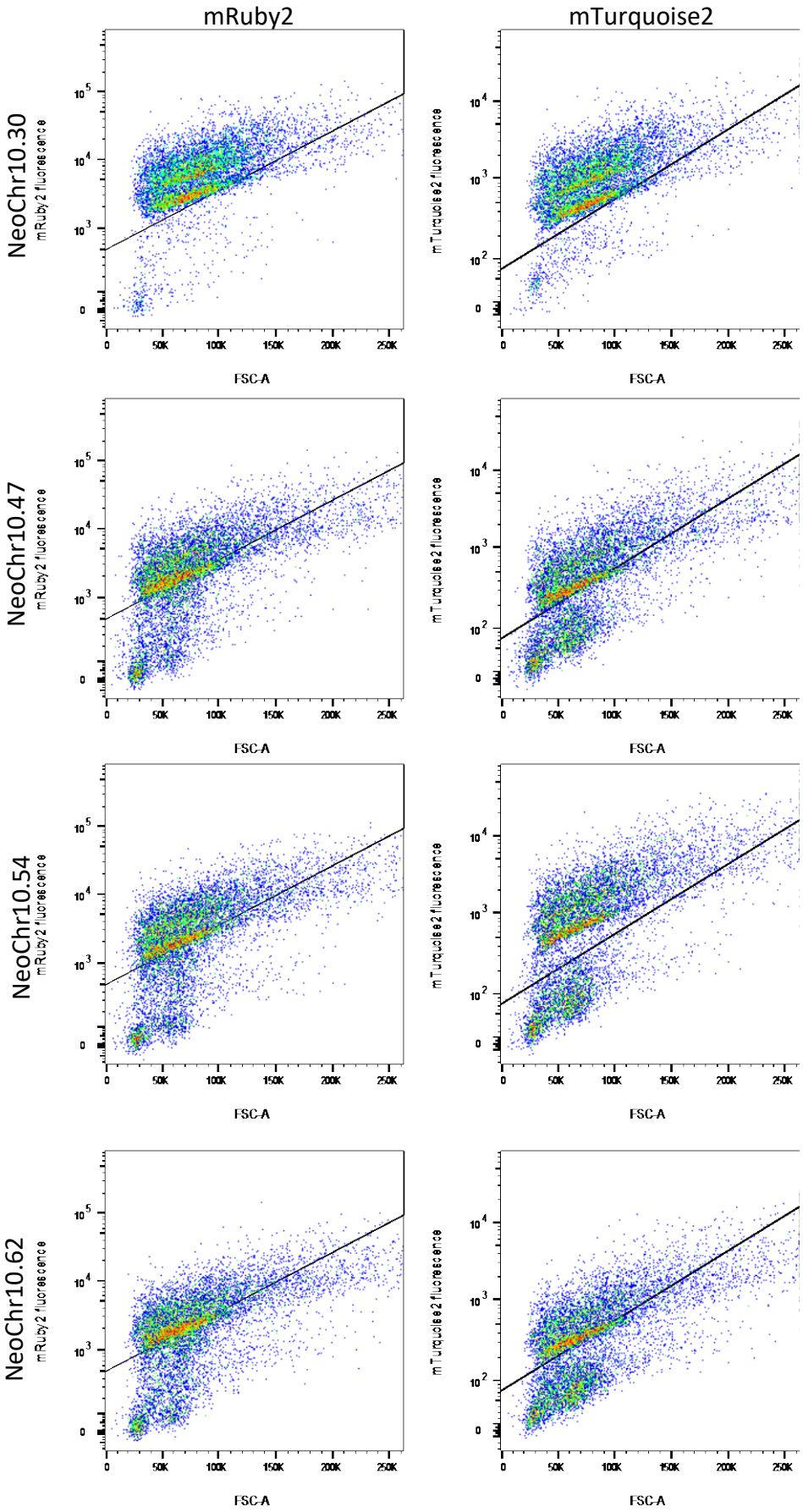
10^2
10^3
10^4
10^5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mTurquoise2
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NeoChr10.47
NeoChr10.54
NeoChr10.62
mRuby2 fluorescence
mTurquoise2 fluorescence
FSC-A
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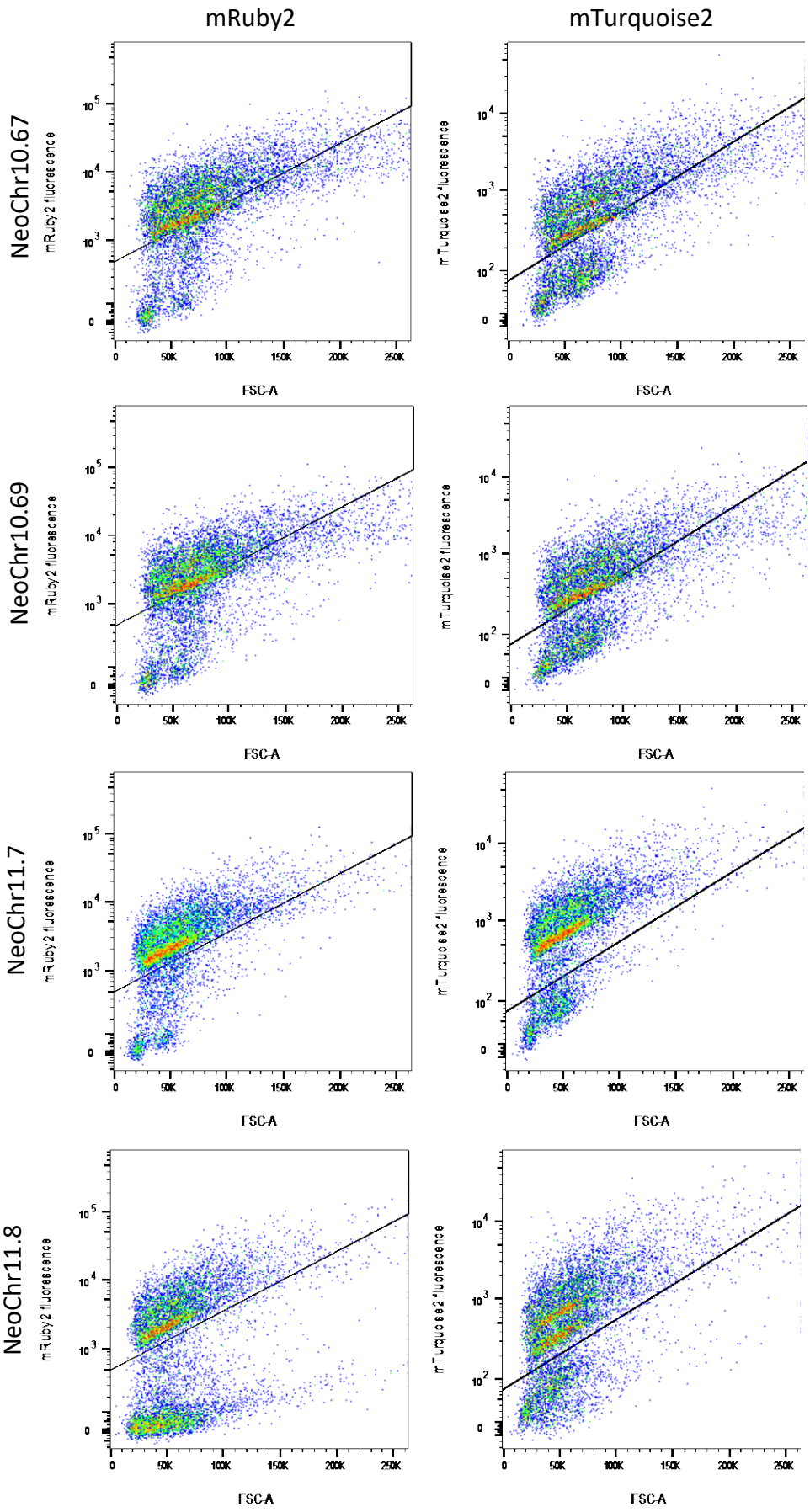
100K
150K
200K
250K
10^2
10^3
10^4
10^5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NeoChr10.69
NeoChr11.7
NeoChr11.8
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mTurquoise2 fluorescence
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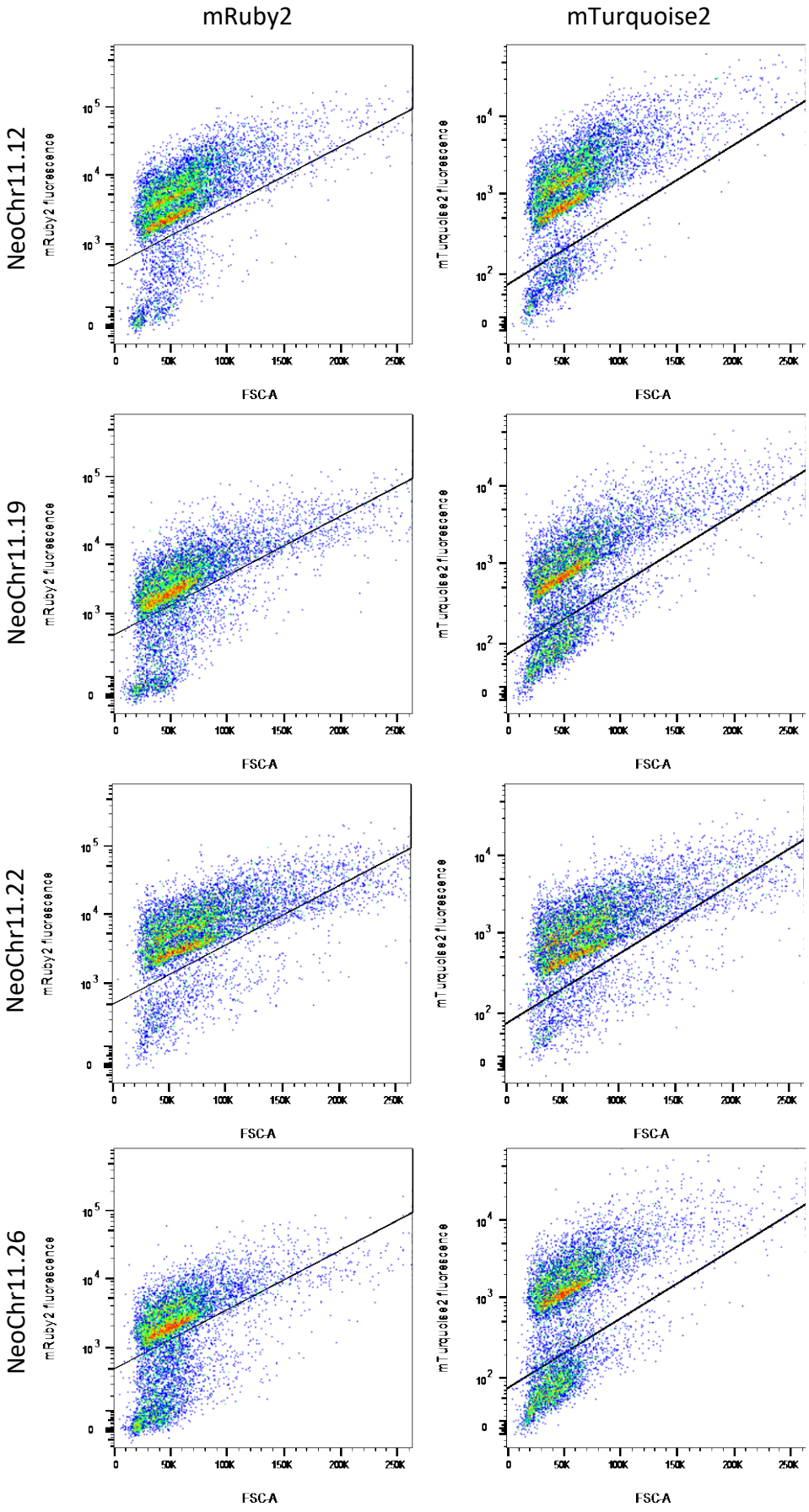
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mTurquoise2
NeoChr11.12
NeoChr11.19
NeoChr11.22
NeoChr11.26
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mTurquoise2 fluorescence
FSC-A
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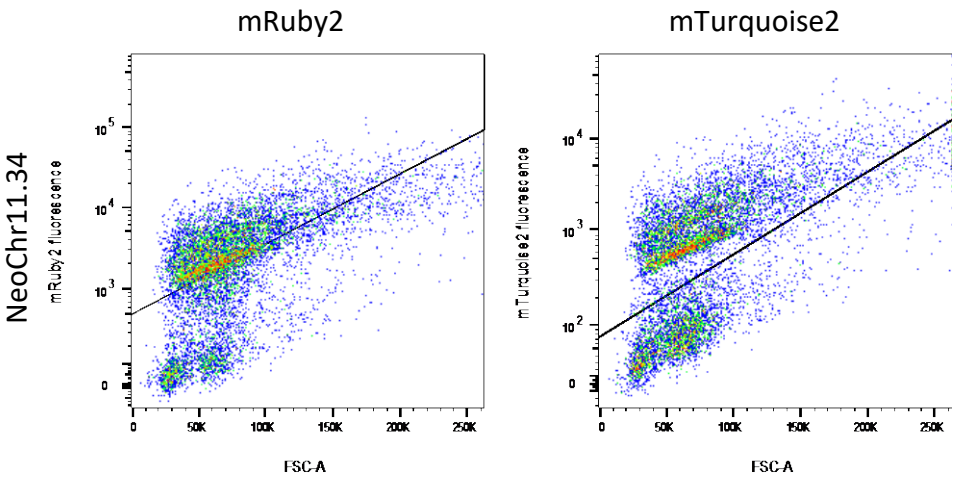
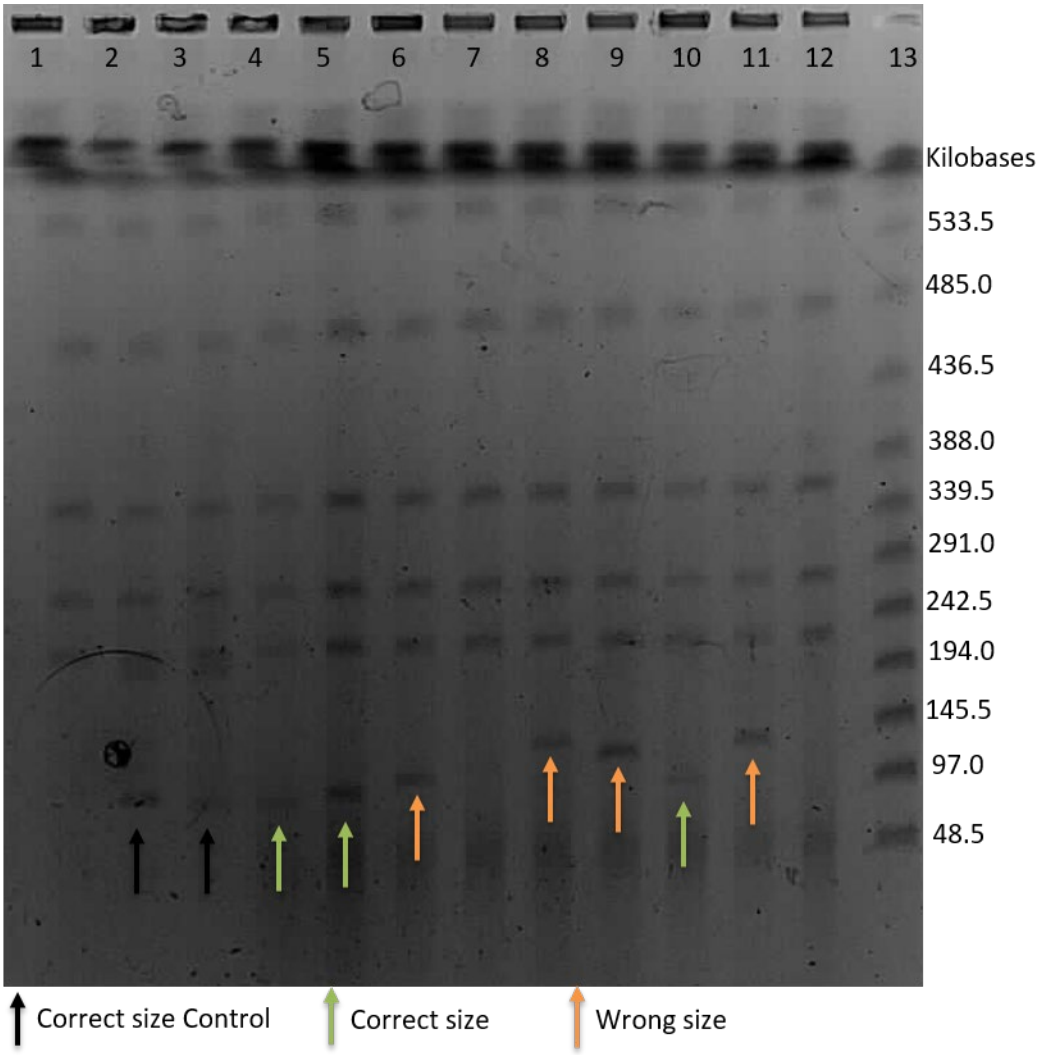
100K
150K
200K
250K
10^2
10^3
10^4
10^5

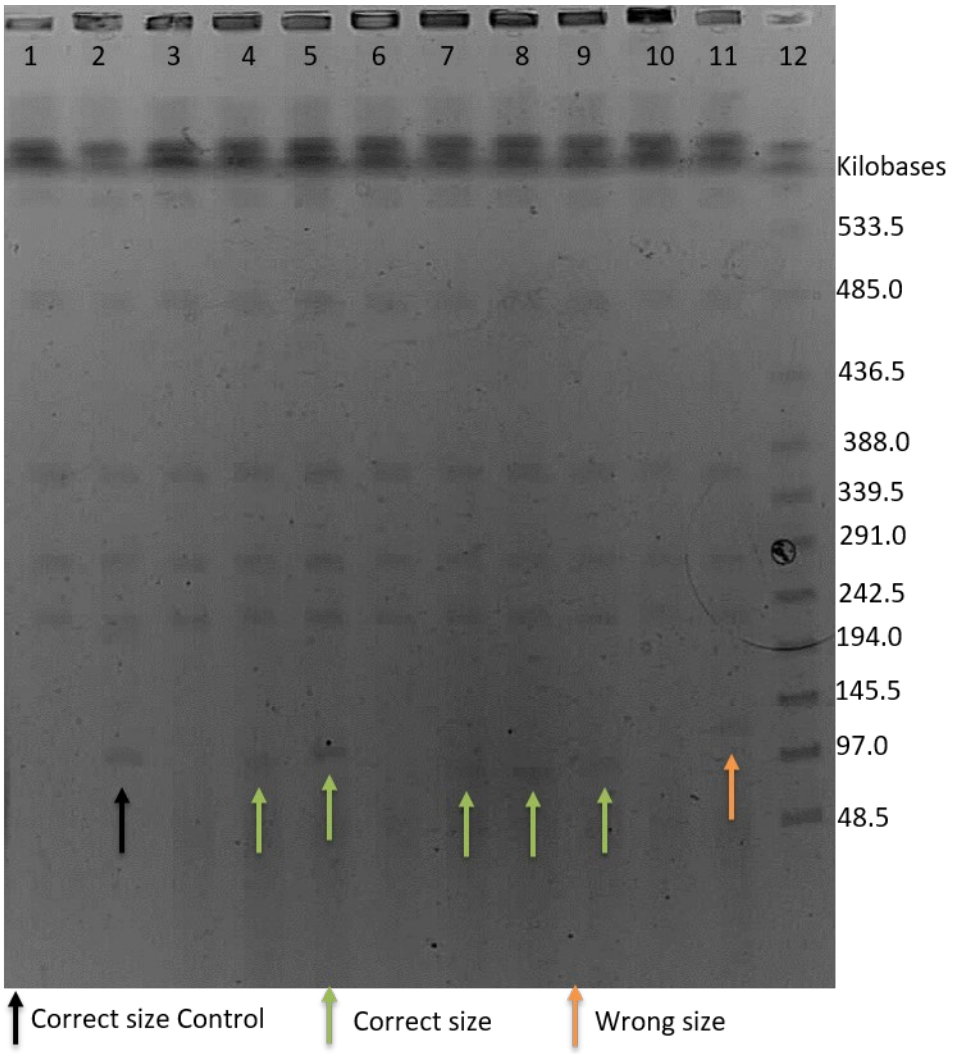

mRuby2
mTurquoise2
NeoChr11.34
mRuby2 fluorescence
mTurquoise2 fluorescence
FSC-A
FSC-A

Supplementary Figure 3 - Separation of the test linear neochromosomes on pulsed-field electrophoresis

A. 1) IMX1338: control strain without neochromosome. 2) IMF6: control strain with 100 kb in plug linearized neochromosome NeoChr1. 3) IMF23: strain with 100 kb in plug linearized NeoChr12. 4) NeoChr10.10: correct size. 5)NeoChr10.13: correct size. 6) NeoChr10.16: wrong size. 7) NeoChr11.7: no visible neochromosome. 8) NeoChr11.8: wrong size. 9) NeoChr11.12: wrong size. 10) NeoChr11.19: correct size. 11)NeoChr11.22: wrong size. 12) NeoChr11.26: no visible neochromosome. 13) Size ladder



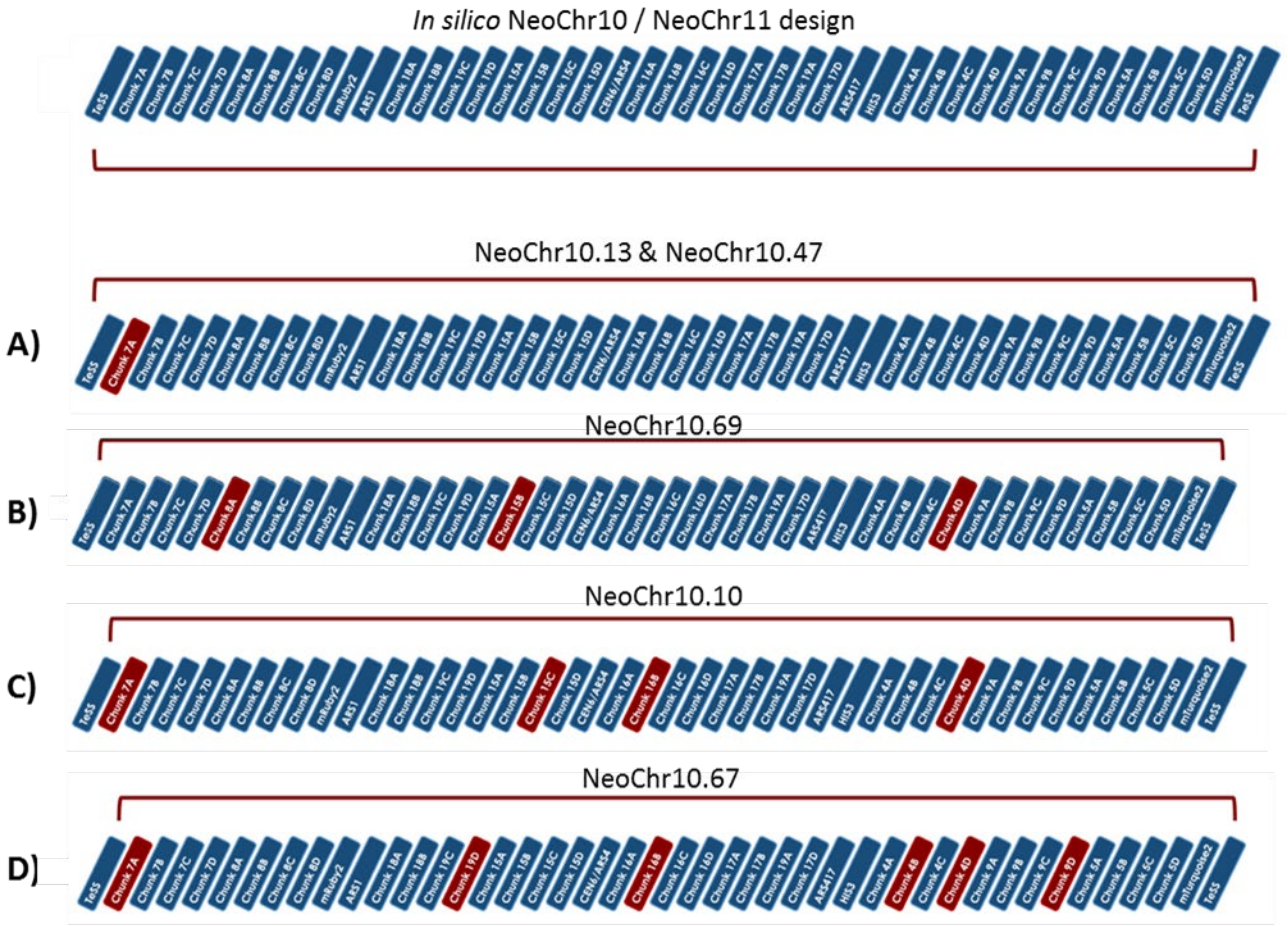
B. 1) IMX1338: control strain without neochromosome. 2) IMF6: control strain with 100 kb in plug linearized neochromosome (NeoChr1). 3) NeoChr10.30: no visible neochromosome. 4) NeoChr10.47: correct size. 5)NeoChr10.54: correct size. 6) NeoChr10.60: no visible neochromosome. 7) NeoChr10.62: correct size. 8) NeoChr10.67: correct size. 9) NeoChr10.69: correct size. 10) NeoChr11.29: no visible neochromosome. 11) NeoChr11.34: wrong size. 12) Size ladder



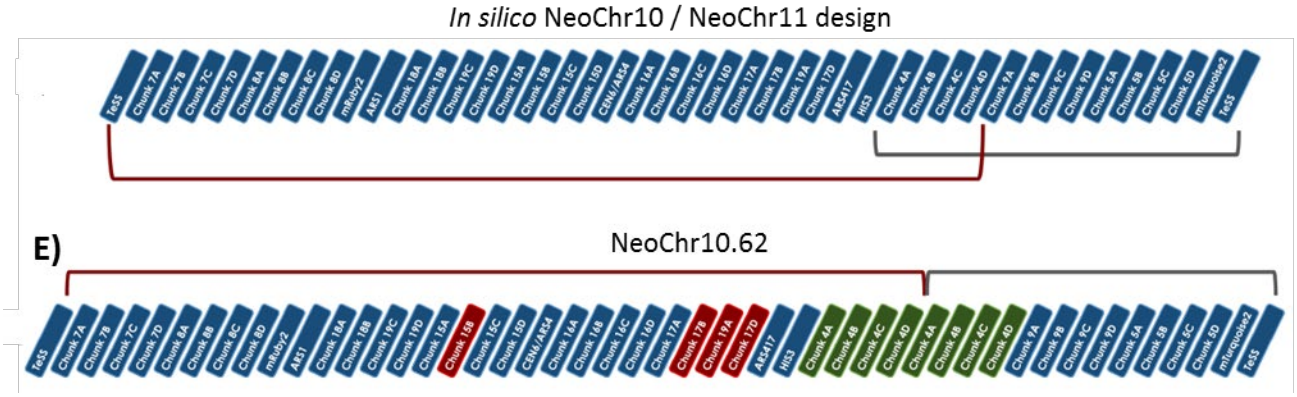
Supplementary Figure 4 - Sequencing results of test linear neochromosomes

in silico fragment configuration in NeoChr10 / NeoChr11 as well as *in vivo* fragment configuration of the neochromosome transformants as measured by long-read nanopore sequencing. The fragments of the *in silico* design which are present in the neochromosome transformants are connected by the same colored line. A dotted line indicates an area which is inverted. Fragments are color coded as follows: blue represents a correctly assembled fragment; red represents a missing fragment; green represents a duplicated fragment; and yellow indicates an inverted fragment.

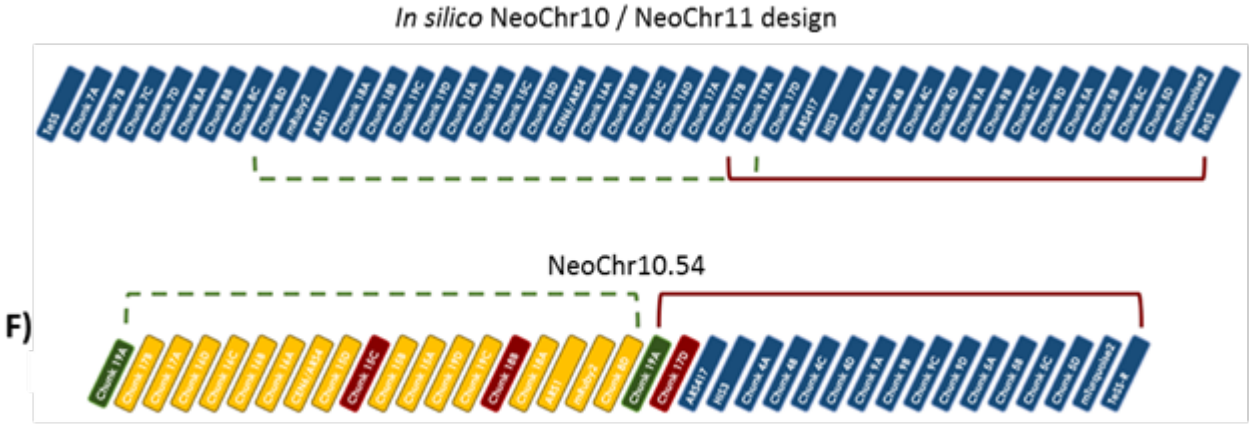
A) NeoChr10.13 and NeoChr10.47 are missing an internal part of chunk 7A. B) NeoChr10.69 is missing 3 chunks: 8A, 15B and 4D. C) NeoChr10.10 is missing 4 chunks: 7A, 15C, 16B and 4D. D) NeoChr10.67 is missing 6 chunks: 7A, 19D, 16B, 4B, 4D and 9D



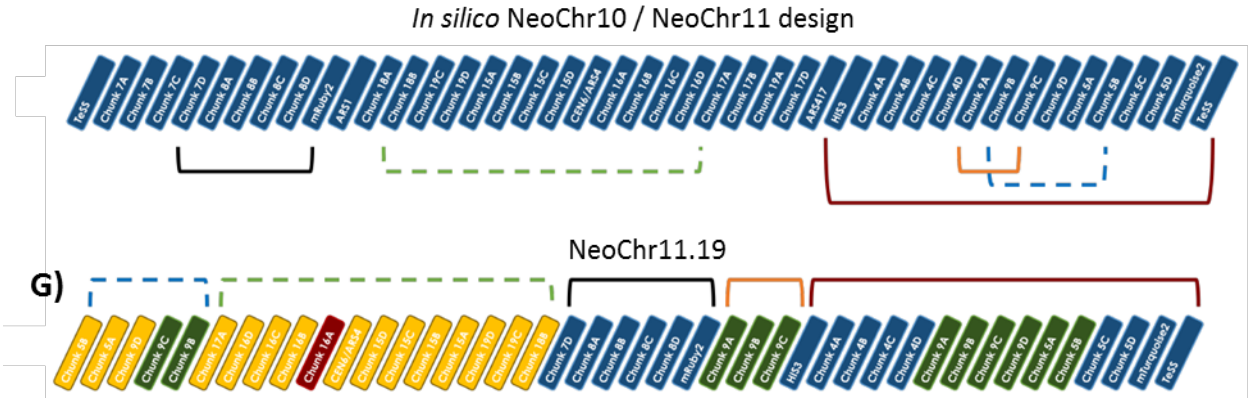
E) NeoChr10.62 is missing 4 chunks: 15B, 17B, 19A and 17D. In addition, a region containing the chunks 4A, 4B, 4C and 4D is duplicated



F) Neochr10.54 has a large inversion from 8D until 19A, from this region 2 chunks are missing: 15C and 18B. This region is link to a region spanning from 19A (which is thus duplicated) until the right telomere. From this region chunk 17D is missing

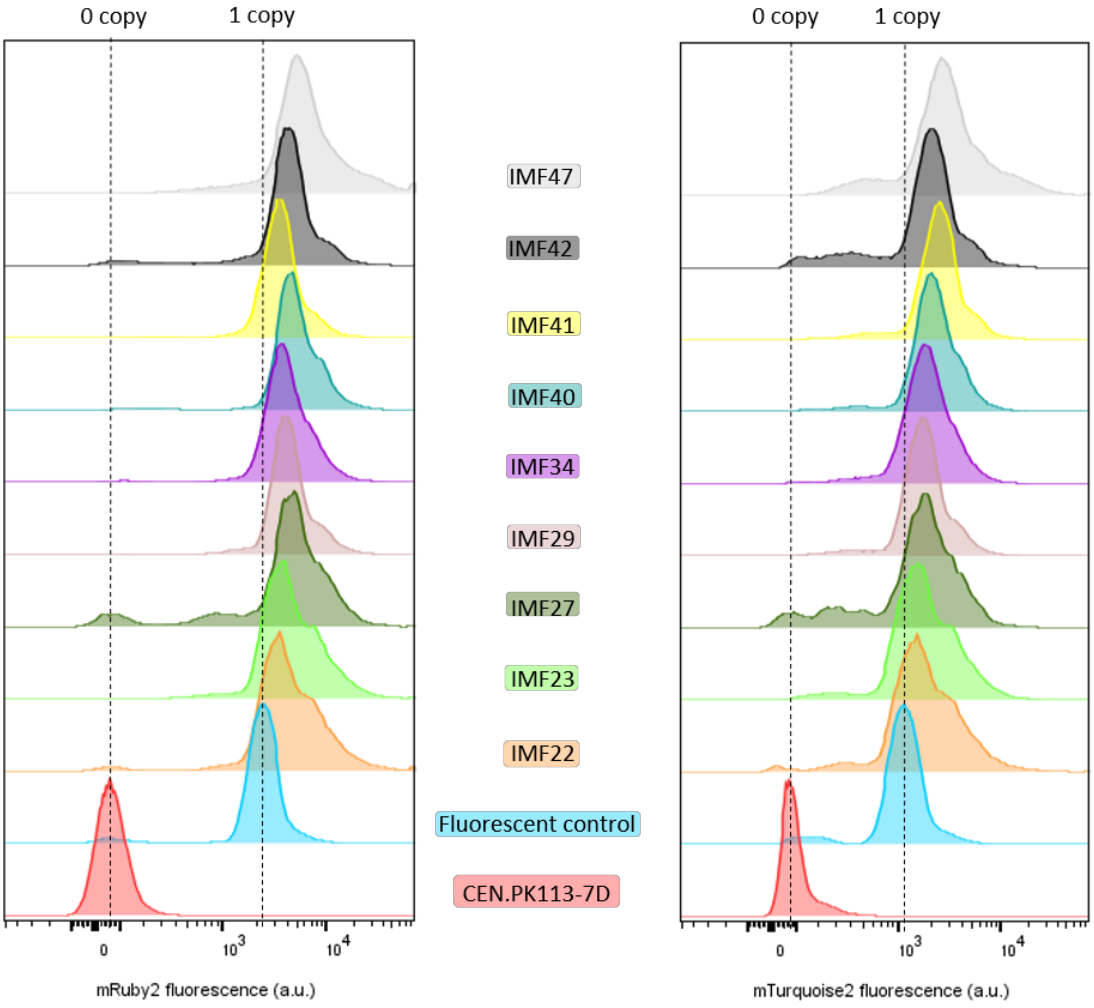


G) NeoChr11.19 contains several duplicated and inverted areas, from one area chunk 16A is missing.



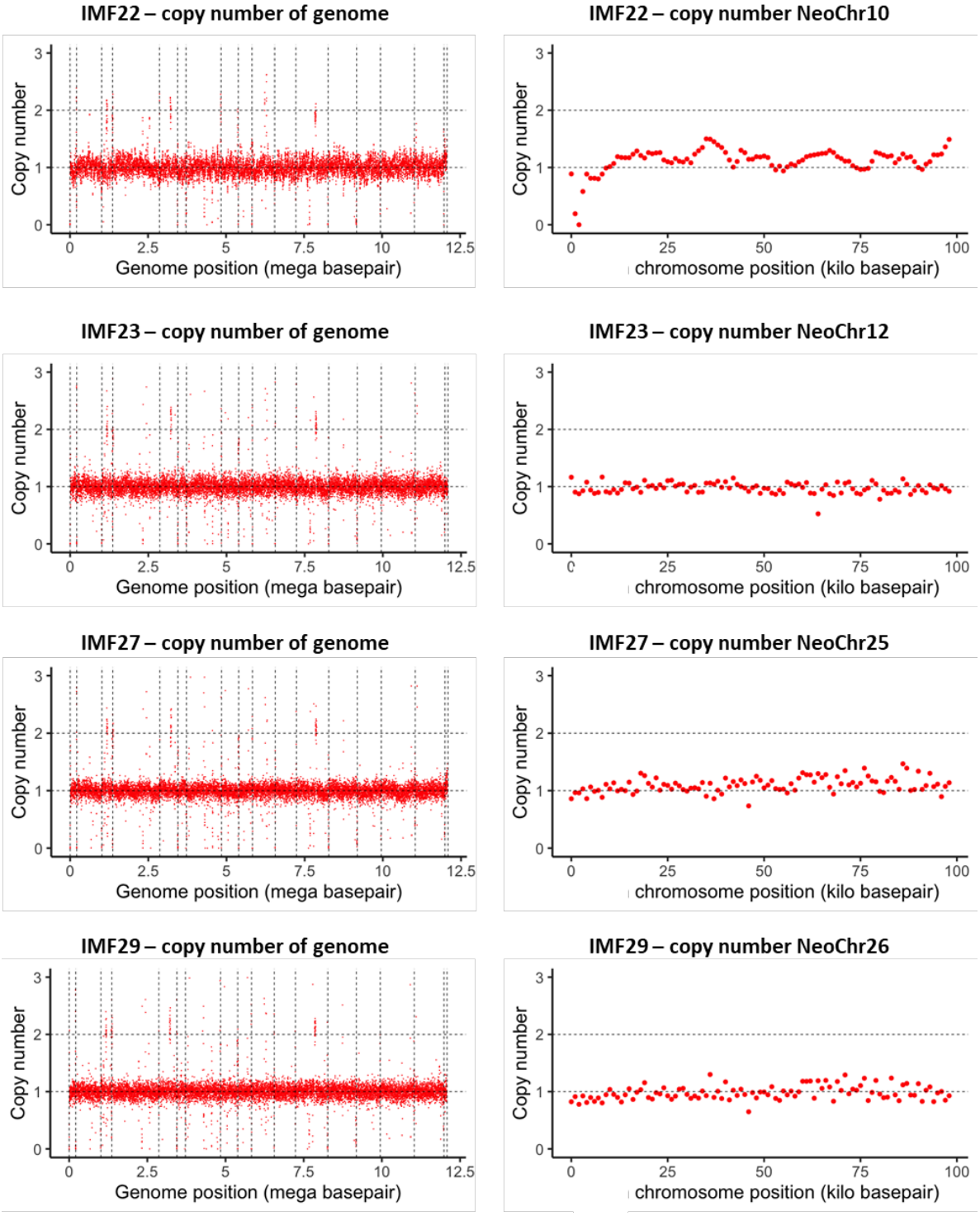
Supplementary Figure 5 - NeoChr copy number estimation based on fluorescence

mRuby2 and mTurquoise2 fluorescence was measured by flow cytometry. CEN.PK113-7D with no fluorescent markers was used as negative control. IMX2224 and IMX2226 with a single copy of *mRuby2* and *mTurquoise2* integrated in the genome, respectively, were used as positive controls. All strains showed a fluorescence corresponding to the expected NeoChr. copy number.

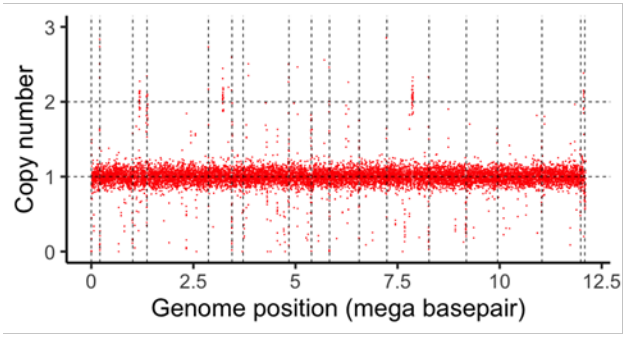


Supplementary Figure 6 - NeoChr copy number estimation based on sequencing

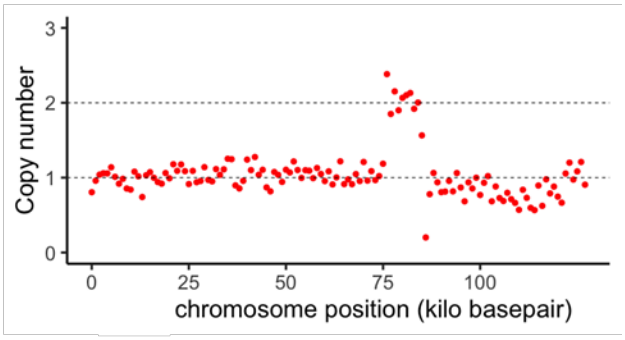
IMF22 and IMF48 were analyzed by long-read Nanopore sequencing and IMF23, IMF41, IMF42 and IFM47 by short-read Miseq sequencing. Plots on the left represent the copy number of native chromosomes, while plots on the right show the NeoChrs copy number



IMF41 – copy number of genome



IMF41 – copy number of NeoChr30



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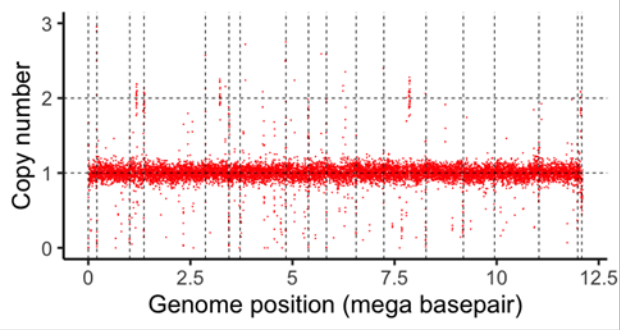

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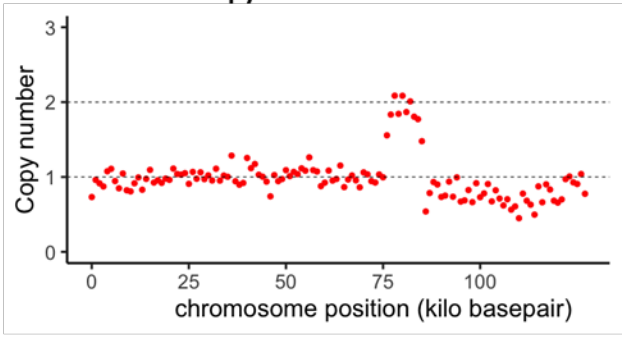

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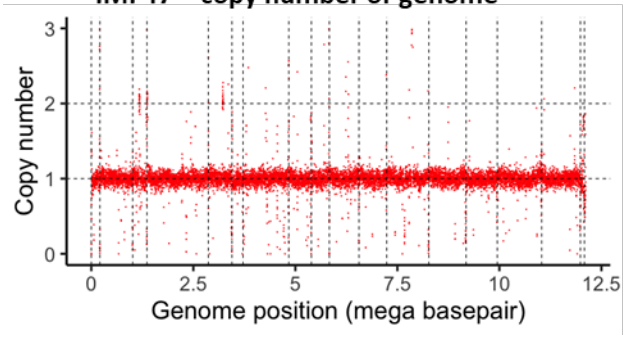

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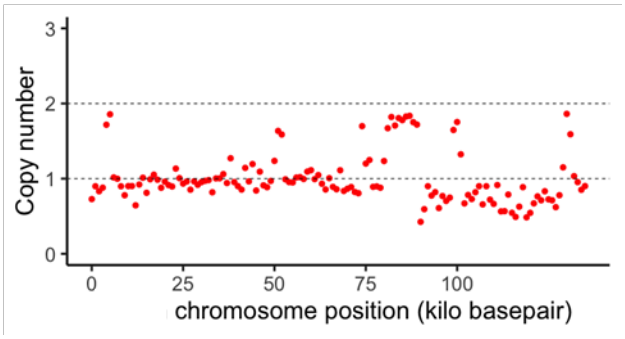
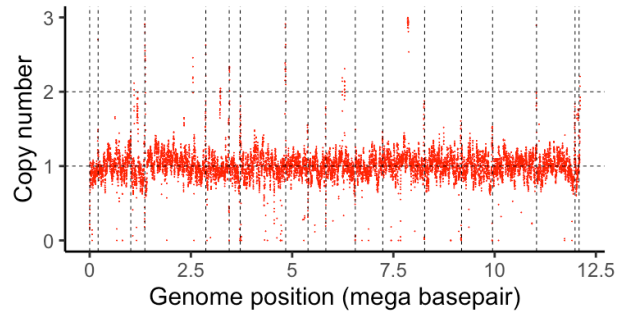
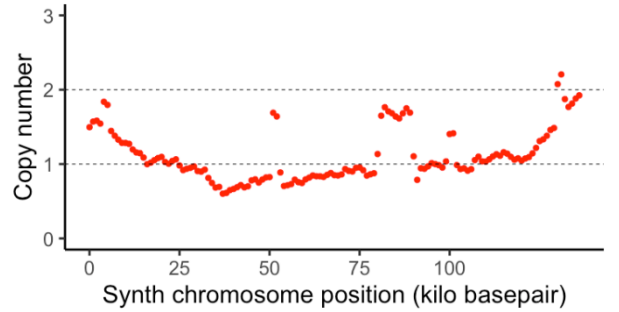


IMF48 – copy number of genome

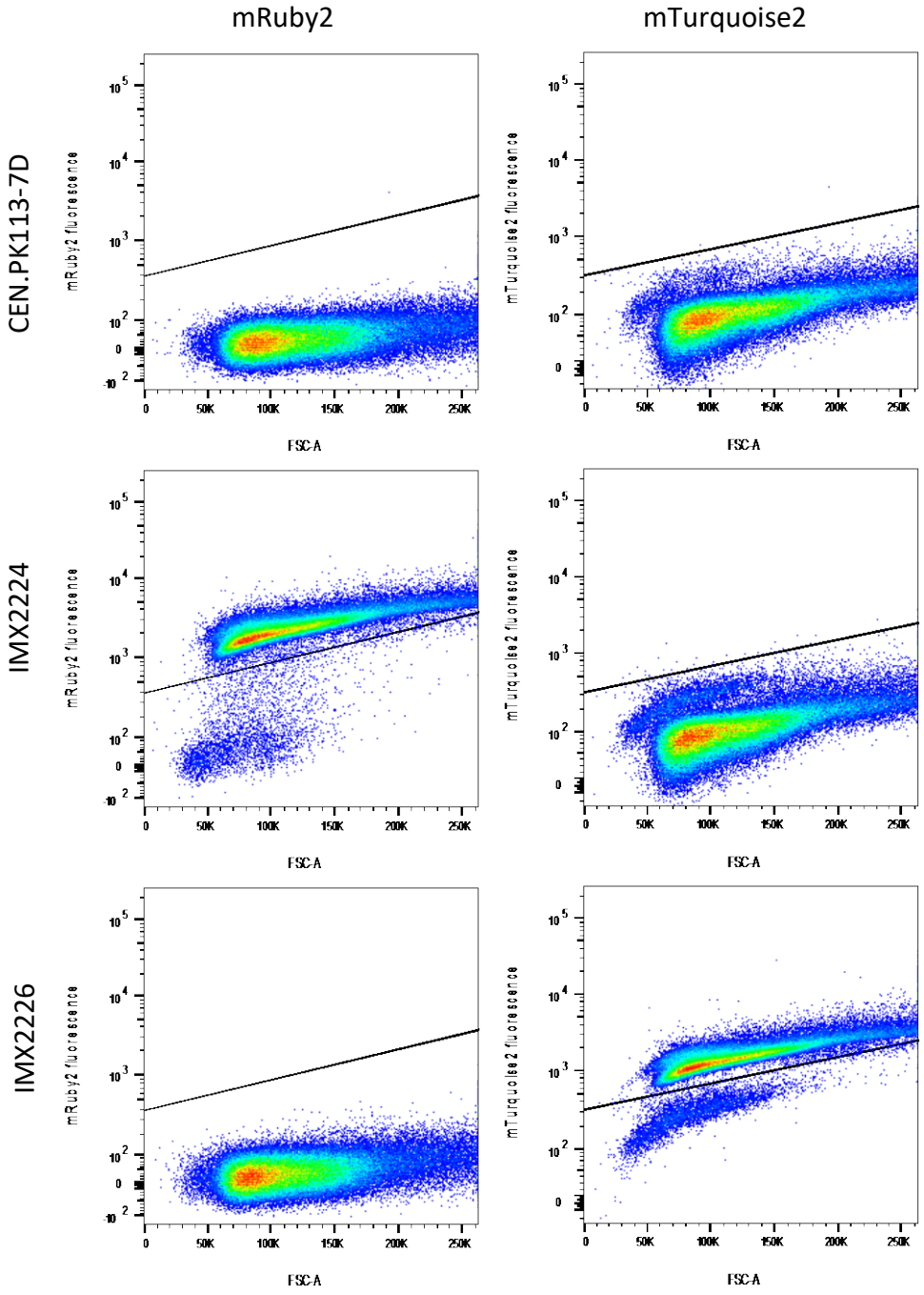


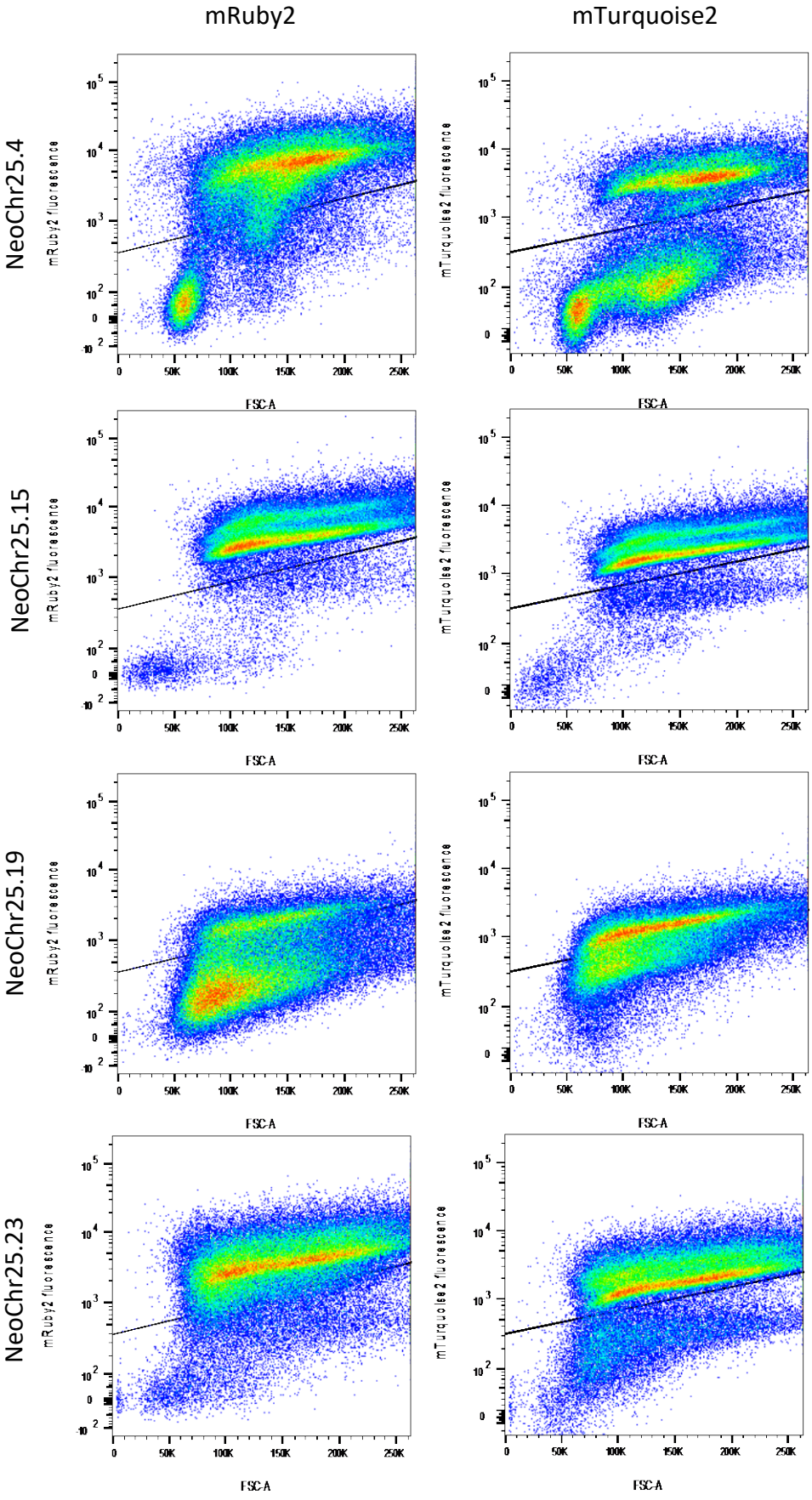
IMF48 – copy number of NeoChr34



Supplementary Figure 7 - Flow cytometric analysis of (linear) NeoChr25 and (circular) NeoChr26 designed for anthocyanin production

Cells from shake flask cultures were analyzed by FACS. The fluorescence is plotted on the y-axis and the FSC-A on the x-axis. Negative control: CEN.PK113-7D. Positive controls: IMX2224 (mRuby2), IMX2226 (mTurquoise2). Gates for fluorescence of the two different fluorescent proteins were drawn based on the IMX2224 and IMX2226 controls. Approximately 10000 or 100000 events are shown for each plot.

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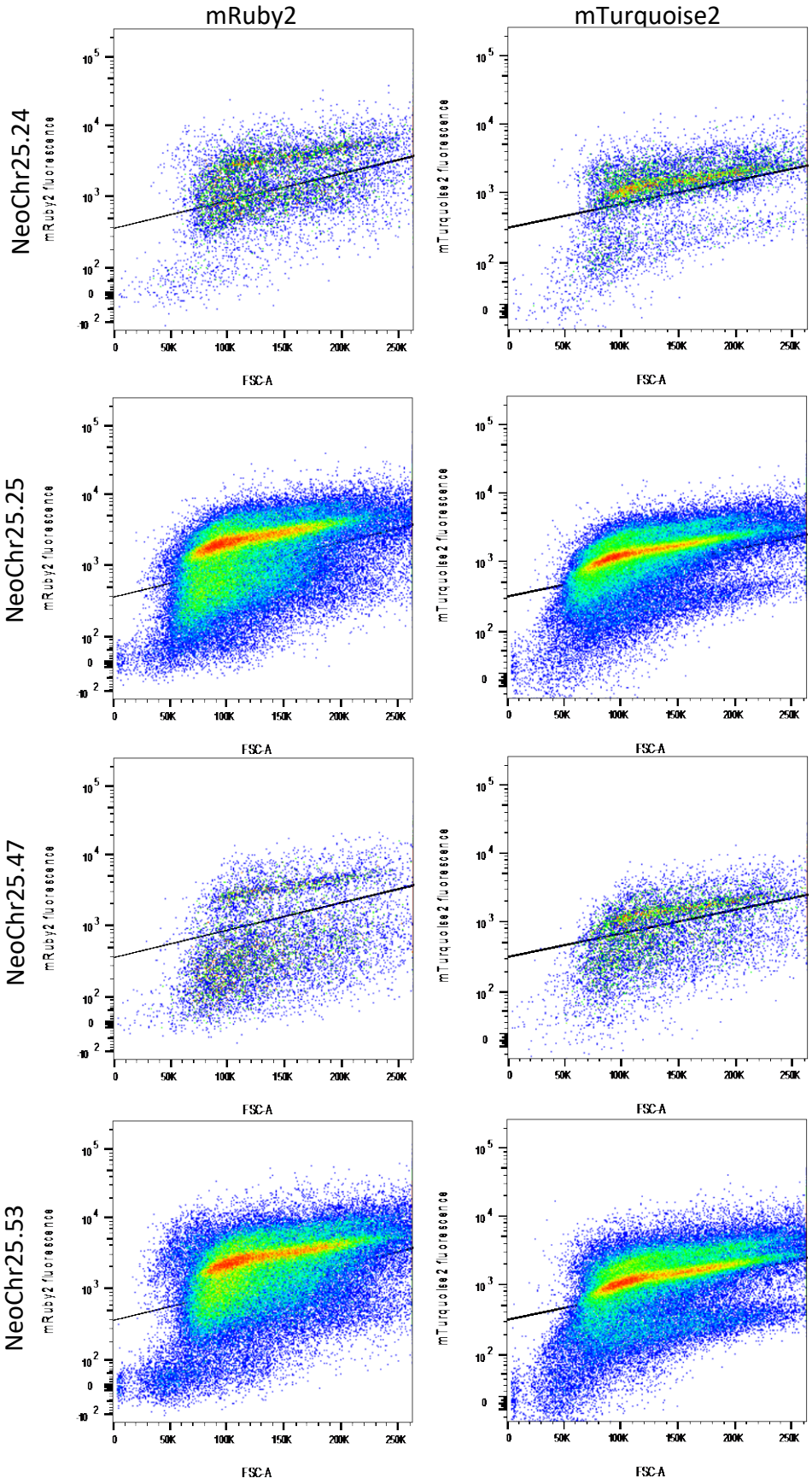
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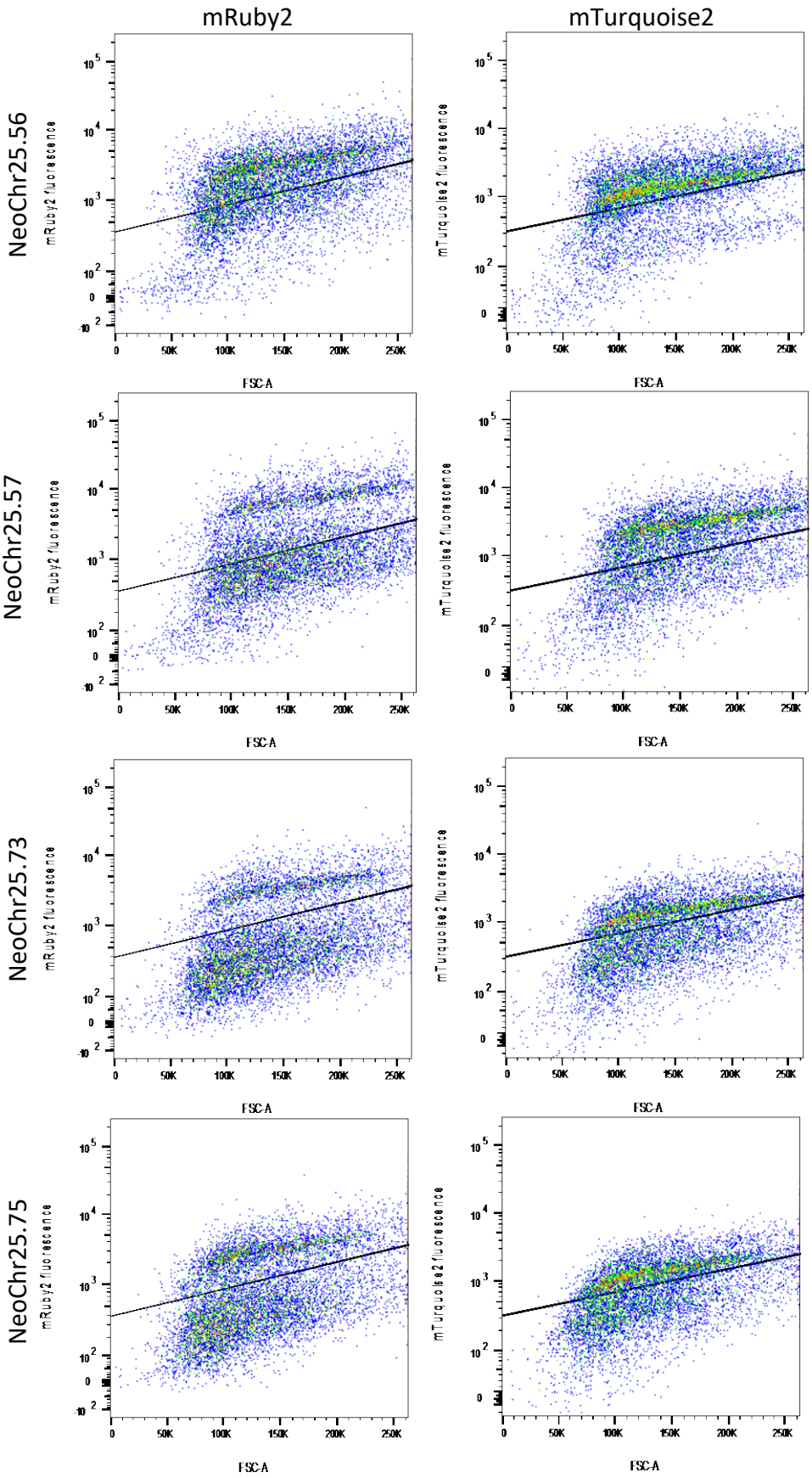
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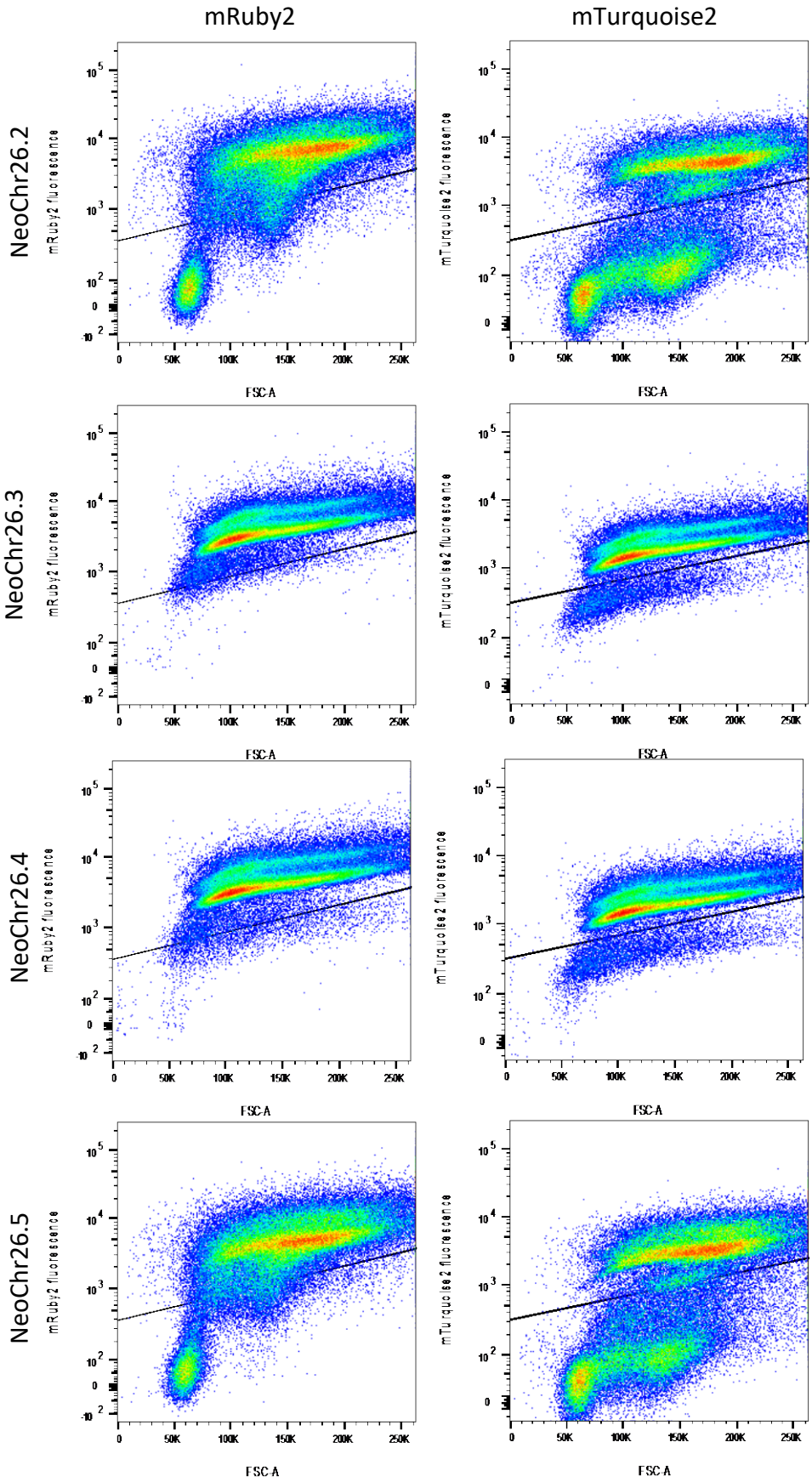
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200K
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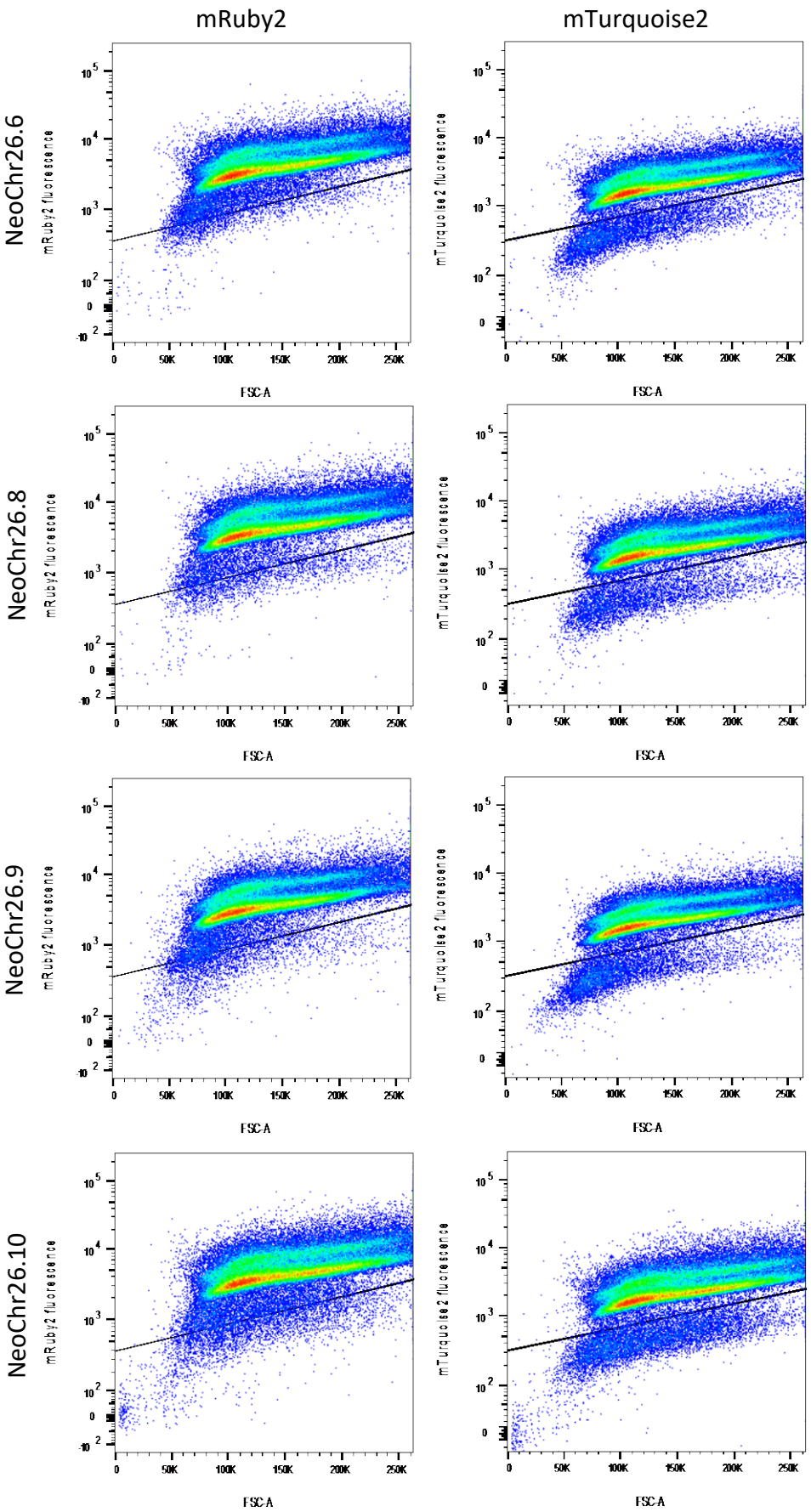
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NeoChr26.8
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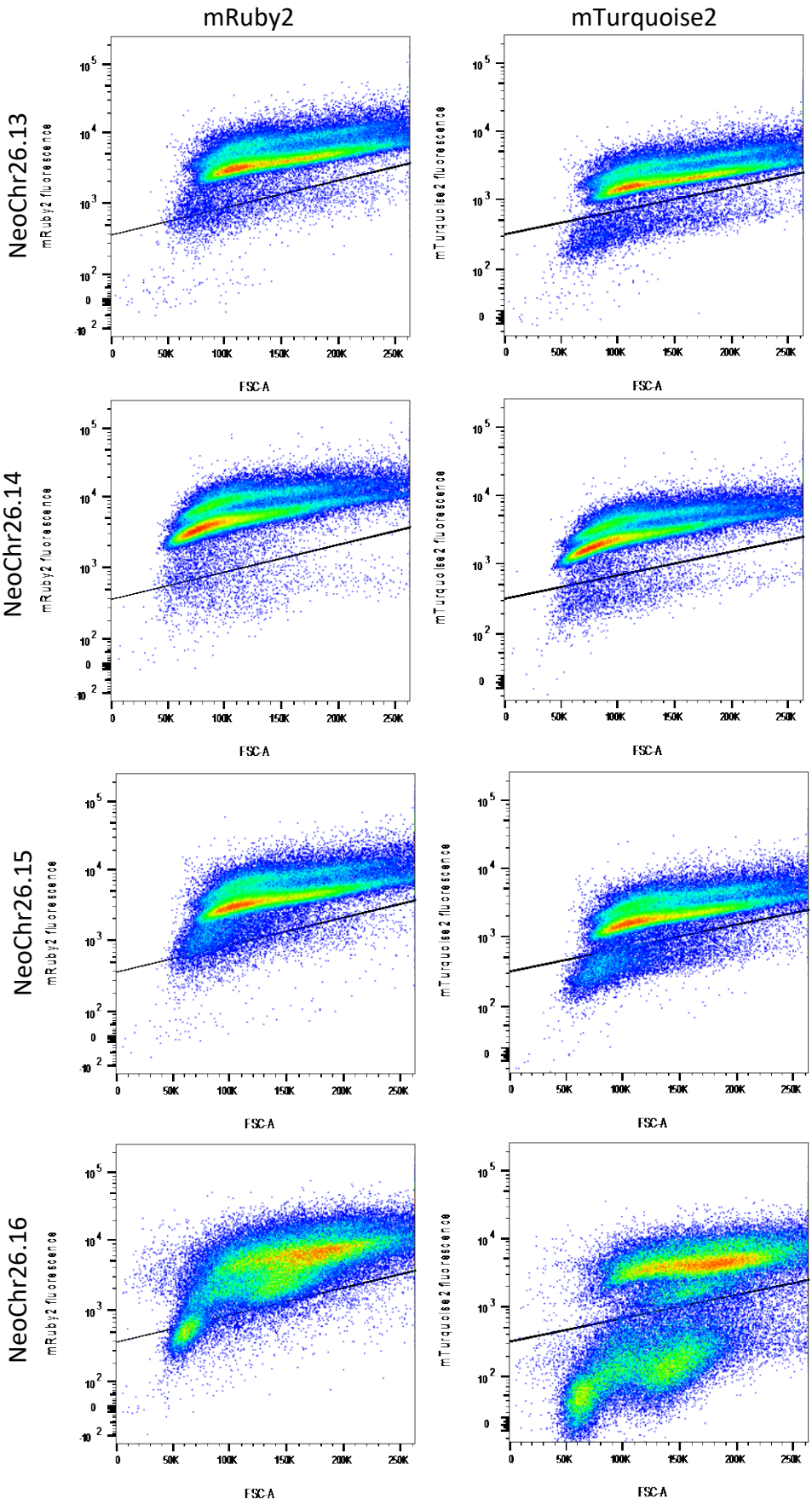
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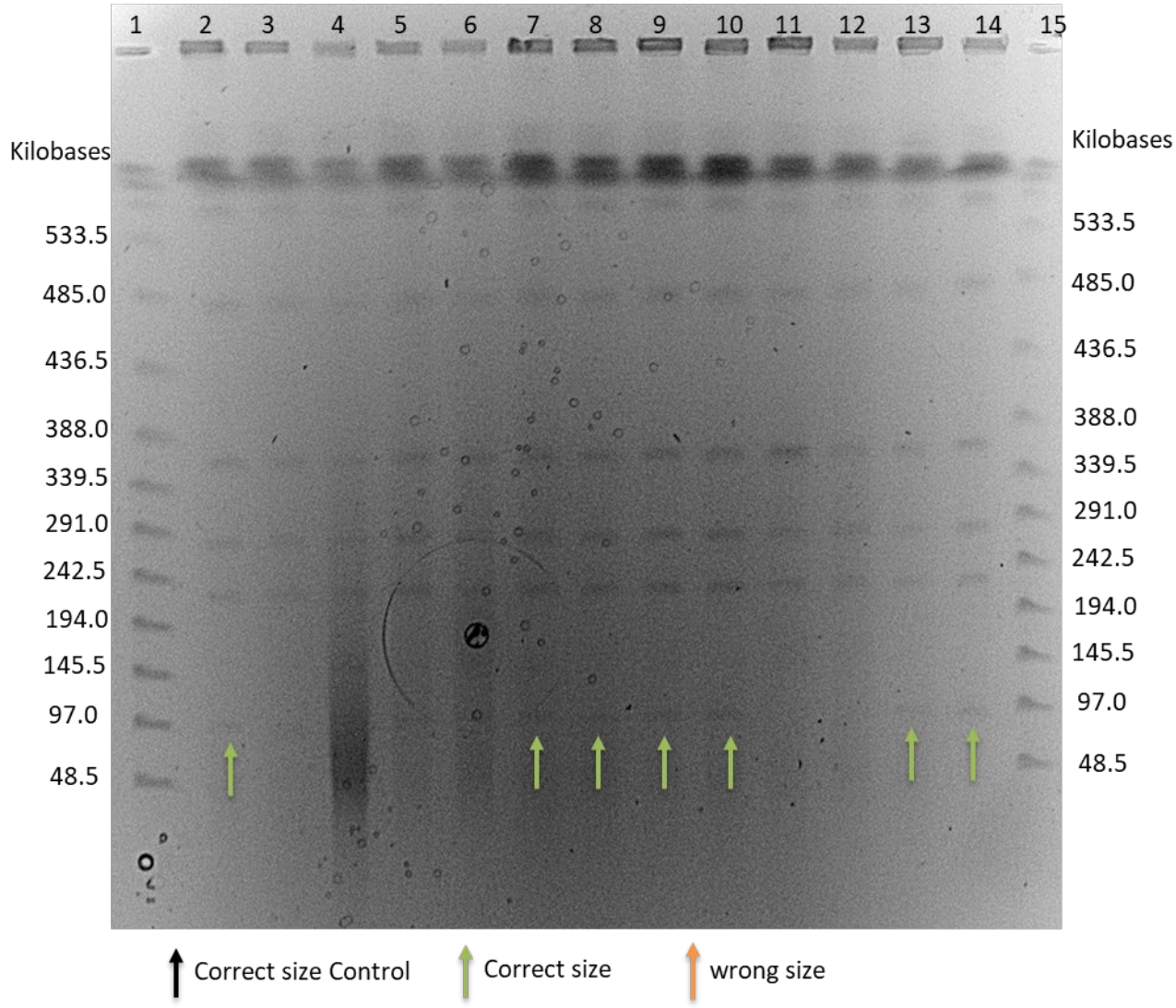
mRuby2 fluorescence
mTurquoise2 fluorescence
FSC-A

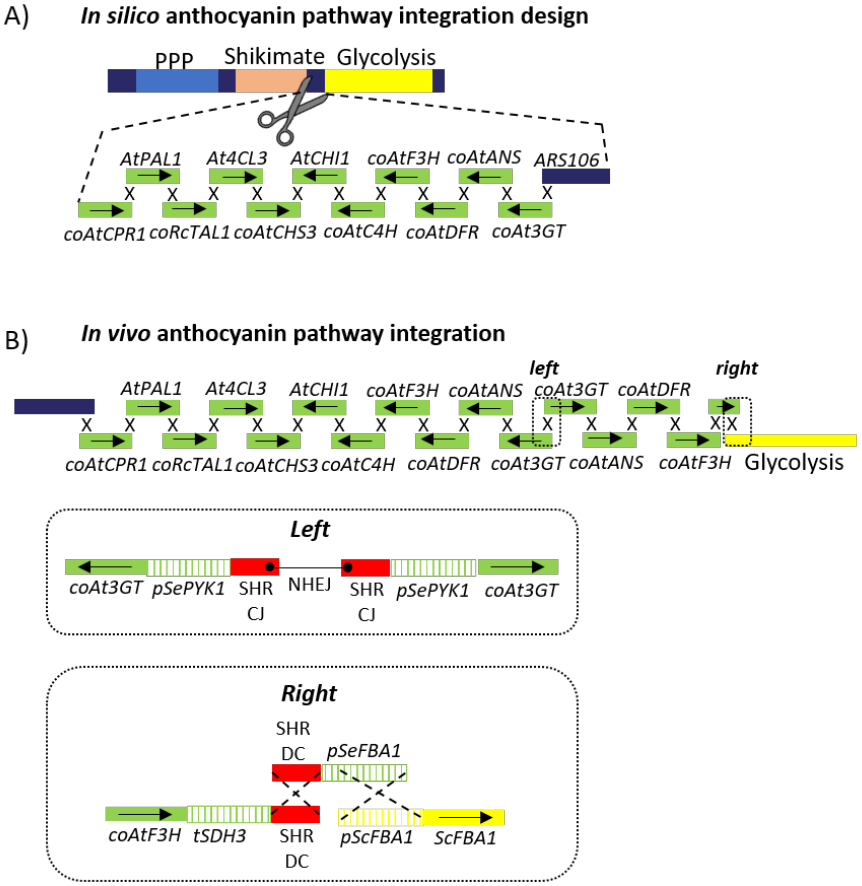
Supplementary Figure 8 - Separation of (linear) NeoChr25 transformants on pulsed-field electrophoresis.

Pulsed-field electrophoresis was used to estimate the size of NeoChr25 in several yeast transformants. 1) Size ladder. 2) NeoChr25.4: correct size. 3) NeoChr25.15: no visible neochromosome. 4) NeoChr25.19: no visible neochromosome. 5) NeoChr25.23: no visible neochromosome. 6) NeoChr25.24: no visible neochromosome. 7) NeoChr25.25: correct size. 8) NeoChr25.47: correct size. 9) NeoChr25.53: correct size. 10) NeoChr25.56: correct size. 11) NeoChr25.57: no visible neochromosome. 12) NeoChr25.73: no visible neochromosome. 13) NeoChr25.75: correct size. 14) IMF22: positive control. 15) Size ladder.



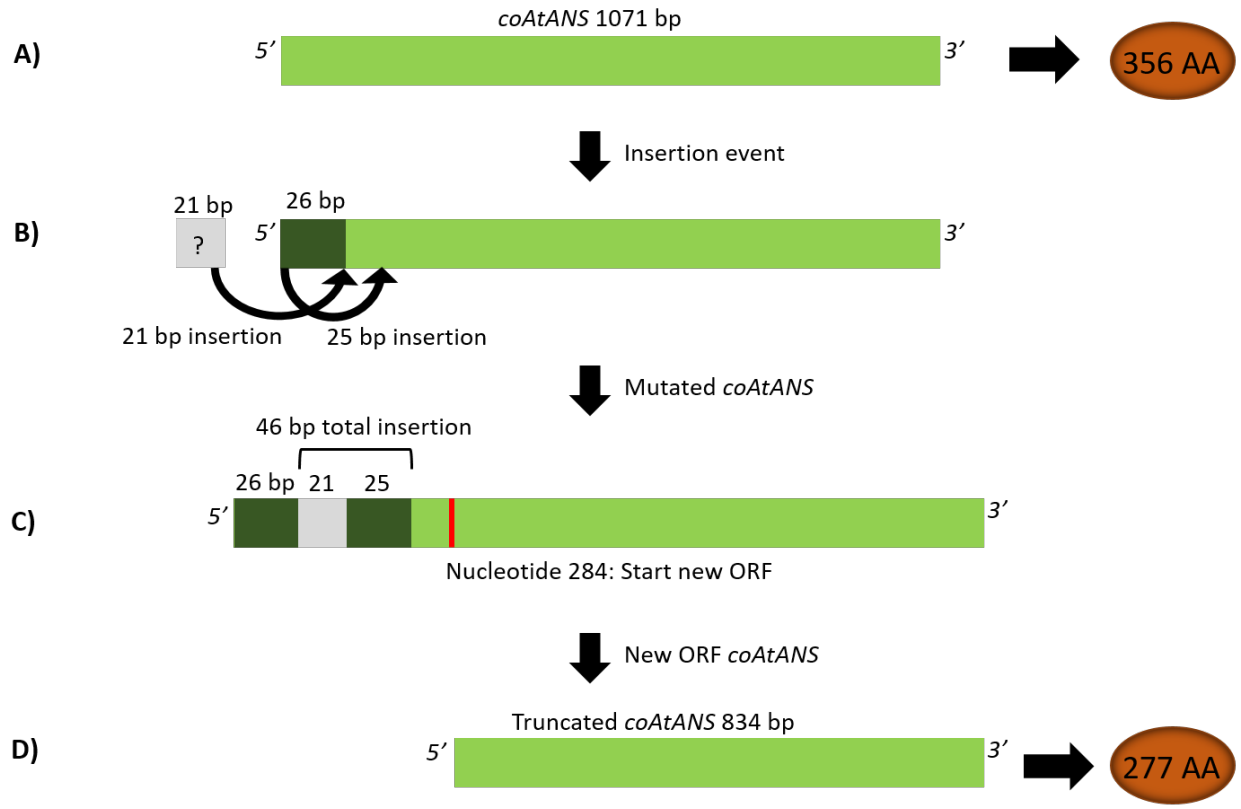
Supplementary Figure 9 – Duplication and inversion of four plant genes in linear NeoChr25 and circular Neochr26.

An unexpected recombination was observed upon integration of the genes encoding the anthocyanin production pathway in the linear and circular NeoChrs. A) Schematic representation of the *in silico* design for the integration of the anthocyanin pathway in the circular NeoChr26 of IMF40 resulting in IMF41 and the linear NeoChr25 of IMF34 resulting in IMF42. B) Schematic representation of the genetic organization observed in IMF41 and IMF42. The last four genes in the anthocyanin pathway (*coAtF3H*, *coGhDFR*, *coAtANS* and *coAt3GT*) were duplicated and inversed, and *ARS106* was absent. The dashed boxes illustrate the recombination events that occurred on the left and right flank of this duplicated region. For the left flank, there was probably an exonuclease and subsequent Non-Homologous End Joining (NHEJ) event between the two SHR CJ, since there was no homology between the inverted and non-inverted sequences. In the sequenced IMF41 strain (circular) 57 bp of SHR CJ was retained and in the sequenced IMF42 (linear) 51 bp of SHR CJ was retained. For the right flank, in the IMF41 strain (circular) the first 649 bp showed exact homology to *pSeFBA1*, while the last 414 bp showed exact homology to *pScFBA1* (100% homology overlap of 7 bp). In the sequenced IMF42 strain (linear) the first 29 bp showed exact homology to *pSeFBA1* and the last 710 bp showed exact homology to *pSceFBA1* (overlap of 100% homology is 24 bp).



Supplementary Figure 10 – schematic representation of *coAtANS* mutation in strains IMF41, IMF42, IMF44 and IMF47

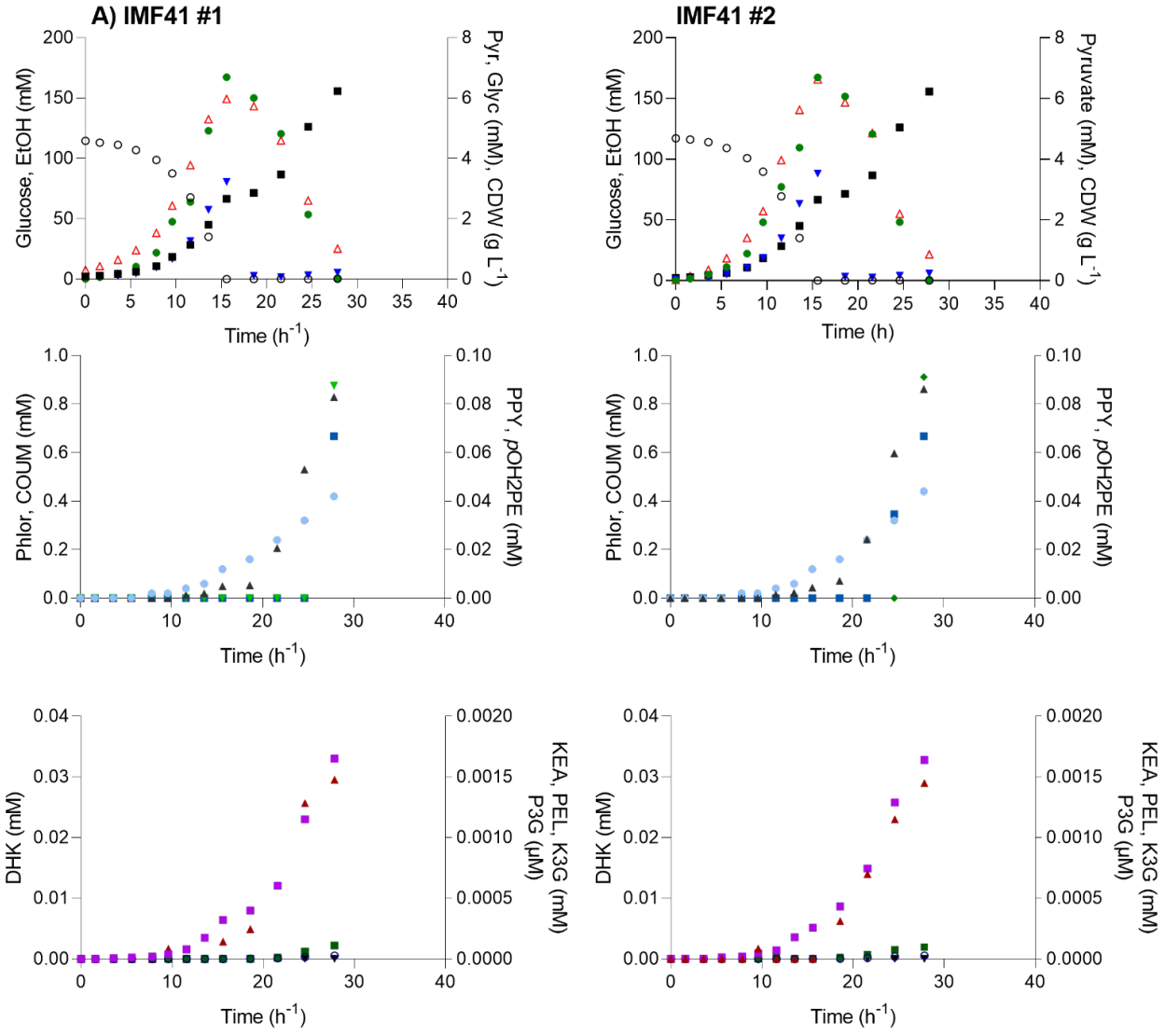
A) The original coAtANS has a length of 1071 bp and encodes for an enzyme consisting of 356 amino acids. **B)** In strains IMF41, IMF42, IMF44 and IMF47, 21 nucleotides of non-homologous DNA (indicated in grey) together with 25 of the first 26 nucleotides of the *coAtANS* gene (indicated in dark green) were inserted right after the 26th nucleotide. **C)** This insertion resulted in a total insertion of 46 nucleotides disrupting the original ORF. However, this also resulted in a new ORF starting from the 284th nucleotide. **D)** The new ORF of the truncated coAtANS has a length of 834 bp and encodes for an enzyme consisting of 277 amino acids.

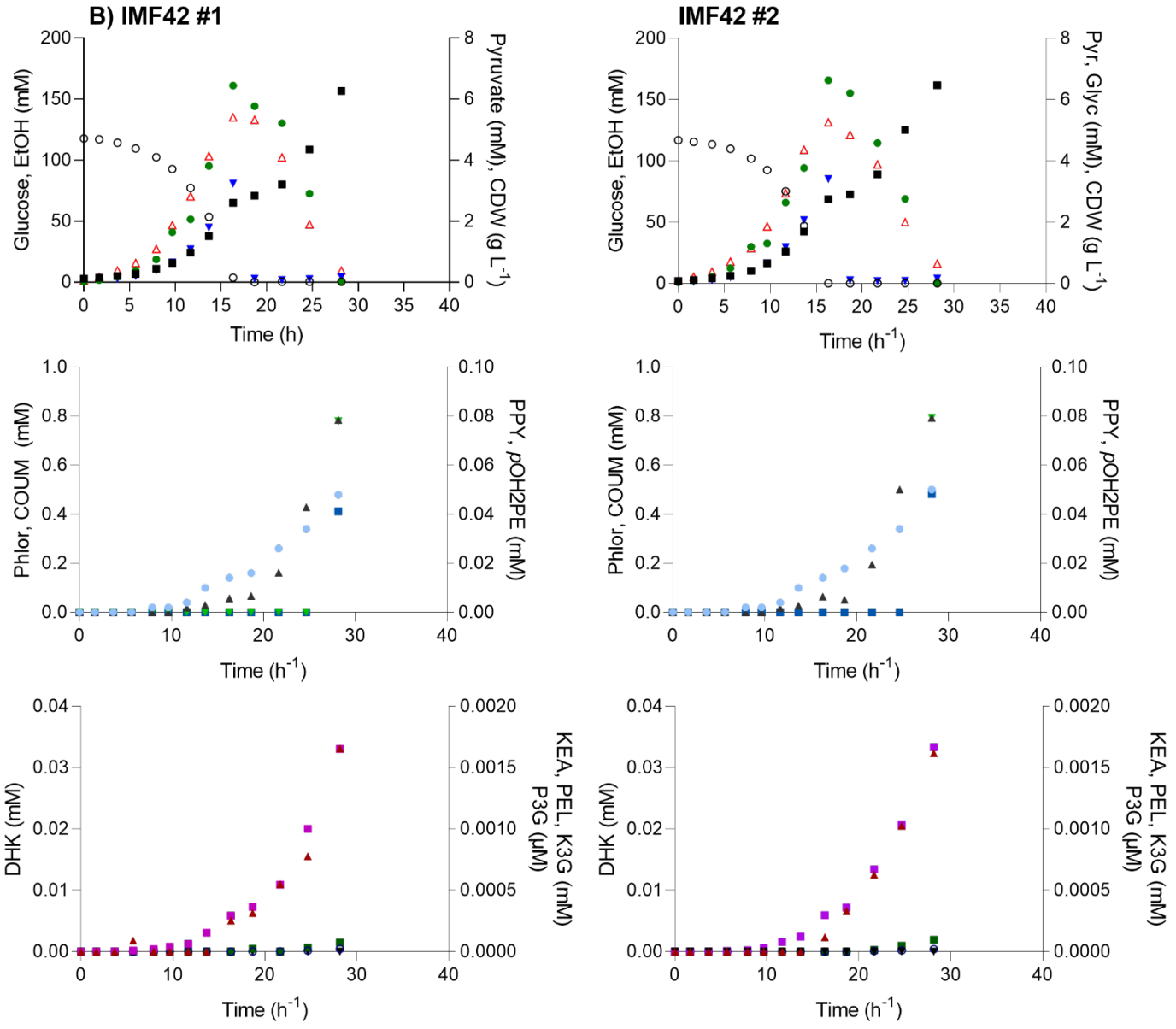


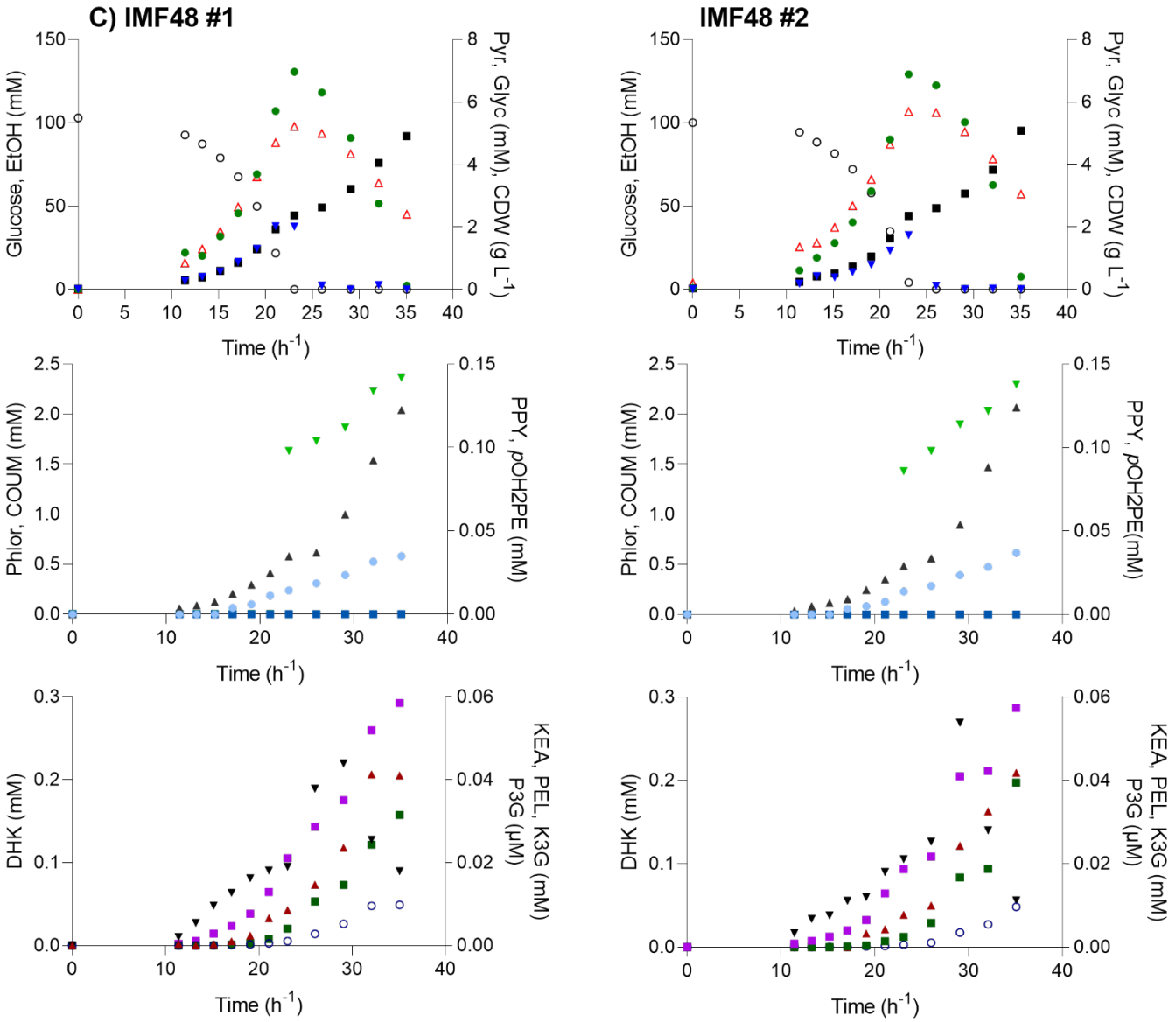
Supplementary Figure 11 - Substrates and products profiles during aerobic batch cultivation in bioreactors of IMF41, IMF42 and IMF48.

A) IMF41 (Cir, 1x *coAtCHS3*), **B)** IMF42 (Lin, 1x *coAtCHS3*), and **C)** IMF48 (Lin, 9x *coAtCHS3, coAtANS*), were grown at 30°C in aerobic batch cultures in bioreactors, in chemically defined medium with 20 g L^{-1} glucose as sole carbon source (SMD). Biological duplicates were performed and are shown in two columns as #1 and #2.

Row 1) ■ CDW (g L^{-1}), ○ Glucose (mM), ● EtOH (mM), ▼ PYR (mM), △ Glyc (mM)
Row 2) ■ PPY (mM), ▲ COUM (mM), ● Phlor (mM), ▼ *p*OH2PE (mM)
Row 3) ■ DHK (mM), ▲ PEL (mM), ■ KEA (mM) ○ K3G (mM) ▼ P3G (µM)




B) IMF42 #1
IMF42 #2
Glucose, EtOH (mM)
Pyruvate (mM), CDW (g L-1)
Pyr, Glyc (mM), CDW (g L-1)
Time (h)
Time (h-1)
Phlor, COUM (mM)
PPY, pOH2PE (mM)
DHK (mM)
KEA, PEL, K3G (mM)
P3G (µM)


C) IMF48 #1
IMF48 #2
Glucose, EtOH (mM)
Pyr, Glyc (mM), CDW (g L-1)
Time (h-1)
Phlor, COUM (mM)
PPY, pOH2PE (mM)
DHK (mM)
KEA, PEL, K3G (mM)
P3G (µM)

Supplementary Figure 12 – Detection and quantification of pelargonidin and pelargonidin 3-O-glucoside by LC-MS/MS

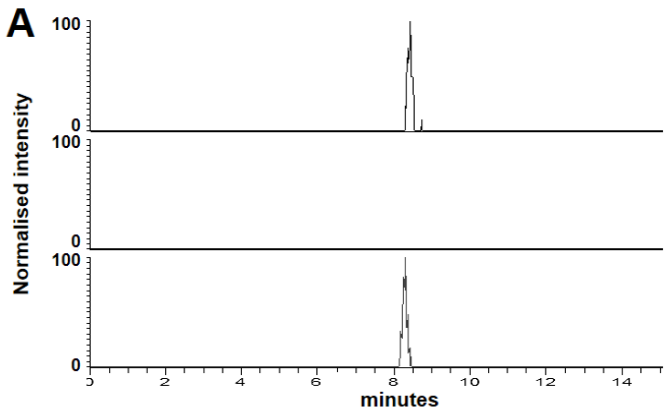
A) Extracted ion chromatogram for the pelargonidin 3-0-glucoside (P3G) mass peak with the composition $C_{21}H_{21}O_{10}^{+}$ and the m/z of 433.1. Data shown for the cell pellet extract of IMF48 duplicate #1 (Table 2), grown in aerobic bioreactor (sample, upper trace), for a blank injection (trace in the middle) analysed just before the sample and for a synthetic P3G standard shown in the lower trace (Pelargonidin 3-O-glucoside chloride, Sigma Aldrich, Cat No PHL89753).

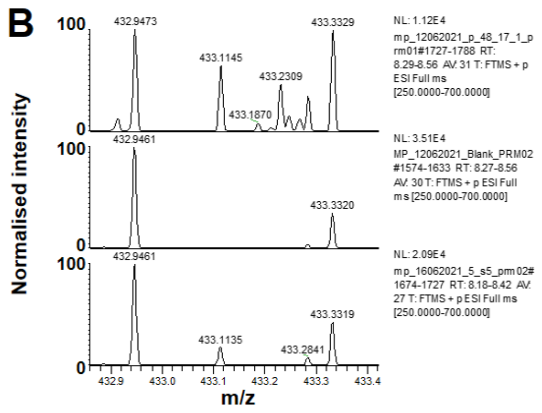
B) The mass spectra show the accurate mass of P3G observed in the sample (upper mass spectrum) and the standard (lower mass spectrum). No corresponding P3G peak was observed for the blank injection (spectrum in the middle) analysed before the sample.

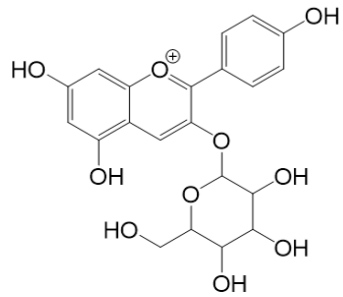
C) Extracted ion chromatogram of the pelargonidin (PEL) fragment with the composition $C_{15}H_{11}O_{5}$+, and a m/z of 271.06 Da. The corresponding fragment was observed in the sample (upper mass spectrum) and the standard (lower mass spectrum). No corresponding PEL fragment peak was observed for the blank injection analysed just before the sample. (Pelargonidin chloride, Sigma Aldrich, Cat No PHL80084).

D) The spectra show the accurate mass of the PEL major fragment with the composition $C_{15}H_{11}O_{5}^{+}$ and a m/z of 271.06 Da, as observed for the sample (upper spectrum) and the standard (lower spectrum). No corresponding fragment mass peak was observed for the blank injection (spectrum in the middle), which was performed just before the sample.

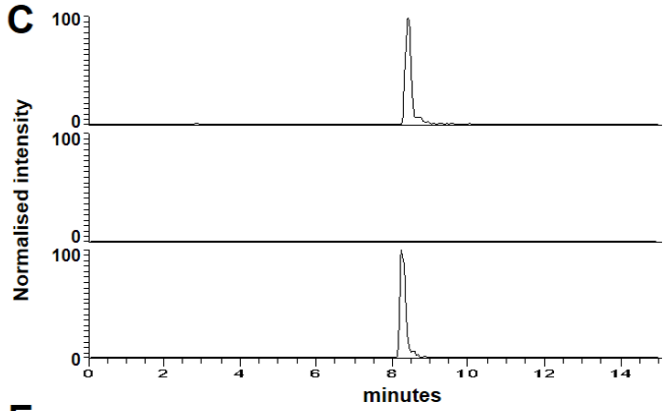
E) The table summarised the chemical compositions of P3G and the major fragment of pelargonidin (PEL) (loss of the sugar unit), the resulting theoretical m/z values, the sobered m/z values and the mass deviations (ppm). The observed mass deviations for standard and sample peaks were <5 ppm compared to their theoretical m/z values.

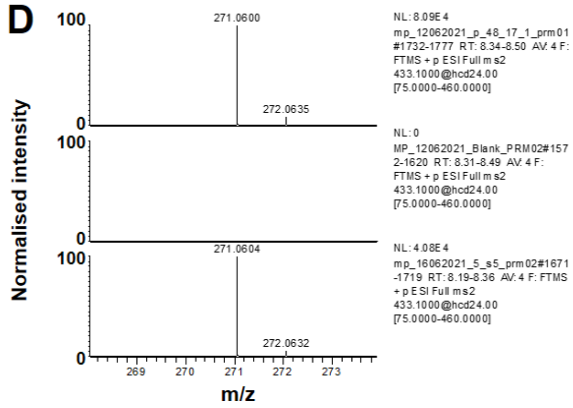


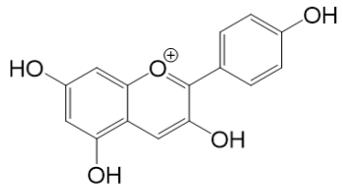




Chemical Formula: $C_{21}H_{21}O_{10}^{+}$
Exact Mass: 433,11







Chemical Formula: $C_{15}H_{11}O_{5}^{+}$
Exact Mass: 271,06

E

COMPOUND	COMPOSITION	THEORETICAL M/Z	SAMPLE M/Z	Δppm	STANDARD M/Z	Δppm
P3G	$C_{21}H_{21}O_{10}^{+}$	433.1129	433.1145	3.69	433.1135	1.39
PEL (fragment)	$C_{15}H_{11}O_{5}^{+}$	271.0601	271.0600	-0.37	271.0604	1.11

Supplementary Table 1 - Promoter-gene-terminator combinations in the NeoChrs.

Promoters, genes or terminators originate from *S. cerevisiae* unless indicated by: *Ec= Escherichia coli*, *At= Arabidopsis thaliana*, *Rc= Rhodobacter capsulatus*, *Gh= Gerbera hybrida Se= Saccharomyces eubayanus*, *Sk= Saccharomyces kudriavzevii*, *co= codon optimized.* Watermarked *S. cerevisiae* genes[1] are indicated with an *.

Promoter	ORF	Terminator
Genes from glycolysis and ethanolic fermentation are expressed from their native promoters and terminators		
pFBA1	*FBA1**	*tFBA1*
pPGM1	*PGM1**	*tPGM1*
pHXK2	*HXK2**	*tHXK2*
pPDC1	*PDC1**	*tPDC1*
pPFK1	*PFK1**	*tPFK1*
pPFK2	*PFK2**	*tPFK2*
pPGK1	*PGK1**	*tPGK1*
pPYK1	*PYK1**	*tPYK1*
pTPI1	*TPI1**	*tTPI1*
pADH1	*ADH1**	*tADH1*
pTDH3	*TDH3**	*tTDH3*
pENO2	*ENO2**	*tENO2*
pPGI1	*PGI1**	*tPGI1*
Genes from the pentose phosphate pathway are expressed from their native promoters and terminators		
pZWF1	*ZWF1**	*tZWF1*
pTKL1	*TKL1**	*tTKL1*
pGND1	*GND1**	*tGND1*
pRKI1	*RKI1**	*tRKI1*
pTAL1	*TAL1**	*tTAL1*
pRPE1	*RPE1**	*tRPE1*
pSOL3	*SOL3**	*tSOL3*
Auxotrophic markers are expressed from their native promoters and terminators		
pHIS3	*HIS3*	*tHIS3*
pURA3	*URA*	*tURA3*
Fluorescent markers are expressed from *S. cerevisiae* promoters and terminators. Promoters identified from [2]		
pCCW12	*mRuby2*	*ENO1*
pTEF2	*mTurquoise2*	*tSSA1*
Genes from the *E.coli* shikimate pathway are expressed from *S. cerevisiae* promoters and terminators. Promoters identified from [2, 3, 4]		
pRPL3	*coEcaroA*	*tSOL4*
pRPL25	*coEcaroD*	*tGPH1*
pRPP0	*coEcaroE*	*tCYC1*
pHHF1	*coEcaroG*^{p150L}	*tTEF1*
pHTB2	*coEcaroL*	*tPGM2*

pRPL10	*coEctyrA$^{M53I\ A354V}$*	*tGDB1*
pCWP2	*coEctyrB*	*tGLC3*
pHHF2	*coEcaroB*	*tTEF2*
pRPL8A	*coEcaroC*	*tGPD2*
pRPL18B	*coEcpheA^{T326P}*	*tGSY2*
One gene from the anthocyanin pathway is expressed from a *S. cerevisiae* promoter and terminator. Promoter identified from [2, 3, 4]		
pTEF1	*coAtCHS3*	*tMDH1*
Genes from the anthocyanin pathway are expressed from a *S. eubayanus* and *S. kudriavzevii* promoters [5] and *S. cerevisiae* terminators.		
pSePDC1	*AtPAL1*	*tLAT1*
pSeGPM1	*coRcTAL1*	*tCIT1*
pSkADH1	*AtCHI1*	*tSDH4*
pSeFBA1	*coAtC4H*	*tADH3*
pSkTDH3	*coAtF3H*	*tSDH3*
pSePGK1	*coGhDFR*	*tACO1*
pSeENO2	*coAtANS*	*tFUM1*
pSePYK1	*coAt3GT*	*tDIC*

Supplementary Table 2 - Sequence fidelity of NeoChrs

Mutation identified in the neochromosomes as compared to the *in silico* design and with the most relevant parental strain. The * indicates mutations which are the same in two separate transformations and therefore probably resulting from the template DNA and not during the *in vivo* assembly. Non-synonymous mutations are indicated in bold.

Position	Region	Mutation type
NeoChr25 (IMF27)		
8648	*pTKL1*	C to CT
14466	SHR BQ	C to CT
20137*	*pTEF2 (mTurquoise 2)**	CAT to C*
26993	*pHHF2 (EcAroB)*	AT to A
46676*	*pCWP2 (EcTyrB)**	A to G*
52732	SHR AE	G to GT
66608	SHR N	CA to C
73854*	*tENO2**	C to A*
86809	*pPFK2*	GA to G
90753	SHR M	GC to G
90762	SHR M	AT to A
NeoChr26 (IMF29)		
14306	*tGND1*	CT to C
15181	***RKI1***	**C to A (Glu-129-Gln)**
20137*	*pTEF2 (mTurquoise2)**	CAT to C*
22220	SHR DF	TC to T
22223	SHR DF	TC to T
46676*	*pCWP2 (EcTyrB)**	A to G*
57795	SHR DL	A to AG
64374	SHR Q	T to TG
66632	*pPYK1*	CT to C
73854*	*tENO2**	C to A*
73864	*tENO2*	GT to G
73925	SHR B	T to C
73926	SHR B	A to T
73928	SHR B	G to A
78556	*pPGI1*	C to A
88608	*pHIS3*	GA to G
90398	*pGPM1*	C to CTA
NeoChr30 (IMF41) as compared to NeoChr26 (IMF29)		
8648	*pTKL1*	C to CT
35602	*tPGM2 (coEcAroL)*	CT to C (In T stretch)
67393	*pSeTPI1 (At4CL3)*	G to GT (In T stretch)
71689	*tMDH1 (coAtCHS3)*	GA to G (In A stretch)
73063*	*pSkADH1 (AtCHI1)**	AT to A*
73209*	*pSkADH1 (AtCHI1)**	CT to C (In T stretch)*
81107	*tFUM1 (coAtANS)*	CG to C
82459*	***coAtANS****	**Insertion of 46 bp***

115179	*pPFK2*	G to GAA (In A stretch)
NeoChr31 (IMF42) as compared to NeoChr25 (IMF27)		
57432	Chunk 16AB	A to AC
64168	*tLAT1 (AtPAL1)*	TAA to T (In A stretch)
67393	*pSeTPI1 (At4CL3)*	G to GT (In T stretch)
67753	***At4CL3***	**A to G (Thr-15-Ala)**
70927	*coAtCHS3*	G to A (Leu-155-Leu)
70930	*coAtCHS3*	A to G (Arg-156-Arg)
73063*	*pSkADH1 (AtCHI1)**	AT to A*
73209*	*pSkADH1 (AtCHI1)**	CT to C (In T stretch)*
82459*	***coAtANS****	**Insertion of 46 bp***
NeoChr33 (IMF47) as compared to NeoChr31 (IMF42)		
48856	*pCWP2 (coEcTyrB)*	A to G
50730	*tMDH1 (coAtCHS3)*	A to AT (In T stretch)
85431	SHR EB	AT to A
85439	SHR EB	TG to T
85458	SHR EB	GA to G
96995	*tPGK1*	T to A

Supplementary Table 3 - Amino acid substitution in native genome of NeoChr strains

Amino acid substitutions identified in the genome of the constructed strains as compared to most relevant parental strain.

Systematic name	Name	Type	Amino acid change
IMF27 compared to IMX589			
YPL283W-A	-	Intron	-
YNL327W	*EGT2*	synonymous	Tyr-583-Tyr
YNL327W	*EGT2*	Non-synonymous	Thr-586-Ser
YNL161W	*CBK1*	Non-synonymous	Ser-711-Ala
IMF29 compared to IMX589			
YPL283W-A	-	intron	-
YPL283W-A	-	intron	-
YPL283W-A	-	intron	-
YPL283W-A	-	Non-synonymous	Gly-132-Ser
YMR160W	-	Non-synonymous	Gln-11-Arg
YNL327W	*EGT2*	Synonymous	Tyr-583-Tyr
YNL327W	*EGT2*	Non-synonymous	Thr-586-Ser
YNL161W	*CBK1*	Non-synonymous	Ser-711-Ala
IMF41 compared to IMF29			
YCR089W	FIG2	Non-synonymous	Thr-1017-Arg
YCR089W	FIG2	Non-synonymous	Ala-1020-Ser
YDR224C	HTB1	Synonymous	Ala-121-Ala
YIL137C	*TMA108*	Non-synonymous	Ser-742-Leu
IMF42 compared to IMF27			
YCR089W	FIG2	Non-synonymous	Thr-1017-Arg
YCR089W	FIG2	Non-synonymous	Ala-1020-Ser
YBL113C	-	Non-synonymous	His-252-Asn
IMF47 compared to IMF42			
YEL075W-A	-	intron	-
YHR016C	*YSC84*	intron	-
YJR143C	*PMT4*	Non-synonymous	Met-1-Ile

Supplementary Table 4 - Extracellular concentration of aromatic compounds produced by engineered *S. cerevisiae* strains in shake flask cultures

Determination of the intermediates of the anthocyanin pathway in *S. cerevisiae* strains IMF41 (Cir NeoChr, 1X *coAtCHS3*), IMF42 (Lin NeoChr, 1X *coAtCHS3*), IMF47 (Lin NeoChr, 9X *coAtCHS3*) and IMF48 (Lin NeoChr, 9X *coAtCHS3* repaired *coAtANS*), grown in aerobic shake flask batch cultures on glucose (20 g L^{-1}) and urea. The data represents the average ± mean deviation of independent biological triplicates. Intermediates of the anthocyanin pathway coumaroyl-CoA, naringenin-chalcone, and leucopelargonidin were not measured. * Indicates statistical significance when comparing IMF47 or IMF48 to IMF42, and # when comparing IMF48 to IMF47 (Student *t*-test, two-tailed, homoscedastic, *p*-value threshold 0.05).

(mM)	IMF41	IMF42	IMF47	IMF48
Phenylpyrurate	2.00E-02 ± 0.00E+00	4.33E-02 ± 4.04E-02	BD[a]	BD[a]
2-Phenylethanol	8.67E-02 ± 1.15E-02	8.67E-02 ± 2.52E-02	3.23E-01 ± 3.51E-02*	1.97E-01 ± 2.62E-03*#
p-Hydroxyphenylethanol	2.33E-02 ±2.08E-02	3.33E-02 ±5.77E-03	1.10E-01 ±1.73E-02*	BD[a]
Cinnamic acid	3.80E-01 ±2.00E-02	1.60E-01 ± 2.00E-02	0.00E+00 ± 0.00E+00*	1.46E-01 ± 3.06E-03#
Coumaric acid	4.27E-01 ± 1.15E-02	5.40E-01 ± 2.00E-02	7.13E-01 ± 1.15E-02*	7.64E-01 ± 6.24E-03*#
Phloretic acid	7.25E-01 ± 1.21E-02	7.18E-01 ± 8.72E-03	5.09E-01 ± 1.15E-03*	1.07E+00 ± 7.66E-03*#
Naringenin	BD[a]	BD[a]	BD[a]	BD[a]
Dihydrokaempferol	2.25E-02 ± 2.57E-03	2.84E-02 ± 1.27E-03	9.49E-02 ± 4.29E-03*	3.83E-01 ± 8.34E-02*#
Kaempferol	6.33E-04 ± 3.44E-05	6.96E-04 ± 3.46E-05	2.14E-03 ± 3.06E-04*	1.39E-02 ± 5.72E-03*#
Pelargonidin	2.45E-03 ± 1.60E-04	1.81E-03 ± 1.98E-04	5.76E-03 ± 4.11E-04*	3.65E-02 ± 7.64E-03*#
Kaempferol 3-O-glucoside	2.24E-04 ± 1.83E-05	2.24E-04 ± 5.98E-06	4.39E-04 ± 2.41E-05*	5.53E-03 ± 1.13E-03*#
Pelargonidin 3-O-glucoside	BD[a]	BD[a]	BD[a]	2.14E-05 ± 3.93E-06*#
Total aromatics before CHS	1.66 ± 0.06	1.58 ± 0.06	1.66 ± 0.05	1.98 ± 0.01*#
Total anthocyanins (after CHS)	0.03 ± 0.00	0.03 ± 0.00	0.1 ± 0.0*	0.45 ± 0.10*#
Total aromatics	1.69 ± 0.06	1.61 ± 0.08	1.76 ± 0.05	2.43 ± 0.11*#

[a]BD: below detection

Supplementary Table 5 - Physiological characterization of anthocyanin-producing strains grown in bioreactors

A) The specific growth rate (μ) and the yield (Y) of biomass (X) and ethanol (ETOH) on glucose (S)

B) The overall yield (Y) of glycerol (GLYC), pyruvate (PYR), coumaric acid (COUM), phloretic acid (PHLOR) and dihydrokaempferol (DHK) on glucose and ethanol (S) during aerobic bioreactor batch cultivation of IMF41 (Cir NeoChr, 1x *coAtCHS3*), IMF42 (Lin NeoChr, 1x *coAtCHS3*),) and IMF48 (Lin NeoChr, 9x *coAtCHS3*, repaired *coAtANS*).

A)	[a] μ_{MAX} h^{-1}	[a] $Y_{X/S}$ (g g^{-1})	[a] $Y_{ETOH/S}$ (mol mol^{-1})
IMF41 (Cir)	0.23 ± 0.00	0.12 ± 0.00	1.44 ± 0.05
IMF42 (Lin)	0.22 ± 0.01	0.12 ± 0.00	1.41 ± 0.02
IMF48 (Lin, 9x *coAtCHS3, coAtANS*)	0.20 ± 0.01	0.13 ± 0.00	1.29 ± 0.05

B)	$Y_{GLYC/S}$ (mol mol^{-1})	$Y_{PYR/S}$ (mol mol^{-1})	$Y_{X/S}$ (mol mol^{-1})	$Y_{coum/S}$ (μmol mol^{-1})	$Y_{PHLOR/S}$ (μmol mol^{-1})	$Y_{DHK/S}$ (μmol mol^{-1})
IMF41 (Cir)	0.056 ± 0.005	0.029 ± 0.001	0.29 ± 0.01	7.30 ± 0.08	4.71 ± 0.20	0.28 ± 0.01
IMF42 (Lin)	0.048 ± 0.002	0.029 ± 0.001	0.30 ± 0.01	6.70 ± 0.12	5.02 ± 0.15	0.28 ± 0.00
IMF48 (Lin, 9x *coAtCHS3, coAtANS*)	0.052 ± 0.003	0.019 ± 0.003	0.27 ± 0.01	20.2 ± 0.59	5.89 ± 0.35	2.85 ± 0.02

[a] Determined for the glucose phase only

Supplementary Table 6 - *S. cerevisiae* strains used in this study

Strains that were short-read or long-read sequenced in this study are marked with a *. SHRs are differently annotated than in Kuijpers *et al.* [6]. SHRs are annotated in bold subscript between de genetic fragments that they join together.

Strain	Relevant Genotype	Source
CEN.PK113-7D	*MATa URA3 HIS3 LEU2 TRP1 MAL2-8c SUC2*	Entian and Kötter [7]
IMC111	*MATa ura3-52 can1Δ::cas9-natNT2 TRP1 LEU2 HIS3* pUDC191 (mRuby2)	Postma, Dashko [8]
IMC112	*MATa ura3-52 can1Δ::cas9-natNT2 TRP1 LEU2 HIS3* pUDC192 (mTurquoise2)	Postma, Dashko [8]
IMX589	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1Δ:: (pAgTEF1-SpHIS5-tAgTEF1) hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ::(* $_{G}$ *tFBA1-FBA1-pFBA1* $_{H}$ *pTPI1-TPI1-tTPI1* $_{P}$ *tPGK1-PGK1-pPGK1* $_{Q}$ *tADH1-ADH1-pADH1* $_{N}$ *pPYK1-PYK1-tPYK1* $_{O}$ *tTDH3-TDH3-pTDH3* $_{A}$ *pENO2-ENO2-tENO2* $_{B}$ *pHXK2-HXK2-tHXK2* $_{C}$ *pPGI-PGI1-tPGI1 D pPFK1-PFK1-tPFK1* $_{J}$ *tPFK2-PFK2-pPFK2* $_{K}$ *pAgTEF1-AmdSYM-tAgTEF1* $_{L}$ *tGPM1-GPM1-pPGM1* $_{M}$ *pPDC1-PDC1-tPDC1-SYN* $_{F}$ *) pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ*	Kuijpers, Solis-Escalante [6]
IMX1338	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1Δ::(pAgTEF1-SpHIS5-tAgTEF1)Δ::(pGAL1-I SceI-tCYC1) hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ::(* $_{G}$ *tFBA1-FBA1-pFBA1* $_{H}$ *pTPI1-TPI1-tTPI1 P tPGK1-PGK1-pPGK1* $_{Q}$ *tADH1-ADH1-pADH1* $_{N}$ *pPYK1-PYK1-tPYK1* $_{O}$ *tTDH3-TDH3-pTDH3* $_{A}$ *pENO2-ENO2-tENO2* $_{B}$ *pHXK2-HXK2-tHXK2* $_{C}$ *pPGI-PGI1-tPGI1* $_{D}$ *pPFK1-PFK1-tPFK1* $_{J}$ *tPFK2-PFK2-pPFK2* $_{K}$ *pAgTEF1-AmdSYM-tAgTEF1* $_{L}$ *tGPM1-GPM1-pPGM1* $_{M}$ *pPDC1-PDC1-tPDC1-SYN* $_{F}$*) pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ*	Postma, Dashko [8]
IMX1433	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1Δ:: (pAgTEF1-SpHIS5-tAgTEF1) hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ::(* $_{G}$ *tFBA1-FBA1-pFBA1* $_{H}$ *pTPI1-TPI1-tTPI1* $_{P}$ *tPGK1-PGK1-pPGK1* $_{Q}$ *tADH1-ADH1-pADH1* $_{N}$ *pPYK1-PYK1-tPYK1* $_{O}$ *tTDH3-TDH3-pTDH3* $_{A}$ *pENO2-ENO2-tENO2* $_{B}$ *pHXK2-HXK2-tHXK2* $_{C}$ *pPGI-PGI1-tPGI1* $_{D}$ *pPFK1-PFK1-tPFK1* $_{J}$ *tPFK2-PFK2-pPFK2* $_{KL}$ *tGPM1-GPM1-pPGM1* $_{M}$ *pPDC1-PDC1-tPDC1-SYN* $_{F}$*) pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ in vivo* recombined pMEL10 backbone with repair oligo 11588/11589	This study
IMX1769	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1Δ:: (pAgTEF1-SpHIS5-tAgTEF1) hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ::(* $_{G}$ *tFBA1-*	This study

	FBA1-pFBA1 $_{H}$ pTPI1-TPI1-tTPI1 $_{P}$ tPGK1-PGK1-pPGK1 $_{Q}$ tADH1-ADH1-pADH1 $_{N}$ pPYK1-PYK1-tPYK1 $_{O}$ tTDH3-TDH3-pTDH3 $_{A}$ pENO2-ENO2-tENO2 $_{B}$ pHXK2-HXK2-tHXK2 $_{C}$ pPGI-PGI1-tPGI1 $_{D}$ pPFK1-PFK1-tPFK1 $_{J}$ tPFK2-PFK2-pPFK2 $_{KL}$ tGPM1-GPM1-pPGM1 $_{M}$ pPDC1-PDC1-tPDC1-SYN $_{F}$) pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ	
IMX2059	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ::(FBA1 $_{GH}$ TPI1 $_{HP}$ PGK1 $_{PQ}$ ADH1 $_{QN}$ PYK1 $_{NO}$ TDH3 $_{OA}$ ENO2 $_{AB}$ HXK2 $_{BC}$ PGI1 $_{CD}$ PFK1 $_{DJ}$ PFK2 $_{JK}$ AmdSYM $_{KL}$ GPM1 $_{LM}$ PDC1-SYN $_{MF}$) pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF-cas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ glk1Δ::Sphis5Δ::(pGAL1-I SceI-tCYC1) x2::pURA3-URA3-tURA3 $_{DT}$ pHIS3-HIS3-tHIS3*	Postma, Dashko [8]
IMX2154	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1Δ:: (pAgTEF1-SpHIS5-tAgTEF1) hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ::($_{G}$ tFBA1-FBA1-pFBA1 $_{H}$ pTPI1-TPI1-tTPI1 $_{P}$ tPGK1-PGK1-pPGK1 $_{Q}$ tADH1-ADH1-pADH1 $_{N}$ pPYK1-PYK1-tPYK1 $_{O}$ tTDH3-TDH3-pTDH3 $_{A}$ pENO2-ENO2-tENO2 $_{B}$ pHXK2-HXK2-tHXK2 $_{C}$ pPGI-PGI1-tPGI1 $_{D}$ pPFK1-PFK1-tPFK1 $_{J}$ tPFK2-PFK2-pPFK2 $_{KL}$ tGPM1-GPM1-pPGM1 $_{M}$ pPDC1-PDC1-tPDC1-SYN $_{F}$) pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ gnd2Δ sol4Δ tkl2Δ nqm1Δ pUDR286 pUDR590*	This study
IMX2204	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1Δ:: (pAgTEF1-SpHIS5-tAgTEF1) hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ::($_{G}$ tFBA1-FBA1-pFBA1 $_{H}$ pTPI1-TPI1-tTPI1 $_{P}$ tPGK1-PGK1-pPGK1 $_{Q}$ tADH1-ADH1-pADH1 $_{N}$ pPYK1-PYK1-tPYK1 $_{O}$ tTDH3-TDH3-pTDH3 $_{A}$ pENO2-ENO2-tENO2 $_{B}$ pHXK2-HXK2-tHXK2 $_{C}$ pPGI-PGI1-tPGI1 $_{D}$ pPFK1-PFK1-tPFK1 $_{J}$ tPFK2-PFK2-pPFK2 $_{KL}$ tGPM1-GPM1-pPGM1 $_{M}$ pPDC1-PDC1-tPDC1-SYN $_{F}$) pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ gnd2Δ sol4Δ tkl2Δ nqm1Δ*	This study
IMX2224	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ::(FBA1 $_{GH}$ TPI1 $_{HP}$ PGK1 $_{PQ}$ ADH1 $_{QN}$ PYK1 $_{NO}$ TDH3 $_{OA}$ ENO2 $_{AB}$ HXK2 $_{BC}$ PGI1 $_{CD}$ PFK1 $_{DJ}$ PFK2 $_{JK}$ AmdSYM $_{KL}$ GPM1 $_{LM}$ PDC1-SYN $_{MF}$) pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF-cas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ glk1Δ::Sphis5Δ::(pGAL1-I SceI-tCYC1) x2::pURA3-URA3-tURA3 $_{DT}$ pHIS3-HIS3-tHIS3 YPRCtau3Δ::pCCW12-mRuby2-tENO1*	Postma, Dashko [8]
IMX2226	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ::(FBA1 $_{GH}$ TPI1 $_{HP}$ PGK1 $_{PQ}$ ADH1 $_{QN}$*	Postma, Dashko [8]

	PYK1 $_{NO}$ TDH3 $_{OA}$ ENO2 $_{AB}$ HXK2 $_{BC}$ PGI1 $_{CD}$ PFK1 $_{DJ}$ PFK2 $_{JK}$ AmdSYM $_{KL}$ GPM1 LM PDC1-SYN $_{MF}$) pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF-cas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ glk1Δ::Sphis5Δ::(pGAL1-I SceI-tCYC1) x2::pURA3-URA3-tURA3-SHR DT-pHIS3-HIS3-tHIS3 YPRCtau3Δ::pTEF1-Venus-tTDH1	
IMX2234	*MATa ura3Δ his3Δ leu2-3,112 MAL2-8c SUC2 glk1Δ hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ::($_{G}$ tFBA1-FBA1-pFBA1 $_{H}$ pTPI1-TPI1-tTPI1 $_{P}$ tPGK1-PGK1-pPGK1 $_{Q}$ tADH1-ADH1-pADH1 $_{N}$ pPYK1-PYK1-tPYK1 $_{O}$ tTDH3-TDH3-pTDH3 $_{A}$ pENO2-ENO2-tENO2 $_{B}$ pHXK2-HXK2-tHXK2 $_{C}$ pPGI-PGI1-tPGI1 $_{D}$ pPFK1-PFK1-tPFK1 $_{J}$ tPFK2-PFK2-pPFK2 $_{KL}$ tGPM1-GPM1-pPGM1 $_{M}$ pPDC1-PDC1-tPDC1-SYN F) pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ gnd2Δ sol4Δ tkl2Δ nqm1Δ*	This study
IMX2270	*MATa ura3Δ his3Δ leu2-3,112 MAL2-8c SUC2 glk1Δ hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ::($_{G}$ tFBA1-FBA1-pFBA1 $_{H}$ pTPI1-TPI1-tTPI1 $_{P}$ tPGK1-PGK1-pPGK1 $_{Q}$ tADH1-ADH1-pADH1 $_{N}$ pPYK1-PYK1-tPYK1 $_{O}$ tTDH3-TDH3-pTDH3 $_{A}$ pENO2-ENO2-tENO2 $_{B}$ pHXK2-HXK2-tHXK2 $_{C}$ pPGI-PGI1-tPGI1 $_{D}$ pPFK1-PFK1-tPFK1 $_{J}$ tPFK2-PFK2-pPFK2 $_{K\,L}$ tGPM1-GPM1-pPGM1 $_{M}$ pPDC1-PDC1-tPDC1-SYN $_{F}$) pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ gnd2Δ sol4Δ tkl2Δ nqm1Δ aro10Δ*	This study
IMF2	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1Δ::(pAgTEF1-SpHIS5-tAgTEF1)Δ::(pGAL1-I SceI-tCYC1) hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ::($_{G}$ tFBA1-FBA1-pFBA1 $_{H}$ pTPI1-TPI1-tTPI1 $_{P}$ tPGK1-PGK1-pPGK1 $_{Q}$ tADH1-ADH1-pADH1 $_{N}$ pPYK1-PYK1-tPYK1 $_{O}$ tTDH3-TDH3-pTDH3 $_{A}$ pENO2-ENO2-tENO2 $_{B}$ pHXK2-HXK2-tHXK2 $_{C}$ pPGI-PGI1-tPGI1 $_{D}$ pPFK1-PFK1-tPFK1 $_{J}$ tPFK2-PFK2-pPFK2 $_{K}$ pAgTEF1-AmdSYM-tAgTEF1 $_{L}$ tGPM1-GPM1-pPGM1 $_{M}$ pPDC1-PDC1-tPDC1-SYN $_{F}$) pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ NeoChr2*	Postma, Dashko [8]
IMF6	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1Δ::(pAgTEF1-SpHIS5-tAgTEF1)Δ::(pGAL1-I SceI-tCYC1) hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ::($_{G}$ tFBA1-FBA1-pFBA1 $_{H}$ pTPI1-TPI1-tTPI1 $_{P}$ tPGK1-PGK1-pPGK1 $_{Q}$ tADH1-ADH1-pADH1 $_{N}$ pPYK1-PYK1-tPYK1 $_{O}$ tTDH3-TDH3-pTDH3 $_{A}$ pENO2-ENO2-tENO2 $_{B}$ pHXK2-HXK2-tHXK2 $_{C}$ pPGI-PGI1-tPGI1 $_{D}$ pPFK1-PFK1-tPFK1 $_{J}$ tPFK2-PFK2-pPFK2 $_{K}$ pAgTEF1-AmdSYM-tAgTEF1 $_{L}$ tGPM1-GPM1-pPGM1 $_{M}$ pPDC1-PDC1-tPDC1-SYN $_{F}$) pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ NeoChr1*	Postma, Dashko [8]

IMF22*	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1Δ::(pAgTEF1-SpHIS5-tAgTEF1)Δ::(pGAL1-I SceI-tCYC1) hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ::(*$_G$ *tFBA1-FBA1-pFBA1*$_H$ *pTPI1-TPI1-tTPI1*$_P$ *tPGK1-PGK1-pPGK1*$_Q$ *tADH1-ADH1-pADH1*$_N$ *pPYK1-PYK1-tPYK1*$_O$ *tTDH3-TDH3-pTDH3*$_A$ *pENO2-ENO2-tENO2*$_B$ *pHXK2-HXK2-tHXK2*$_C$ *pPGI-PGI1-tPGI1*$_D$ *pPFK1-PFK1-tPFK1*$_J$ *tPFK2-PFK2-pPFK2*$_K$ *pAgTEF1-AmdSYM-tAgTEF1*$_L$ *tGPM1-GPM1-pPGM1*$_M$ *pPDC1-PDC1-tPDC1-SYN*$_F$*) pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ NeoChr10*	This study
IMF23*	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1Δ::(pAgTEF1-SpHIS5-tAgTEF1)Δ::(pGAL1-I SceI-tCYC1) hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ::(*$_G$ *tFBA1-FBA1-pFBA1*$_H$ *pTPI1-TPI1-tTPI1*$_P$ *tPGK1-PGK1-pPGK1*$_Q$ *tADH1-ADH1-pADH1*$_N$ *pPYK1-PYK1-tPYK1*$_O$ *tTDH3-TDH3-pTDH3*$_A$ *pENO2-ENO2-tENO2*$_B$ *pHXK2-HXK2-tHXK2*$_C$ *pPGI-PGI1-tPGI1*$_D$ *pPFK1-PFK1-tPFK1*$_J$ *tPFK2-PFK2-pPFK2*$_K$ *pAgTEF1-AmdSYM-tAgTEF1*$_L$ *tGPM1-GPM1-pPGM1*$_M$ *pPDC1-PDC1-tPDC1-SYN*$_F$*) pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ NeoChr12*	Postma, Dashko [8]
IMF27*	*MATa ura3Δ his3Δ leu2-3,112 MAL2-8c SUC2 glk1Δ hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ::(*$_G$ *tFBA1-FBA1-pFBA1*$_H$ *pTPI1-TPI1-tTPI1*$_P$ *tPGK1-PGK1-pPGK1*$_Q$ *tADH1-ADH1-pADH1*$_N$ *pPYK1-PYK1-tPYK1*$_O$ *tTDH3-TDH3-pTDH3*$_A$ *pENO2-ENO2-tENO2*$_B$ *pHXK2-HXK2-tHXK2*$_C$ *pPGI-PGI1-tPGI1*$_D$ *pPFK1-PFK1-tPFK1*$_J$ *tPFK2-PFK2-pPFK2*$_{K\,L}$ *tGPM1-GPM1-pPGM1*$_M$ *pPDC1-PDC1-tPDC1-SYN*$_F$*) pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ gnd2Δ sol4Δ tkl2Δ nqm1Δ aro10Δ NeoChr25*	This study
IMF29*	*MATa ura3Δ his3Δ leu2-3,112 MAL2-8c SUC2 glk1Δ hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ::(*$_G$ *tFBA1-FBA1-pFBA1*$_H$ *pTPI1-TPI1-tTPI1*$_P$ *tPGK1-PGK1-pPGK1*$_Q$ *tADH1-ADH1-pADH1*$_N$ *pPYK1-PYK1-tPYK1*$_O$ *tTDH3-TDH3-pTDH3*$_A$ *pENO2-ENO2-tENO2*$_B$ *pHXK2-HXK2-tHXK2*$_C$ *pPGI-PGI1-tPGI1*$_D$ *pPFK1-PFK1-tPFK1*$_J$ *tPFK2-PFK2-pPFK2*$_{K\,L}$ *tGPM1-GPM1-pPGM1*$_M$ *pPDC1-PDC1-tPDC1-SYN*$_F$*) pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ gnd2Δ sol4Δ tkl2Δ nqm1Δ aro10Δ NeoChr26*	This study
IMF31	*MATa ura3Δ his3Δ leu2-3,112 MAL2-8c SUC2 glk1Δ hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ::pKlURA3-KlURA3-tKlURA3 pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ gnd2Δ sol4Δ tkl2Δ nqm1Δ aro10Δ NeoChr25*	This study

IMF32	*MATa ura3Δ his3Δ leu2-3,112 MAL2-8c SUC2 glk1Δ hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ gnd2Δ sol4Δ tkl2Δ nqm1Δ aro10Δ NeoChr26*	This study
IMF33	*MATa ura3Δ his3Δ leu2-3,112 MAL2-8c SUC2 glk1Δ hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ::pKlURA3-KlURA3-tKlURA3 pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ gnd2Δ sol4Δ tkl2Δ nqm1Δ aro10Δ NeoChr25 zwf1Δ sol3Δ gnd1Δ rki1Δ*	This study
IMF34	*MATa ura3Δ his3Δ leu2-3,112 MAL2-8c SUC2 glk1Δ hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ::pKlURA3-KlURA3-tKlURA3 pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ gnd2Δ sol4Δ tkl2Δ nqm1Δ aro10Δ NeoChr25 zwf1Δ sol3Δ gnd1Δ rki1Δ tkl1Δ tal1Δ rpe1Δ*	This study
IMF35	*MATa ura3Δ his3Δ leu2-3,112 MAL2-8c SUC2 glk1Δ hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ gnd2Δ sol4Δ tkl2Δ nqm1Δ aro10Δ NeoChr26::(rki1::RKI1)*	This study
IMF36	*MATa ura3Δ his3Δ leu2-3,112 MAL2-8c SUC2 glk1Δ hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ gnd2Δ sol4Δ tkl2Δ nqm1Δ aro10Δ NeoChr26::(rki1::RKI1) zwf1Δ sol3Δ gnd1Δ rki1Δ*	This study
IMF40	*MATa ura3Δ his3Δ leu2-3,112 MAL2-8c SUC2 glk1Δ hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ gnd2Δ sol4Δ tkl2Δ nqm1Δ aro10Δ NeoChr26::(rki1::RKI1) zwf1Δ sol3Δ gnd1Δ rki1Δ tkl1Δ tal1Δ rpe1Δ*	This study
IMF41*	*MATa ura3Δ his3Δ leu2-3,112 MAL2-8c SUC2 glk1Δ hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ gnd2Δ sol4Δ tkl2Δ nqm1Δ aro10Δ zwf1Δ sol3Δ gnd1Δ rki1Δ tkl1Δ tal1Δ rpe1Δ NeoChr30*	This study
IMF42*	*MATa ura3Δ his3Δ leu2-3,112 MAL2-8c SUC2 glk1Δ hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ::pKlURA3-KlURA3-tKlURA3 pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ gnd2Δ sol4Δ tkl2Δ nqm1Δ aro10Δ zwf1Δ sol3Δ gnd1Δ rki1Δ tkl1Δ tal1Δ rpe1Δ NeoChr31*	This study

IMF44	*MATa ura3Δ his3Δ leu2-3,112 MAL2-8c SUC2 glk1Δ hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ::pKlURA3-KlURA3-tKlURA3 pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ gnd2Δ sol4Δ tkl2Δ nqm1Δ aro10Δ zwf1Δ sol3Δ gnd1Δ rki1Δ tkl1Δ tal1Δ rpe1Δ x2Δ::pTEF1-coAtCHS3-tMDH1 yprctau3Δ::pTEF1-coAtCHS3-tMDH1 spr3Δ::pTEF1-coAtCHS3-tMDH1 can1::pTEF1-coAtCHS3-tMDH NeoChr31*	This study
IMF47*	*MATa ura3Δ his3Δ leu2-3,112 MAL2-8c SUC2 glk1Δ hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ::pKlURA3-KlURA3-tKlURA3 pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ gnd2Δ sol4Δ tkl2Δ nqm1Δ aro10Δ zwf1Δ sol3Δ gnd1Δ rki1Δ tkl1Δ tal1Δ rpe1Δ x2Δ::pTEF1-coAtCHS3-tMDH1 yprctau3Δ::pTEF1-coAtCHS3-tMDH1 spr3Δ::pTEF1-coAtCHS3-tMDH1 can1::pTEF1-coAtCHS3-tMDH NeoChr33*	This study
IMF48*	*MATa ura3Δ his3Δ leu2-3,112 MAL2-8c SUC2 glk1Δ hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ::pKlURA3-KlURA3-tKlURA3 pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ gnd2Δ sol4Δ tkl2Δ nqm1Δ aro10Δ zwf1Δ sol3Δ gnd1Δ rki1Δ tkl1Δ tal1Δ rpe1Δ x2Δ::pTEF1-coAtCHS3-tMDH1 yprctau3Δ::pTEF1-coAtCHS3-tMDH1 spr3Δ::pTEF1-coAtCHS3-tMDH1 can1::pTEF1-coAtCHS3-tMDH NeoChr34*	This study

Supplementary Table 7 - Neochromosome configurations

SHRs are differently annotated than in Kuijpers *et al.* [6]. SHRs are annotated in subscript between the genetic fragments that they join together.

Name	Size	Notes	Stocked name	Neochromosome configuration
NeoChr1	100 kb	Circular	IMF6	$_{AO'}$ 7A $_{BJ}$ 7B $_{BK}$ 7C $_{BL}$ 7D $_{AP}$ 8A $_{BM}$ 8B $_{BN}$ 8C $_{BO}$ 8D $_{AC}$ *pCCW12-mRuby2-tENO1* $_{AD}$ *CEN6/ARS4*$_{AE}$ 1A $_{AT}$ 1B $_{AS}$ 1C $_{AU}$ 1D $_{AF}$ 2A $_{AV}$ 2B $_{AW}$2C $_{AX}$ 2D $_{AG}$ 3A $_{AY}$ 3B $_{AZ}$ 3C $_{BA}$ 3D $_{AH}$ *pTEF2-mTurquoise2-tSSA 1*$_{AI}$ ARS417 $_{BU}$ *pHIS3-HIS3-tHIS3*$_{AJ}$ 4A $_{BC}$ 4B $_{BD}$ 4C $_{BE}$4D $_{AK}$ 9A $_{BF}$9B $_{BS}$9C $_{BT}$9D $_{AQ}$5A $_{BG}$ 5B $_{BH}$ 5C $_{BI}$ 5D $_{AL}$ *pTEF1-Venus-tTDH1*$_{AM}$ ARS1 $_{AN}$ 6A $_{BP}$ 6B $_{BQ}$ 6C $_{BR}$ 6D $_{AO`}$ Telomerator $_{AO`}$
NeoChr2	50 kb	Circular	IMF2	$_{AO'}$ 7A $_{BJ}$ 7B $_{BK}$ 7C $_{BL}$ 7D $_{AC}$ *pCCW12-mRuby2-tENO1* $_{AD}$ *CEN6/ARS4* $_{AE}$ 1A $_{AT}$ 1B $_{AS}$ 1C $_{AU}$ 1Ds $_{AH}$ *pTEF2 - mTurquoise2 - tSSA1*$_{AI}$ *pHIS3-HIS3-tHIS3* $_{AJ}$ 4A $_{BC}$ 4B $_{BD}$ 4C $_{BE}$ 4D $_{AL}$ *pTEF1 - Venus - tTDH1* $_{AM}$ *ARS1* $_{AN}$ 6A $_{BP}$ 6B $_{BQ}$ 6C $_{BR}$ 6D $_{AO'}$ Telomerator $_{AO'}$
NeoChr10	100 kb	Linear	IMF22	Telomere $_{AO}$ 7A $_{BJ}$ 7B $_{BK}$ 7C $_{BL}$ 7D $_{AP}$ 8A $_{BM}$ 8B $_{BN}$ 8C $_{BO}$ 8D $_{AC}$ *pCCW12-mRuby2-tENO1* $_{AD}$ *ARS1* $_{AN}$ 18A $_{BP}$ 18B $_{BQ}$ 19C $_{BR}$ 19D $_{DE}$ 15A $_{DF}$ 15B $_{DH}$ 15C $_{DI}$ 15D $_{DJ}$ *CEN6/ARS4* $_{AE}$ 16A $_{DK}$ 16B $_{DL}$ 16C $_{DM}$ 16D $_{DN}$ 17A $_{DO}$ 17B $_{DP}$ 19A $_{DQ}$ 17D $_{DR}$ *ARS417* $_{BU}$ *pHIS3-HIS3-tHIS3* $_{AJ}$ 4A $_{BC}$ 4B $_{BD}$ 4C $_{BE}$ 4D $_{AK}$ 9A $_{BF}$ 9B $_{BS}$ 9C $_{BT}$ 9D $_{AQ}$ 5A $_{BG}$ 5B $_{BH}$ 5C $_{BI}$ 5D $_{AL}$ *tSSA1-mTurquoise2- pTEF2* $_{DS}$ Telomere
NeoChr11	100 kb	Linear. Few bases changed in right telomere to prevent recirculari-sation	-	Telomere $_{AO}$ 7A $_{BJ}$ 7B $_{BK}$ 7C $_{BL}$ 7D $_{AP}$ 8A $_{BM}$ 8B $_{BN}$ 8C $_{BO}$ 8D $_{AC}$ *pCCW12-mRuby2-tENO1* $_{AD}$ *ARS1* $_{AN}$ 18A $_{BP}$ 18B $_{BQ}$ 19C $_{BR}$ 19D $_{DE}$ 15A $_{DF}$ 15B $_{DH}$ 15C $_{DI}$ 15D $_{DJ}$ *CEN6/ARS4* $_{AE}$ 16A $_{DK}$ 16B $_{DL}$ 16C $_{DM}$ 16D $_{DN}$ 17A $_{DO}$ 17B $_{DP}$ 19A $_{DQ}$ 17D $_{DR}$ *ARS417* $_{BU}$ *pHIS3-HIS3-tHIS3* $_{AJ}$ 4A $_{BC}$ 4B $_{BD}$ 4C $_{BE}$ 4D $_{AK}$ 9A $_{BF}$ 9B $_{BS}$ 9C $_{BT}$ 9D $_{AQ}$ 5A $_{BG}$ 5B $_{BH}$ 5C $_{BI}$ 5D $_{AL}$ *tSSA1-mTurquoise2- pTEF2* $_{DS}$ Telomere
NeoChr12	100 kb	Circular	IMF23	$_{AO}$ 7A $_{BJ}$ 7B $_{BK}$ 7C $_{BL}$ 7D $_{AP}$ 8A $_{BM}$ 8B $_{BN}$ 8C $_{BO}$ 8D $_{AC}$ *pCCW12-mRuby2-tENO1* $_{AD}$ *ARS1*$_{AN}$ 18A $_{BP}$ 18B $_{BQ}$ 19C $_{BR}$ 19D $_{DE}$ 15A $_{DF}$ 15B $_{DH}$ 15C $_{DI}$ 15D $_{DJ}$ *CEN6/ARS4*$_{AE}$ 16A $_{DK}$ 16B $_{DL}$ 16C $_{DM}$ 16D $_{DN}$ 17A $_{DO}$ 17B $_{DP}$ 19A $_{DQ}$ 17D $_{DR}$ *ARS417* $_{BU}$ *pHIS3-HIS3-tHIS3*$_{AJ}$ 4A $_{BC}$ 4B $_{BD}$ 4C $_{BE}$ 4D $_{AK}$ 9A $_{BF}$ 9B $_{BS}$ 9C $_{BT}$ 9D $_{AQ}$ 5A $_{BG}$ 5B $_{BH}$ 5C $_{BI}$ 5D $_{AL}$ *tSSA1-mTurquoise2- pTEF2* $_{DS}$ Telomerator $_{AO}$
NeoChr25	100 kb	Linear	IMF27, IMF31, IMF33, IMF34	telomere $_{BJ}$ 7BC $_{BL}$ *ARS1* $_{AN}$ *pZWF1-ZWF1-tZWF1* $_{BP}$ *pTKL1-TKL1-tTKL1* $_{DE}$ *pGND1-GND1-tGND1* $_{BQ}$ *tRKI1-RKI1-pRKI1* $_{BR}$ *tTAL1-TAL1-pTAL1* $_{AL}$ *tSSA1-mTurquoise2-pTEF2* $_{DS}$ *tRPE1-RPE1-pRPE1* $_{DF}$ *tSOL3-SOL3-pSOL3* $_{DI}$ *ARS417* $_{BE}$ *pHHF1-coEcaroG*$^{(P150L)}$*-tTEF1* $_{DK}$ *pHHF2-coEcaroB-tTEF2* $_{AC}$ *pCCW12-mRuby2-tENO1* $_{AD}$ *pRPL25-coEcaroD-tGPH1* $_{DM}$

				pRPP0-coEcaroE-tCYC1 DN *pHTB2-coEcaroL-tPGM2* DO *pRPL3-coEcaroA-tSOL4* DP *tGPD2-coEcaroC-pRPL8A* DQ *tGDB1-coEctyrA*$^{(M53I,A354V)}$*-pRPL10* DR *tGSY2-coEcpheA*$^{(T326P)}$ *-pRPL18B* AJ *tGLC3-coEctyrB-pCWP2* DH 15CD DJ *CEN6/ARS4* AE 16AB DL *pFBA1-FBA1-tFBA1* H *pTPI1-TPI1-tTPI1* P *pPGK1-PGK1-tPGK1* Q *pADH1-ADH1-tADH1* N *pPYK1-PYK1-tPYK1* O *pTDH3-TDH3-tTDH3* A *pENO2-ENO2-tENO2* B *tHXK2-HXK2-pHXK2* C *tPGI1-PGI1-pPGI1* D *tPFK1-PFK1-pPFK1* J *tPFK2-PFK2-pPFK2* BU *tHIS3-HIS3-pHIS3* L *tGPM1-GPM1-pGPM1* M *tPDC1-PDC1-pPDC1* AR *ARS1211* BS 9CD AQ telomere
NeoChr26	100 kb	Circular	IMF29, IMF32, IMF35, IMF36, IMF40	BJ 7BC BL *ARS1* AN *pZWF1-ZWF1-tZWF1* BP *pTKL1-TKL1-tTKL1* DE *pGND1-GND1-tGND1* BQ *tRKI1-RKI1-pRKI1* BR *tTAL1-TAL1-pTAL1* AL *tSSA1-mTurquoise2-pTEF2* DS *tRPE1-RPE1-pRPE1* DF *tSOL3-SOL3-pSOL3* DI *ARS417* BE *pHHF1-coEcaroG*$^{(P150L)}$*-tTEF1* DK *pHHF2-coEcaroB-tTEF2* AC *pCCW12-mRuby2-tENO1* AD *pRPL25-coEcaroD-tGPH1* DM *pRPP0-coEcaroE-tCYC1* DN *pHTB2-coEcaroL-tPGM2* DO *pRPL3-coEcaroA-tSOL4* DP *tGPD2-coEcaroC-pRPL8A* DQ *tGDB1-coEctyrA*$^{(M53I,A354V)}$*-pRPL10* DR *tGSY2-coEcpheA*$^{(T326P)}$ *-pRPL18B* AJ *tGLC3-coEctyrB-pCWP2* DH 15CD DJ *CEN6/ARS4* AE 16AB DL *pFBA1-FBA1-tFBA1* H *pTPI1-TPI1-tTPI1* P *pPGK1-PGK1-tPGK1* Q *pADH1-ADH1-tADH1* N *pPYK1-PYK1-tPYK1* O *pTDH3-TDH3-tTDH3* A *pENO2-ENO2-tENO2* B *tHXK2-HXK2-pHXK2* C *tPGI1-PGI1-pPGI1* D *tPFK1-PFK1-pPFK1* J *tPFK2-PFK2-pPFK2* BU *tHIS3-HIS3-pHIS3* L *tGPM1-GPM1-pGPM1* M *tPDC1-PDC1-pPDC1* AR *ARS1211* BS 9CD AQ telomerator
NeoChr30	128 kb	Circular. Insertion of anthocyanin pathway in NeoChr26 of strain IMF40	IMF41	BJ 7BC BL *ARS1* AN *pZWF1-ZWF1-tZWF1* BP *pTKL1-TKL1-tTKL1* DE *pGND1-GND1-tGND1* BQ *tRKI1-RKI1-pRKI1* BR *tTAL1-TAL1-pTAL1* AL *tSSA1-mTurquoise2-pTEF2* DS *tRPE1-RPE1-pRPE1* DF *tSOL3-SOL3-pSOL3* DI *ARS417* BE *pHHF1-coEcaroG*$^{(P150L)}$*-tTEF1* DK *pHHF2-coEcaroB-tTEF2* AC *pCCW12-mRuby2-tENO1* AD *pRPL25-coEcaroD-tGPH1* DM *pRPP0-coEcaroE-tCYC1* DN *pHTB2-coEcaroL-tPGM2* DO *pRPL3-coEcaroA-tSOL4* DP *tGPD2-coEcaroC-pRPL8A* DQ *tGDB1-coEctyrA*$^{(M53I,A354V)}$*-pRPL10* DR *tGSY2-coEcpheA*$^{(T326P)}$ *-pRPL18B* AJ *tGLC3-coEctyrB-pCWP2* DH 15CD DJ *CEN6/ARS4* AE 16AB *pRPS3-coAtCPR1-tIDH2* F *pSePDC1-AtPAL1-tLAT1* DW *pSeGPM1-coRcTAL1-tCIT1* DX *pSeTPI1-At4CL3-tSDH2* DY *pTEF1-coAtCHS3-tMDH1* AM *tSDH4-AtCHI1-pSkADH1* AB *tADH3-coAtC4H-pSeFBA1* DC *tSDH3-coAtF3H-pSkTDH3* EA *tACO1-coGhDFR-pSePGK1* EB *tFUM1-coAtANS-pSeENO2* EC *tDIC1-coAt3GT-pSePYK1* CJ *ARS106* DL *pFBA1-FBA1-tFBA1* H *pTPI1-TPI1-tTPI1* P *pPGK1-PGK1-tPGK1* Q *pADH1-ADH1-tADH1* N *pPYK1-PYK1-tPYK1* O *pTDH3-TDH3-tTDH3* A *pENO2-ENO2-tENO2* B *tHXK2-HXK2-pHXK2* C *tPGI1-PGI1-pPGI1* D *tPFK1-PFK1-*

				pPFK1 $_{J}$ *tPFK2-PFK2-pPFK2* $_{BU}$ *tHIS3-HIS3-pHIS3* $_{L}$ *tGPM1-GPM1-pGPM1* $_{M}$ *tPDC1-PDC1-pPDC1* $_{AR}$ *ARS1211* $_{BS}$ 9CD $_{AQ}$ Telomerator
NeoChr31	128 kb	Linear. Insertion of anthocyanin pathway in NeoChr25 of strain IMF34	IMF42, IMF44	Telomere $_{BJ}$ 7BC $_{BL}$ *ARS1* $_{AN}$ *pZWF1-ZWF1-tZWF1* $_{BP}$ *pTKL1-TKL1-tTKL1* $_{DE}$ *pGND1-GND1-tGND1* $_{BQ}$ *tRKI1-RKI1-pRKI1* $_{BR}$ *tTAL1-TAL1-pTAL1* $_{AL}$ *tSSA1-mTurquoise2-pTEF2* $_{DS}$ *tRPE1-RPE1-pRPE1* $_{DF}$ *tSOL3-SOL3-pSOL3* $_{DI}$ *ARS417* $_{BE}$ *pHHF1-coEcaroG*$^{(P150L)}$*-tTEF1* $_{DK}$ *pHHF2-coEcaroB-tTEF2* $_{AC}$ *pCCW12-mRuby2-tENO1* $_{AD}$ *pRPL25-coEcaroD-tGPH1* $_{DM}$ *pRPP0-coEcaroE-tCYC1* $_{DN}$ *pHTB2-coEcaroL-tPGM2* $_{DO}$ *pRPL3-coEcaroA-tSOL4* $_{DP}$ *tGPD2-coEcaroC-pRPL8A* $_{DQ}$ *tGDB1-coEctyrA*$^{(M53I,A354V)}$*-pRPL10* $_{DR}$ *tGSY2-coEcpheA*$^{(T326P)}$ *-pRPL18B* $_{AJ}$ *tGLC3-coEctyrB-pCWP2* $_{DH}$ 15CD $_{DJ}$ *CEN6/ARS4* $_{AE}$ 16AB *pRPS3-coAtCPR1-tIDH2* $_{F}$ *pSePDC1-AtPAL1-tLAT1* $_{DW}$ *pSeGPM1-coRcTAL1-tCIT1* $_{DX}$ *pSeTPI1-At4CL3-tSDH2* $_{DY}$ *pTEF1-coAtCHS3-tMDH1* $_{AM}$ *tSDH4-AtCHI1-pSkADH1* $_{AB}$ *tADH3-coAtC4H-pSeFBA1* $_{DC}$ *tSDH3-coAtF3H-pSkTDH3* $_{EA}$ *tACO1-coGhDFR-pSePGK1* $_{EB}$ *tFUM1-coAtANS-pSeENO2* $_{EC}$ *tDIC1-coAt3GT-pSePYK1* $_{CJ}$ *ARS106* $_{DL}$ *pFBA1-FBA1-tFBA1* $_{H}$ *pTPI1-TPI1-tTPI1* $_{P}$ *pPGK1-PGK1-tPGK1* $_{Q}$ *pADH1-ADH1-tADH1* $_{N}$ *pPYK1-PYK1-tPYK1* $_{O}$ *pTDH3-TDH3-tTDH3* $_{A}$ *pENO2-ENO2-tENO2* $_{B}$ *tHXK2-HXK2-pHXK2* $_{C}$ *tPGI1-PGI1-pPGI1* $_{D}$ *tPFK1-PFK1-pPFK1* $_{J}$ *tPFK2-PFK2-pPFK2* $_{BU}$ *tHIS3-HIS3-pHIS3* $_{L}$ *tGPM1-GPM1-pGPM1* $_{M}$ *tPDC1-PDC1-pPDC1* $_{AR}$ *ARS1211* $_{BS}$ 9CD $_{AQ}$ Telomere
NeoChr33	137 kb	Insertion of *AtCHS* at Chunk 7BC, chunk 15CD, SHR N and chunk 9CD in NeoChr31 of strain IMF42 and IMF44.	IMF47 (from IMF44)	Telomere $_{BJ}$ 7BC *tMDH1-coAtCHS3-pTEF1* 7BC $_{BL}$ *ARS1* $_{AN}$ *pZWF1-ZWF1-tZWF1* $_{BP}$ *pTKL1-TKL1-tTKL1* $_{DE}$ *pGND1-GND1-tGND1* $_{BQ}$ *tRKI1-RKI1-pRKI1* $_{BR}$ *tTAL1-TAL1-pTAL1* $_{AL}$ *tSSA1-mTurquoise2-pTEF2* $_{DS}$ *tRPE1-RPE1-pRPE1* $_{DF}$ *tSOL3-SOL3-pSOL3* $_{DI}$ *ARS417* $_{BE}$ *pHHF1-coEcaroG*$^{(P150L)}$*-tTEF1* $_{DK}$ *pHHF2-coEcaroB-tTEF2* $_{AC}$ *pCCW12-mRuby2-tENO1* $_{AD}$ *pRPL25-coEcaroD-tGPH1* $_{DM}$ *pRPP0-coEcaroE-tCYC1* $_{DN}$ *pHTB2-coEcaroL-tPGM2* $_{DO}$ *pRPL3-coEcaroA-tSOL4* $_{DP}$ *tGPD2-coEcaroC-pRPL8A* $_{DQ}$ *tGDB1-coEctyrA*$^{(M53I,A354V)}$*-pRPL10* $_{DR}$ *tGSY2-coEcpheA*$^{(T326P)}$ *-pRPL18B* $_{AJ}$ *tGLC3-coEctyrB-pCWP2* $_{DH}$ 15CD *tMDH1-coAtCHS3-pTEF1 15 CD* $_{DJ}$ *CEN6/ARS4* $_{AE}$ 16AB *pRPS3-coAtCPR1-tIDH2* $_{F}$ *pSePDC1-AtPAL1-tLAT1* $_{DW}$ *pSeGPM1-coRcTAL1-tCIT1* $_{DX}$ *pSeTPI1-At4CL3-tSDH2* $_{DY}$ *pTEF1-coAtCHS3-tMDH1* $_{AM}$ *tSDH4-AtCHI1-pSkADH1* $_{AB}$ *tADH3-coAtC4H-pSeFBA1* $_{DC}$ *tSDH3-coAtF3H-pSkTDH3* $_{EA}$ *tACO1-coGhDFR-pSePGK1* $_{EB}$ *tFUM1-coAtANS-pSeENO2* $_{EC}$ *tDIC1-coAt3GT-pSePYK1* $_{CJ}$ *ARS106* $_{DL}$ *pFBA1-FBA1-tFBA1* $_{H}$ *pTPI1-TPI1-tTPI1* $_{P}$ *pPGK1-PGK1-tPGK1* $_{Q}$ *pADH1-ADH1-tADH1* $_{N}$ *pTEF1-coAtCHS3-tMDH1 pPYK1-PYK1-tPYK1* $_{O}$ *pTDH3-TDH3-tTDH3* $_{A}$ *pENO2-ENO2-tENO2* $_{B}$ *tHXK2-HXK2-pHXK2* $_{C}$ *tPGI1-PGI1-pPGI1* $_{D}$ *tPFK1-PFK1-pPFK1* $_{J}$

				tPFK2-PFK2-pPFK2 $_{BU}$ *tHIS3-HIS3-pHIS3* $_{L}$ *tGPM1-GPM1-pGPM1* $_{M}$ *tPDC1-PDC1-pPDC1* $_{AR}$ *ARS1211* $_{BS}$ 9CD *pTEF1-coAtCHS3-tMDH1 9CD* $_{AQ}$ Telomere
NeoChr34	137 kb	Insertion of *CoAtANS* in *mTurquoise*	IMF48 (from IMF47)	Telomere $_{BJ}$ 7BC *tMDH1-coAtCHS3-pTEF1* 7BC $_{BL}$ *ARS1* $_{AN}$ *pZWF1-ZWF1-tZWF1* $_{BP}$ *pTKL1-TKL1-tTKL1* $_{DE}$ *pGND1-GND1-tGND1* $_{BQ}$ *tRKI1-RKI1-pRKI1* $_{BR}$ *tTAL1-TAL1-pTAL1* $_{AL}$ *tSSA1-mTurquoise2-pTEF2Δ::tFUM1-CoAtANS-pSeENO2* $_{DS}$ *tRPE1-RPE1-pRPE1* $_{DF}$ *tSOL3-SOL3-pSOL3* $_{DI}$ *ARS417* $_{BE}$ *pHHF1-coEcaroG*$^{(P150L)}$*-tTEF1* $_{DK}$ *pHHF2-coEcaroB-tTEF2* $_{AC}$ *pCCW12-mRuby2-tENO1* $_{AD}$ *pRPL25-coEcaroD-tGPH1* $_{DM}$ *pRPP0-coEcaroE-tCYC1* $_{DN}$ *pHTB2-coEcaroL-tPGM2* $_{DO}$ *pRPL3-coEcaroA-tSOL4* $_{DP}$ *tGPD2-coEcaroC-pRPL8A* $_{DQ}$ *tGDB1-coEctyrA*$^{(M53I,A354V)}$*-pRPL10* $_{DR}$ *tGSY2-coEcpheA*$^{(T326P)}$ *-pRPL18B* $_{AJ}$ *tGLC3-coEctyrB-pCWP2* $_{DH}$ 15CD *tMDH1-coAtCHS3-pTEF1 15 CD* $_{DJ}$ *CEN6/ARS4* $_{AE}$ 16AB *pRPS3-coAtCPR1-tIDH2* $_{F}$ *pSePDC1-AtPAL1-tLAT1* $_{DW}$ *pSeGPM1-coRcTAL1-tCIT1* $_{DX}$ *pSeTPI1-At4CL3-tSDH2* $_{DY}$ *pTEF1-coAtCHS3-tMDH1* $_{AM}$ *tSDH4-AtCHI1-pSkADH1* $_{AB}$ *tADH3-coAtC4H-pSeFBA1* $_{DC}$ *tSDH3-coAtF3H-pSkTDH3* $_{EA}$ *tACO1-coGhDFR-pSePGK1* $_{EB}$ *tFUM1-coAtANS-pSeENO2* $_{EC}$ *tDIC1-coAt3GT-pSePYK1* $_{CJ}$ *ARS106* $_{DL}$ *pFBA1-FBA1-tFBA1* $_{H}$ *pTPI1-TPI1-tTPI1* $_{P}$ *pPGK1-PGK1-tPGK1* $_{Q}$ *pADH1-ADH1-tADH1* $_{N}$ *pTEF1-coAtCHS3-tMDH1 pPYK1-PYK1-tPYK1* $_{O}$ *pTDH3-TDH3-tTDH3* $_{A}$ *pENO2-ENO2-tENO2* $_{B}$ *tHXK2-HXK2-pHXK2* $_{C}$ *tPGI1-PGI1-pPGI1* $_{D}$ *tPFK1-PFK1-pPFK1* $_{J}$ *tPFK2-PFK2-pPFK2* $_{BU}$ *tHIS3-HIS3-pHIS3* $_{L}$ *tGPM1-GPM1-pGPM1* $_{M}$ *tPDC1-PDC1-pPDC1* $_{AR}$ *ARS1211* $_{BS}$ 9CD *pTEF1-coAtCHS3-tMDH1 9CD* $_{AQ}$ Telomere

Supplementary Table 8 - Plasmids

Table 8A gRNA plasmids

Plasmid	Relevant characteristics	Primer(s) used for gRNA	Source
pMEL10	2µm ampR *KlURA3* gRNA-*CAN1*.Y	N.A.	Mans, *et al.* [9]
pROS10	2µm ampR *URA3* gRNA-*CAN1.Y* gRNA-*ADE2.Y*	N.A.	Mans, *et al.* [9]
pROS11	2µm ampR *amdSYM* gRNA-*CAN1*.Y gRNA-*ADE2*.Y	N.A.	Mans, *et al.* [9]
pROS12	2µm ampR *hphNT1* gRNA-*CAN1.Y* gRNA-*ADE2.Y*	N.A.	Mans, *et al.* [9]
pROS13	2µm ampR *kanMX* gRNA-*CAN1*.Y gRNA-*ADE2*.Y	N.A.	Mans, *et al.* [9]
pUDR286	2µm ampR *URA3* gRNA-*TKL2* gRNA-*SOL4*	9508 & 9503	Postma, *et al.*[10]
pUDR400	2µm ampR *hphNT1* gRNA-*mTurquoise*	12911 & 12912	This study
pUDR406	2µm ampR *URA3* gRNA-*ARO10* gRNA-*PDC5/6*	13614 & 7246	This study
pUDR413	2µm ampR *kanMX* gRNA- *RECYCLE SinLoG*	N.A.	Boonekamp, *et al.* [1]
pUDR426	2µm ampR *KanMX* gRNA-*spHIS5 (2x)*	10641	This study
pUDR546	2µm ampR *hphNT1* gRNA-*URA3* gRNA-*HIS3*	14756 & 8314	This study
pUDR590	2µm ampR *amdS* gRNA-*NQM1* gRNA-*GND2*	12569 &7231	This study
pUDR700	2µm ampR *KanMX* gRNA-*GND1* gRNA-*RKI1*	16895 & 16897	This study
pUDR701	2µm ampR *KanMX* gRNA-*TAL1 (2x)*	16909	This study
pUDR702	2µm ampR *hphNT1* gRNA-*RPE1* gRNA-*TKL1*	16903 & 16905	This study
pUDR703	2µm ampR *hphNT1* gRNA-*ZWF1* gRNA-*SOL3*	8564 & 16889	This study
pUDR756	2µm ampR *hphNT1* gRNA-*RKI1_WM (2x)*	17613	This study
pUDR765	2µm ampR *hphNT1* gRNA-*Chunk16AB (2x)*	17868	This study
pUDR771	2µm ampR *hphNT1* gRNA-*CAN1* gRNA-*X2*	6008 & 10866	This study
pUDR772	2µm ampR *kanMX* gRNA-*YPRCtau3* gRNA-*SPR3*	12985 & 12034	This study
pUDR780	2µm ampR *hphNT1* gRNA-*Chunk7BC* gRNA-*Chunk15CD)*	18226 & 18277	This study
pUDR781	2µm ampR *kanMX* gRNA-*shrN* gRNA-*Chunk9CD)*	18228 & 18299	This study

Table 8B in-house golden gate part plasmids

Plasmid	Relevant characteristics	Source
pUD565	camR *GFP* entry vector	Boonekamp, *et al.*[1]
pYTK012	camR *pHHF2*	Lee, *et al.* [2]
pYTK013	camR *pTEF1*	Lee, *et al.* [2]
pYTK015	camR *pHHF1*	Lee, *et al.* [2]
pYTK016	camR *pHTB2*	Lee, *et al.* [2]
pYTK017	camR *pRPL18B*	Lee, *et al.* [2]
pGGKp038	camR *tTEF2*	Hassing, *et al.* [11]
pGGKp039	camR *tTEF1*	Hassing, *et al.* [11]
pGGKp062	kanR *pSkADH1*	Hassing, *et al.* [11]
pGGKp063	kanR *pSkTDH3*	Hassing, *et al.* [11]
pGGkp074	camR *pSePDC1*	Hassing, *et al.* [11]
pGGKp075	camR *pSeFBA1*	Hassing, *et al.* [11]
pGGkp095	camR *pSeGPM1*	Hassing, *et al.* [11]
pGGKp113	camR *tADH3*	Hassing, *et al.* [11]
pGGKp119	camR *coEcaroG*$^{(p150L)}$	Hassing, *et al.* [11]

pGGKp120	camR *coEcaroB*	Hassing, *et al.* [11]
pGGKp121	camR *coEcaroD*	Hassing, *et al.* [11]
pGGKp122	camR *coEcaroE*	Hassing, *et al.* [11]
pGGKp123	camR *coEcaroL*	Hassing, *et al.* [11]
pGGKp124	camR *coEcaroA*	Hassing, *et al.* [11]
pGGKp125	camR *coEcaroC*	Hassing, *et al.* [11]
pGGKp126	camR *coEcpheA*^{T326P}	Hassing, *et al.* [11]
pGGKp182	camR *tCYC1*	This study
pGGKp327	camR *coRcTAL1*	This study

Table 8C Part plasmids subcloned by GeneArt in entry vector pUD565

Plasmid	Relevant characteristics	Source
pGGKp131	camR *AtPAL1*	GeneArt
pGGKp135	camR *coEctyrA*$^{M53I\ A354V}$	GeneArt
pGGKp245	ampR *coEctyrB*	GeneArt
pUD262	camR *pCWP2*	GeneArt
pUD819	camR *tACO1*	GeneArt
pUD826	camR *tCIT1*	GeneArt
pUD829	camR *tDIC1*	GeneArt
pUD832	camR *tIDH2*	GeneArt
pUD836	camR *tFUM1*	GeneArt
pUD841	camR *tGDB1*	GeneArt
pUD842	camR *tGLC3*	GeneArt
pUD844	camR *tTAL1*	GeneArt
pUD845	camR *tGPD2*	GeneArt
pUD846	camR *tGPH1*	GeneArt
pUD848	camR *tGSY2*	GeneArt
pUD850	camR *tSDH2*	GeneArt
pUD851	camR *tSDH3*	GeneArt
pUD852	camR *tSDH4*	GeneArt
pUD858	camR *tLAT1*	GeneArt
pUD862	camR *tMDH1*	GeneArt
pUD873	camR *tPGM2*	GeneArt
pUD875	camR *tSOL3*	GeneArt
pUD876	camR *tRKI1*	GeneArt
pUD879	camR *tGND1*	GeneArt
pUD880	camR *tSOL4*	GeneArt
pUD881	camR *tRPE1*	GeneArt
pUD882	camR *tTKL1*	GeneArt
pUD884	camR *tZWF1*	GeneArt
pUD911	camR *pTAL1*	GeneArt
pUD933	camR *pSOL3*	GeneArt
pUD934	camR *pRKI1*	GeneArt
pUD944	camR *pGND1*	GeneArt
pUD946	camR *pRPE1*	GeneArt
pUD947	camR *pTKL1*	GeneArt
pUD949	camR *pZWF1*	GeneArt

Table 8D Part plasmids ordered from GeneArt and subcloned in house in entry vector pUD565

Plasmid	Relevant characteristics	Source
pUD1038	ampR *ZWF1 **	GeneArt
pUD1039	ampR *TKL1 **	GeneArt
pUD1040	kanR *TAL1 **	GeneArt
pUD1041	ampR *SOL3 **	GeneArt
pUD1042	ampR *RPE1 **	GeneArt
pUD1043	ampR *RKI1 **	GeneArt
pUD1044	kanR *GND1 **	GeneArt
pGGKp245	Amp *coEctyrB*	GeneArt
pGGKp247	camR *ZWF1 **	This study
pGGKp248	camR *TKL1 **	This study
pGGKp249	camR *TAL1 **	This study
pGGKp250	camR *SOL3 **	This study
pGGKp251	camR *RPE1 **	This study
pGGKp252	camR *RKI1 **	This study
pGGKp253	camR *GND1 **	This study
pGGKp293	camR *coEctyrB*	This study

Table 8E Part plasmids made in house by PCR

*****For *pSEPYK1* and initially the *coAtANS* no correct *E.coli* part plasmid transformant was found and expression cassettes were thus assembled with PCR fragments with yeast toolkit flanks.

Plasmid	Relevant characteristics	Template	Primers	Source
pUD257	camR *pRPP0*	CEN.PK113-7D genomic DNA	16294 & 16295	This study
pUD258	camR *pRPL3*	CEN.PK113-7D genomic DNA	16300 & 16301	This study
pUD259	camR *pRPL8A*	CEN.PK113-7D genomic DNA	16298 & 16299	This study
pUD260	camR *pRPL10*	CEN.PK113-7D genomic DNA	16296 & 16297	This study
pUD261	camR *pRPL25*	CEN.PK113-7D genomic DNA	16292 & 16293	This study
pGGKp324	camR *AtCHI1*	pUDI065	17829 & 17830	This study
pGGKp325	camR *coAtCHS3*	pUDE185	17827 & 17828 & 17834 & 17835	This study
pGGKp326	camR *coAtC4H*	pUDE172	17823 & 17824	This study
pGGKp328	camR *coGhDFR*	Genomic DNA PATW076 [12]	17872 & 17873	This study
pGGKp329	camR *coAt3GT*	Genomic DNA PATW076 [12]	17876 & 17877	This study
pGGKp330	camR *pSePGK1*	pUDI102	9413 & 9414	This study
pGGKp331	camR *pSeENO2*	*Saccharomyces eubayanus* genomic DNA	9743 & 9744	This study
pGGKp332	CamR *coAtF3H*	Genomic DNA PATW076	17870 & 17871	This study
pGGKp340	camR *coAtANS*	Genomic DNA PATW076	17874 & 17875	This study
PCR fragment*	*coAtANS*	Genomic DNA PATW076	17874 & 17875	This study

PCR fragment*	*pSePYK1*	pUDI129	10610 & 10611	This study

Table 8F in house expression plasmids described in other studies

Plasmid	Relevant characteristics	Source
pGGKd005	CEN6/ARS4 ampR *hphNT1 GFP*	Hassing, *et al.* [11]
pGGKd012	CEN6/ARS4 *cloNAT^R GFP*	Boonekamp, *et al.* [1]
pUDC212	CEN6/ARS4 *ampR natNT2 pFBA1-FBA1*-tFBA1*	Boonekamp, *et al.* [1]
pUDC213	CEN6/ARS4 *ampR natNT2 pPGM1-PGM1*-tPGM1*	Boonekamp, *et al.* [1]
pUDC214	CEN6/ARS4 *ampR natNT2 pHXK2-HXK2*-tHXK2*	Boonekamp, *et al.*[1]
pUDC215	CEN6/ARS4 *ampR natNT2 pPDC1-PDC1*-tPDC1*	Boonekamp, *et al.* [1]
pUDC216	CEN6/ARS4 *ampR natNT2 pPFK1-PFK1*-tPFK1*	Boonekamp, *et al.*[1]
pUDC217	CEN6/ARS4 *ampR natNT2 pPFK2-PFK2*-tPFK2*	Boonekamp, Dashko [1]
pUDC219	CEN6/ARS4 *ampR natNT2 pPGK1-PGK1*-tPGK1*	Boonekamp, *et al.* [1]
pUDC220	CEN6/ARS4 *ampR natNT2 pPYK1-PYK1*-tPYK1*	Boonekamp, *et al.*[1]
pUDC222	CEN6/ARS4 *ampR natNT2 pTPI1-TPI1*-tTPI1*	Boonekamp, *et al.*[1]
pUDC229	CEN6/ARS4 *ampR natNT2 pADH1-ADH1*-tADH1*	Boonekamp, *et al.*[1]
pUDC230	CEN6/ARS4 *ampR natNT2 pTDH3-TDH3*-tTDH3*	Boonekamp, *et al.*[1]
pUDC231	CEN6/ARS4 *ampR natNT2 pENO2-ENO2*-tENO2*	Boonekamp, *et al.*[1]
pUDC232	CEN6/ARS4 *ampR natNT2 pPGI1-PGI1*-tPGI1*	Boonekamp, *et al.*[1]

Table 8G Expression plasmids constructed in this study

***For *pSEPYK1* and initially *coAtANS* no correct *E.coli* part plasmid transformant was found and expression cassettes were thus assembled with PCR fragments with yeast toolkit flanks

** the ORF of pUDC357 turned out to be mutated after sanger sequencing.

Plasmid	Relevant characteristics	Parts used	Source
pUDC275	CEN6/ARS4 ampR *natNT2 pZWF1-ZWF1*-tZWF1*	pGGKd012, pUD949, pGGKp247, pUD884	This study
pUDC276	CEN6/ARS4 ampR *natNT2 pTKL1-TKL1*-tTKL1*	pGGKd012, pUD947, pGGKp248, pUD882	This study
pUDC277	CEN6/ARS4 ampR *natNT2 pGND1-GND1*-tGND1*	pGGKd012, pUD911, pGGKp253, pUD844	This study
pUDC278	CEN6/ARS4 ampR *natNT2 pRKI1-RKI1*-tRKI1*	pGGKd012, pUD933, pGGKp252, pUD875	This study
pUDC279	CEN6/ARS4 ampR *natNT2 pTAL1-TAL1*-tTAL1*	pGGKd012, pUD946, pGGKp249, PUD881	This study
pUDC280	CEN6/ARS4 ampR *natNT2 pRPE1-RPE1*-tRPE1*	pGGKd012, pUD934, pGGKp251, pUD876	This study
pUDC281	CEN6/ARS4 ampR *natNT2 pSOL3-SOL3*-tSOL3*	pGGKd012, pUD944, pGGKp250, pUD879	This study
pUDC293	CEN6/ARS4 ampR *natNT2 pRPL3-coEcaroA-tSOL4*	pGGKd012, pUD258, pGGKp124, pUD880	This study
pUDC296	CEN6/ARS4 ampR *natNT2 pRPL25-coEcaroD-tGPH1*	pGGKd012, pUD261, pGGKp121, pUD846	This study

pUDC297	CEN6/ARS4 ampR *natNT2 pRPP0-coEcaroE-tCYC1*	pGGKd012, pUD257, pGGKp122, pGGKp182	This study
pUDC298	CEN6/ARS4 ampR *natNT2 pHHF1-coEcaroG$^{(p150L)}$-tTEF1*	pGGKd012, pYTK015, pGGKp119, pGGKp039	This study
pUDC299	CEN6/ARS4 ampR *natNT2 pHTB2-coEcaroL-tPGM2*	pGGKd012, pYTK016, pGGKp123, pUD873	This study
pUDC301	CEN6/ARS4 ampR *natNT2 pRPL10-coEctyrA$^{M53I\ A354V}$-tGDB1*	pGGKd012, pUD260, pGGKp135, pUD841	This study
pUDC302	CEN6/ARS4 ampR *natNT2 pCWP2-coEctyrB-tGLC3*	pGGKd012, pUD262, pGGKp293, pUD842	This study
pUDC294	CEN6/ARS4 ampR *natNT2 pHHF2-coEcaroB-tTEF2*	pGGKd012, pYTK012, pGGKp120, pGGKp038	This study
pUDC295	CEN6/ARS4 ampR *natNT2 pRPL8A-coEcaroC-tGPD2*	pGGKd012, pUD259, pGGKp125, pUD845	This study
pUDC300	CEN6/ARS4 ampR *natNT2 pRPL18B-coEcpheA^{T326P}-tGSY2*	pGGKd012, pYTK017, pGGKp126, pUD848	This study
pUDC349	CEN6/ARS4 ampR *natNT2 pSePDC1-AtPAL1-tLAT1*	pGGKd012, pGGkp074, pGGKp131, pUD858	This study
pUDC350	CEN6/ARS4 ampR *natNT2 pSeGPM1-coRcTAL1-tCIT1*	pGGKd012, pGGkp095, pGGKp327, pUD826	This study
pUDC352	CEN6/ARS4 ampR *natNT2 pTEF1-coAtCHS3-tMDH1*	pGGKd012, pYTK013, pGGKp325, pUD862	This study
pUDC353	CEN6/ARS4 ampR *natNT2 pSkADH1-AtCHI-tSDH4*	pGGKd012, pGGKp062, pGGKp324, pUD852	This study
pUDC354	CEN6/ARS4 ampR *natNT2 pSeFBA1-coAtC4H-tADH3*	pGGKd012, pGGKp075, pGGKp326, pGGKp113	This study
pUDC355	CEN6/ARS4 ampR *natNT2 pSkTDH3-coAtF3H-tSDH3*	pGGKd012, pGGKp063, pGGKp332, pUD851	This study
pUDC356	CEN6/ARS4 ampR *natNT2 pSePGK1-coGhDFR-tACO1*	pGGKd012, PGGKp330, pGGKp328, pUD819	This study
pUDC357**	CEN6/ARS4 ampR *natNT2 pSeENO2-coAtANS-tFUM1*	pGGKd012, pGGKp331, *coAtANS*	This study

		PCR fragment*, pUD836	
pUDC358	CEN6/ARS4 ampR *natNT2 pSePYK1-coAt3GT-tDIC*	pGGKd012, *coAt3GT* PCR fragment *, pGGKp329, pUD829	This study
pUDC398	CEN6/ARS4 ampR *natNT2 pSeENO2-coAtANS-tFUM1*	pGGKd012, pGGKp331, pGGKp340, pUD836	This study

Table 8H Expression plasmids made by Gibson assembly in this study

Plasmid	Relevant characteristics	Source
pUDC348	CEN6/ARS4 ampR *natNT2 pRPS3-coAtCPR1-tIDH2*	This study
pUDC351	CEN6/ARS4 ampR *natNT2 pSeTPI1-At4CL3-tSDH2*	This study

Part	Source	Primers
	pUDC348	
pRPS3	CEN.PK113-7D genomic DNA	17811 &17812
coAtCPR1	pUDE172	17813 & 17814
tIDH2	pUD832	17815 & 17816
Backbone	pGGKd012	12377 & 12378
	pUDC351	
pSeTPI1	pUDI116	17817, 17818
At4CL3	pUDI065	17819, 17820
tSDH2	pUD850	17821, 17822
Backbone	pGGKd012	12377, 12378

Table 8I Other plasmids

Plasmid	Relevant characteristics	Source
pUDI065	Integration plasmid *LEU2 pTDH3-AtCHI1-tCYC1 pTPI-AtCHS3-tADH pTEF-At4CL3-tTEF PYK2*(1-710)	Koopman, *et al.*[13]
pUDI102	*pSePGK1-mRuby2-tENO2*	Boonekamp, *et al.*[5]
pUDI116	*pSeTPI1-mRuby2-tENO2*	Boonekamp, *et al.*[5]
pUDI129	*pSePYK1-mRuby2-tENO2*	Boonekamp, *et al.*[5]
pUDE172	*CEN6/ARS4, URA3, pTDH3-AtPAL1-tCYC1, pTPI-coC4H-tADH, pPGI-coCPR1-tPGI*	Koopman, *et al.*[13]
pUDE185	2 µm *HIS3 pTDH3-coCHS3-tCYC1*	Koopman, *et al.*[13]
pLM092	CEN6/ARS4, ampR, *HIS3*, 5'*URA3-ACT1*intron[Tess-I-SceI-Tess]-3'*URA3*	Mitchell and Boeke [14]
pUDC191	CEN6/ARS4, ampR, *URA3, pCCW12-mRuby2-tENO1*	Postma, *et al.*[8]
pUDC192	CEN6/ARS4, ampR, *URA3, pTEF2-mTurquoise2-tSSA1*	Postma, *et al.*[8]

Supplementary Table 9 - pROS/pMEL gRNA primers.

gRNA sequence is underlined.

Primer number	Primer name	Sequence (5' to 3')
6008	CAN1_targetRNA FW	GTGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGAT AAATGATCGATACGTTCTCTATGGAGGAGTTTTAGAGCTAGAA ATAGCAAGTTAAAATAAG
9508	TKL2_targetRNA FW	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATA AATGATCTCAAAAACTTAATGAGGAATGTTTTAGAGCTAGAAA TAGCAAGTTAAAATAAG
7231	RV_gnd2_gRNA	GTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTC TAAAACTATGATCTGGCAGCTTCGCGGATCATTTATCTTTCACT GCGGAGAAGTTTCGAACGCCGAAACATGCGCA
7246	ARO10 CRISPR KO seq	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATA AATGATCATTTACAAGTATTCTAAACCGTTTTAGAGCTAGAAAT AGCAAGTTAAAATAAGGCTAGTCCGTTATCAAC
8314	Sc_URA3_2gRNA_primer	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATA AATGATCTTGACTGATTTTTCCATGGAGTTTTAGAGCTAGAAA TAGCAAGTTAAAATAAG
8564	ZWF1_targetRNA FW	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATA AATGATCTTAGATTCAGATCTGTGACTGTTTTAGAGCTAGAAA TAGCAAGTTAAAATAAG
9503	SOL4_targetRNA FW	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATA AATGATCACATTTTTCCACATATTAAGGTTTTAGAGCTAGAAAT AGCAAGTTAAAATAAG
10641	Sphis5_targetRNA FW	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATA AATGATCTTCCAAGCATGCAAACCAAAGTTTTAGAGCTAGAAA TAGCAAGTTAAAATAAGGCTAGTCCGTTATCAAC
10866	X2_targetRNA FW	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATA AATGATCGGCGACTAGGAAGAGAGTAGGTTTTAGAGCTAGAAA TAGCAAGTTAAAATAAG
12034	SPR3_targetRNA FW	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATA AATGATCATGCTTTTATAACGAATAATGTTTTAGAGCTAGAAA TAGCAAGTTAAAATAAGGCTAGTCCGTTATCAAC
12569	NQM1_targetRNA RV	GTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTC TAAAACTCTAGAACAGTTATATGAATGATCATTTATCTTTCACT GCGGAGAAGTTTCGAACGCCGAAACATGCGCA
12911	mTurquoise2_gRNA1_fw	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATA AATGATCTACTGCTGCTGGTATTACCTGTTTTAGAGCTAGAAA TAGCAAGTTAAAATAAG
12912	mTurquoise2_gRNA2_fw	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATA AATGATCCCTTAGTCACTACTTTATCTGTTTTAGAGCTAGAAA TAGCAAGTTAAAATAAG
12985	YPRCtau3_targetRNA FW	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATA AATGATCAAACATTCAAATATATTCCAGTTTTAGAGCTAGAAA TAGCAAGTTAAAATAAG

13614	PDC5_PDC6_targetRNA FW	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATA AATGATCATTGTTGTTGCATCATACCTGTTTTAGAGCTAGAAAT AGCAAGTTAAAATAAG
14756	HIS3_targetRNA2 FW	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATA AATGATCTTAACGTCCACACAGGTATAGTTTTAGAGCTAGAAA TAGCAAGTTAAAATAAG
16889	SOL3_targetRNA FW	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATA AATGATCCTCATGCATTATATTTTGTTGTTTTAGAGCTAGAAAT AGCAAGTTAAAATAAGGCTAGTCCGTTATCAAC
16895	GND1_targetRNA FW	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATA AATGATCTTACGAAGAATTGAAGAAGAGTTTTAGAGCTAGAA ATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAAC
16897	RKI1_targetRNA FW	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATA AATGATCAATGCGAGGATACTGTTCAAGTTTTAGAGCTAGAAA TAGCAAGTTAAAATAAGGCTAGTCCGTTATCAAC
16903	RPE1_targetRNA FW	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATA AATGATCCGACTTGGATATTCAAATGGGTTTTAGAGCTAGAAA TAGCAAGTTAAAATAAGGCTAGTCCGTTATCAAC
16905	TKL1_targetRNA FW	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATA AATGATCTAACCCAGATATTATTTTAGGTTTTAGAGCTAGAAAT AGCAAGTTAAAATAAGGCTAGTCCGTTATCAAC
16909	TAL1_targetRNA FW	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATA AATGATCAACTAACCCATCATTGATCTGTTTTAGAGCTAGAAAT AGCAAGTTAAAATAAGGCTAGTCCGTTATCAAC
17613	RKI1_targetRNA_SNP1_FW	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATA AATGATCATGTTTTGGGGACTTTTTTCGTTTTAGAGCTAGAAAT AGCAAGTTAAAATAAGGCTAGTCCGTTATCAAC
18010	SHR AM_targetRNA _FW	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATA AATGATCACTCGTATCTTACATGACGTGTTTTAGAGCTAGAAA TAGCAAGTTAAAATAAGGCTAGTCCGTTATCAAC
18266	Chunk7BC_targetRNA _FW	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATA AATGATCTTTGCGGAATATCGACCACGGTTTTAGAGCTAGAAA TAGCAAGTTAAAATAAG
18227	Chunk15CD_targetRNA _FW	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATA AATGATCATATAAGTGTCCAGCCAGAGGTTTTAGAGCTAGAAA TAGCAAGTTAAAATAAG
18228	shrN_targetRNA _FW	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATA AATGATCTCTTCGTTAGGACTCAATCGGTTTTAGAGCTAGAAA TAGCAAGTTAAAATAAG
18229	Chunk9CD_targetRNA _FW	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATA AATGATCCAGATCAAAATCCACCAGTGGTTTTAGAGCTAGAAA TAGCAAGTTAAAATAAG

Supplementary Table 10 - Primers to check correct construction of gRNA plasmids

Primer number	Primer name	Sequence (5' to 3')
7257	RV_gnd2_gRNA_check	TATGATCTGGCAGCTTCGCG
9708	TKL2_beta_dg rv	ATTCCTCATTAAGTTTTTGA
9709	SOL4_alpha_dg rv	CTTAATATGTGGAAAAATGT
12684	DG spHIS5 targetRNA	TTTGGTTTGCATGCTTGGAAG
12729	NQM1_pMEL_dg fw	ATTCATATAACTGTTCTAGA
13040	YPRCtau3_pROS_dg rv	CTGGAATATATTTGAATGTTTGAT
13263	PDC5 and 6 gRNA dg	AGGTATGATGCAACAACAATG
13264	ARO10 gRNA DG	CGGTTTAGAATACTTGTAAAT
14602	zwf1 gRNA dg RV	AGTCACAGATCTGAATCTAAG
14757	HIS3_pROS_dg rv	TATACCTGTGTGGACGTTAA
14758	URA3_pROS_dg fw	TCCATGGAAAAATCAGTCAA
15821	X2_pROS_dg rv	CTACTCTCTTCCTAGTCGCC
15968	SPR3_pROS_dg rv	CATTATTCGTTATAAAAGCATGAT
16890	SOL3_pROS_dg rv	AACAAAATATAATGCATGAGGATC
16896	GND1_pROS_dg rv	TCTTCTTCAATTCTTCGTAAGAT
16898	RKI1_pROS_dg rv	TTGAACAGTATCCTCGCATT
16904	RPE1_pROS_dg rv	CCATTTGAATATCCAAGTCGG
16906	TKL1_pROS_dg rv	CTAAAATAATATCTGGGTTAGATCA
16910	TAL1_pROS_dg rv	AGATCAATGATGGGTTAGTTG
17621	RKI1_SNP1_pROS_dg rv	CGAAAAAAGTCCCCAAAACATG
18010	SHR_AM_pROS_dg rv	CACGTCATGTAAGATACGAGTG
18012	CAN1_pROS_dg rv	CTCCTCCATAGAGAACGTATCG
18230	diag_gRNA_chunk7BC rv	CGTGGTCGATATTCCGCAAAG
18231	diag_gRNA_chunk15CD rv	CCTCTGGCTGGACACTTATATG
18232	diag_gRNA_SHR-N rv	CCGATTGAGTCCTAACGAAGAG
18233	diag_gRNA_chunk9CD rv	CCACTGGTGGATTTTGATCTGG

Supplementary Table 11 - Primers to make golden gate part plasmids and expression plasmids with Gibson assembly

Primer number	Primer name	Sequence (5' to 3')
9413	PGK1 se prom fw Ytoolkit	AAGCATCGTCTCATCGGTCTCAAACGGCTTCAATTCAAGATACACAGATATAC
9414	PGK1 se prom rev Ytoolkit	TTATGCCGTCTCAGGTCTCACATATGTTTTATATTTGTTGCAAAAAGTAG
9743	ENO1 se prom fw Ytoolkit	AAGCATCGTCTCATCGGTCTCAAACGCCAAGAAGATGCCGGCTAC
9744	ENO1 se prom rev Ytoolkit	TTATGCCGTCTCAGGTCTCACATATATTATTGTTTGATATAGTATTAGTTGCTTGGT
10610	PYK1 se prom fw Ytoolkit	AAGCATCGTCTCATCGGTCTCAAACGTGTAAATACCGGTTTTAGCC
10611	PYK1 se prom rv Ytoolkit	TTATGCCGTCTCAGGTCTCACATATGTGATGATGTTTTATTTGTTTTG
16292	GG_pRPL25_fw	GCATCGTCTCATCGGTCTCAAACGAGGTATGTTAGTGCTAAAAGCAAAATG
16293	GG_pRPL25_rv	ATGCCGTCTCAGGTCTCACATATTTATCTTATTGATCTTCTTTGTTTAGCCTTTTC
16294	GG_pRPP0_fw	GCATCGTCTCATCGGTCTCAAACGTTCAACAATTCGTTATATATATGGTAGGCT
16295	GG_pRPP0_rv	ATGCCGTCTCAGGTCTCACATATTCAAACTTATTATACGTATTTATTAGACTGTT
16296	GG_pRPL10_fw	GCATCGTCTCATCGGTCTCAAACGTCACTTGTCTGTGTGTTTAACTGCC
16297	GG_pRPL10_rv	ATGCCGTCTCAGGTCTCACATACTTGAATTAGTTATTTGATATACTGTACTT
16298	GG_pRPL8A_fw	GCATCGTCTCATCGGTCTCAAACGACATAAATAATTTCTATTAACAATGTAATTTC
16299	GG_pRPL8A_rv	ATGCCGTCTCAGGTCTCACATATTCGAATTAGTTGTTTTGATGTG
16300	GG_pRPL3_fw	GCATCGTCTCATCGGTCTCAAACGAGAGTCTTGGAGATTTTCGACCTG
16301	GG_pRPL3_rv	ATGCCGTCTCAGGTCTCACATAGATTGATTGTTGTAGTAACTGTGTTGTTC
12377	Backbone pGGKd017 FW	AAATCTGCTCGTCAGTGGTG
12378	Backbone pGGKd017 REV	ATTGCGACGAATTGCCACG
17811	pGGKd012-pRPS3 GA fw	CGACAACGTGGCAATTCGTCGCAATTCTGCTACTTTCCATTATCTGG
17812	coAtCPR1-pRPS3 GA rv	AGCAGAAGTCATTTTTGTAGTTTGTTTGCTGTTTTATTTTC
17813	pRPS3-coAtCPR1 GA fw	ACAAACTACAAAAATGACTTCTGCTTTGTACGC
17814	tIDH2-coAtCPR1 GA rv	AAGAATAGGACTTTTACCAAACGTCTCTCAAGTATC
17815	*ATCPR1*-tIDH2 GA fw	GACGTTTGGTAAAAGTCCTATTCTTTTCCCTCTC

17816	pGGKd012-tIDH2 GA rv	GTGAGCACCACTGACGAGCAGATTTTCCACTGAGGGACATTTTG
17817	pGGKd012-pSeTPI1 GA fw	CGACAACGTGGCAATTCGTCGCAATGGATGTCGTTGTTCTTGTTAC
17818	At4CL3-pSeTPI1 GA rv	TGCAGTGATCATTTTTAGTGTATGTGTATGTGTGTTTG
17819	SeTPI1-At4CL3 GA fw	ACATACACTAAAAATGATCACTGCAGCTCTAC
17820	tSDH2-At4CL3 GA rv	TTTTTCTGATAGTTCAACAAAGCTTAGCTTTGAG
17821	At4CL3-tSDH2 GA fw	AAGCTTTGTTGAACTATCAGAAAAACAGCTAGCC
17822	pGGkd012-tSDH2 GA rv	GTGAGCACCACTGACGAGCAGATTTAAGCCAAAAGGCCCTTCAAAAAC
17823	coAtC4H YTKpart fw	GCATCGTCTCATCGGTCTCATATGGACTTGTTGTTGTTGGAAAAGTC
17824	coAtC4H YTKpart rv	ATGCCGTCTCAGGTCTCAGGATTTAACAGTTTCTTGGCTTCATAACG
17827	coAtCHS3 YTKpart fw	GCATCGTCTCATCGGTCTCATATGGTTATGGCTGGTGCTTCTTC
17828	coAtCHS3 YTKpart rv	ATGCCGTCTCAGGTCTCAGGATTTACAATGGAACAGAGTGCAAAAC
17829	AtCHI1 YTKpart fw	GCATCGTCTCATCGGTCTCATATGATGTCTTCATCCAACGCCTGCG
17830	AtCHI1 YTKpart rv	ATGCCGTCTCAGGTCTCAGGATTCAGTTCTCTTTGGCTAGTTTTTCCTC
17834	CHS4 internal BsaI removal RV	CACGTCTCACCTTAAACCCAACAACTTAGTCAA
17835	CHS4 internal BsaI removal FW	TTCGTCTCTAAGGCCATCTGTTAAGAGATTGATGATGTA
17870	coAtF3H YTKpart fw	GCATCGTCTCATCGGTCTCATATGGCTCCAGGTACTTTGAC
17871	coAtF3H YTKpart rv	ATGCCGTCTCAGGTCTCAGGATTTAAGCGAAGATTTGGTCAACTG
17872	coGhDFR YTKpart fw	GCATCGTCTCATCGGTCTCATATGGAAGAAGACTCTCCAGCTACTGTTTG
17873	coGhDFR YTKpart rv	ATGCCGTCTCAGGTCTCAGGATCTATTGACCTTCCTTAGAACAACACAAC
17874	*coAtANS* YTKpart fw	GCATCGTCTCATCGGTCTCATATGGTTGCTGTTGAAAGAGTTGAATC
17875	*coAtANS* YTKpart rv	ATGCCGTCTCAGGTCTCAGGATTTAGTCGTTCTTTTCAGAAACCAATTC
17876	coAt3GT YTKpart fw	GCATCGTCTCATCGGTCTCATATGACTAAGCCATCTGACCCAACTAGAG
17877	coAt3GT YTKpart rv	ATGCCGTCTCAGGTCTCAGGATTTAGATGATGTTAACAACAGCGTCCAAC

Supplementary Table 12 - Diagnostic primers to check golden gate part plasmids

Primer number	Primer name	Sequence (5’ to 3’)
1642	MF fbas	TTTCCCAGTCACGACGTTG
2012	m132	GGAAACAGCTATGACCATG
2397	FW pMA-RQ	AGACCGAGATAGGGTTGAGTG
4941	I-sceI inside rv n	
5394	TKL1 fw (ol pTHD3)	CGAATAAACACACATAAACAAACAAAATGACTCAATTCACTGACATTGATAAGC
7613	FW_gnd1_inside	GATTGGTTTGGCCGTCATGG
7868	RPE1_F	CAACTTGGGTTGCGAATGTC
7869	RKI1_F	CTTTGGGCAATCCTTTGGAG
7871	TAL1_F	GGTGATTTCGGCTCTATTGC
8953	FW_pADH1_ZWF1	CCAAGCATACAATCAACTATCTCATATACAATGAGTGAAGGCCCCGTCAA
8988	FW_diag_3'_SOL3	TTGGGCTGTGGTCCTGATGG
12611	pUD565_fw1	

Supplementary Table 13 - Diagnostic primers to check golden gate and Gibson assembly expression plasmids, for PCR and Sanger sequencing

Primer number	Primer name	Sequence (5' to 3')
1047	TAL1Fw1	CTGTACACTAGGAAGCCCTGTT
1858	FK050	CGGATGGATGTCTCAAAC
2122	BG26-DF	GCTGCAGTATTGTTCCTGAG
2123	BG26-DR	CCTGTTTGCCTTTCCTTACG
2557	FK117-MP1	GTGGACGCTATGTTATGC
2558	FK118-MP2	AGTCTCACCACCAAGATTC
2559	FK119-MP3	CCAGGTCCAATCCCAATC
2560	FK120-MP4	CAGTGTTCACCGTAAACAG
2561	FK121-MP5	TCTGCTTTGTACGCTTCTG
2562	FK122-MP6	CGTATTGGTCGTCGTCAG
2564	FK124-MP8	GATGAAGAACGCTGTTCC
4494	RPE1 DG fw	TATCCAAGTCGAGCTGGGAAAG
4495	RPE1 DG rv	CCCATGAGTTAGGCACTTACG
5598	TKL1 DG fw	CGTTCCGTTCGCAATCTC
5599	TKL1 DG rv	GGTGTGATTCTCTCGAAGG
5807	DT2	ATGTCTTCATCCAACGCC
6023	CHI knockout cassette rv	CAGTTCTCTTTGGCTAGTTTTTC
8566	FW_zwf1_outside	GGGTGGCGAATTCTTCAATG
10335	ConRE Rv	GGCTGTCTTGCTTAGTTGTG
12220	ABZ1_SNO1_THI4 REV	GTGTGGTTCATGGGTGCGTTAGTCATCGGTATGATCTGTACATG
12612	PAL1_fw2	CAGTTCTCTTTGGCTAGTTTTTC
12614	PAL1_rv1	TCGAATCTAACCGCTTCGAG
12615	PAL1_rv2	ACAAATCGCAACGAGGAACG
13483	ConL_pGGK_fw	TCTCCAGGACCATCTGAATC
13668	pGPM1 s.e dg fw	GAGGGCGGTTCTCATATTTC
14788	nadABhigh_seq2_fw	GCCGATAATTGCAGACGAAC
17634	TDH3p pGGKd017 fw	CTGGCCGATAATTGCAGACG
17636	CYC1t pGGKd017 rv	GATTTCCGTCTCATGCTCAG
17819	SeTPI1-At4CL3 GA fw	ACATACACTAAAAATGATCACTGCAGCTCTAC
17820	tSDH2-At4CL3 GA rv	TTTTTCTGATAGTTCAACAAAGCTTAGCTTTGAG
17948	coAtCHS3 dg fw	TATCGACGGTCACTTGAGAG
17949	coAtCHS3 dg rv	CGTGTCTAGTAGCTCTCATC
17975	coGhDFR dg rv1	AAACCAGCAGCACCAGTAAC
17976	coGhDFR dg rv2	CACAAGTCGTCCAAGTGAAC
17977	coGhDFR dg fw	AGTTGTGGAAGGCTGACTTG
17978	pSkTDH3 dg fw1	CGGACATAACCTCAATGGAGTG
17979	coAtF3H dg rv1	GTCTGGTTGTGGACACTTTG
17980	coAtF3H dg fw1	ACGCTTGTGTTGACATGG

Supplementary Table 14 - List of NeoChr10 and NeoChr11 chromosome parts

* Size of the fragments does not include the SHR sequences.

SHR 5'	part	SHR 3'	Size (bp)*	Template	Primer Fw	Primer Rv
	Telomere left	AO	813	pLM092	13395	13396
AO	Chunk 7A	BJ	2472	*E.coli* BL21/*E.coli* XL1 Blue	10004	11535
BJ	Chunk 7B	BK	2435	*E.coli* BL21/*E.coli* XL1 Blue	11536	11537
BK	Chunk 7C	BL	2478	*E.coli* BL21/*E.coli* XL1 Blue	11538	11539
BL	Chunk 7D	AP	2526	*E.coli* BL21/*E.coli* XL1 Blue	11540	10005
AP	Chunk 8A	BM	2477	*E.coli* BL21/*E.coli* XL1 Blue	10006	11541
BM	Chunk 8B	BN	2567	*E.coli* BL21/*E.coli* XL1 Blue	11542	11543
BN	Chunk 8C	BO	2454	*E.coli* BL21/*E.coli* XL1 Blue	11544	11545
BO	Chunk 8D	AC	2452	*E.coli* BL21/*E.coli* XL1 Blue	11546	10007
AC	*mRuby2*	AD	1667	pUDC191	11365	11366
AD	*ARS1*	AN	56	Annealing of complementary primers	13397	9989
AN	Chunk 18A	BP	2525	*E.coli* BL21/*E.coli* XL1 Blue	13398	13399
BP	Chunk 18B	BQ	2510	*E.coli* BL21/*E.coli* XL1 Blue	13400	13401
BQ	Chunk 19C	BR	2495	*E.coli* BL21/*E.coli* XL1 Blue	13509	13510
BR	Chunk 19D	DE	2496	*E.coli* BL21/*E.coli* XL1 Blue	13511	13512
DE	Chunk 15A	DF	2515	*E.coli* BL21/*E.coli* XL1 Blue	13406	13407
DF	Chunk 15B	DH	2504	*E.coli* BL21/*E.coli* XL1 Blue	13408	13409
DH	Chunk 15C	DI	2489	*E.coli* BL21/*E.coli* XL1 Blue	13410	13411
DI	Chunk 15D	DJ	2497	*E.coli* BL21/*E.coli* XL1 Blue	13412	13413
DJ	*CEN6/ARS4*	AE	519	pLM092	13414	9991
AE	Chunk 16A	DK	2520	*E.coli* BL21/*E.coli* XL1 Blue	13415	13416
DK	Chunk 16B	DL	2470	*E.coli* BL21/*E.coli* XL1 Blue	13417	13418
DL	Chunk 16C	DM	2517	*E.coli* BL21/*E.coli* XL1 Blue	13419	13420
DM	Chunk 16D	DN	2520	*E.coli* BL21/*E.coli* XL1 Blue	13421	13422
DN	Chunk 17A	DO	2499	*E.coli* BL21/*E.coli* XL1 Blue	13423	13424
DO	Chunk 17B	DP	2498	*E.coli* BL21/*E.coli* XL1 Blue	13425	13426
DP	Chunk 19A	DQ	2509	*E.coli* BL21/*E.coli* XL1 Blue	13427	13428
DQ	Chunk 17D	DR	2543	*E.coli* BL21/*E.coli* XL1 Blue	13429	13430
DR	*ARS417*	BU	60	Annealing of complementary primers	13431	11508
BU	*HIS3*	AJ	1250	pLM092	11509	11032
AJ	Chunk 4A	BC	2526	*E.coli* BL21/*E.coli* XL1 Blue	9998	11510
BC	Chunk 4B	BD	2488	*E.coli* BL21/*E.coli* XL1 Blue	11511	11512
BD	Chunk 4C	BE	2470	*E.coli* BL21/*E.coli* XL1 Blue	11513	11514
BE	Chunk 4D	AK	2419	*E.coli* BL21/*E.coli* XL1 Blue	11515	9999
AK	Chunk 9.2A	BF	2405	*E.coli* BL21/*E.coli* XL1 Blue	10008	11516
BF	Chunk 9.2B	BS	2565	*E.coli* BL21/*E.coli* XL1 Blue	11517	11518

BS	Chunk 9.2C	BT	2487	*E.coli* BL21/*E.coli* XL1 Blue	11519	11520
BT	Chunk 9.2D	AQ	2502	*E.coli* BL21/*E.coli* XL1 Blue	11521	11522
AQ	Chunk 5A	BG	2485	*E.coli* BL21/*E.coli* XL1 Blue	10000	11523
BG	Chunk 5B	BH	2521	*E.coli* BL21/*E.coli* XL1 Blue	11524	11525
BH	Chunk 5C	BI	2513	*E.coli* BL21/*E.coli* XL1 Blue	11526	11527
BI	Chunk 5D	AL	2441	*E.coli* BL21/*E.coli* XL1 Blue	11528	10001
AL	*mTurquoise2*	DS	1683	pUDC192	13432	13433
DS	Telomerator		807	pLM092	13434	13435 / 13436

Supplementary Table 15 - List of primers for amplifying NeoChr10 and NeoChr11 chromosome parts

Primers number	Primer Name	Sequence 5' - 3'
9989	ARS1_rv	ATACATCATGCACGCCTGAAAGCATCCCTGACGCGAGTATGACGCAGTTCACACA TCTTACTTGTTATTTTACAGATTTTATGTTTAGATCTTTTATGCTTGCTTTTCAAAA
9991	CEN6_ARS4_ rv	CAACGCATGAGGATGATGACAGCAGCACTCGTACCAGATAGAGACAGCTCTTCC GAACATGGACGGATCGCTTGCCTGTAAC
9998	Ecoli_ch4_fw	CACACGCACGAATTTGCATACAGATAGTTTGAGACACTCGCACGATGGCCGATAT TGCGTCCGCGCATATTCCAGCAAGGAG
9999	Ecoli_ch4_rv	TCAGACAATTCTATACGCGGACTGATATGGCAGAAGCTAGGAGACGTTATGCGAT CTTAGTGGTACGGTTTACTCCTTCACCTGTG
10000	Ecoli_ch5_fw	GCGCAGAAGGCAATGCTATACATCTGATTGAAGCAGCGTCGCGCGTGCATCATTC CTTATCTGGGGAAACTGGCGAGCG
10001	Ecoli_ch5_rv	GCGTCCTCTGTATTAAGATGTCATGGTGTTGAGTCTGCACATGCAATGGCAATGA GTCAGTTAGCCCGCAGTGGATCCTCC
10004	Ecoli_ch7_fw	TCATTGATGCCAGGTACGTGGCTAATCTGAAATCCCAGCAACATGGTTGAAAGCG CGTCAATGGTTAGCTTCCCGCTCATTCTCC
10005	Ecoli_ch7_rv	CTCAAGGCTGTGCTGACGTAGGACTGATTCGAGCTATCTCTTGGTGTATTTGAGC AGACCCAGACATGGCGTAACCCCGTG
10006	Ecoli_ch8_fw	GGTCTGCTCAAATACACCAAGAGATAGCTCGAATCAGTCCTACGTCAGCACAGCC TTGAGGCATTTACGCTGTACGGACACCTT
10007	Ecoli_ch8_rv	GCTACATCTTCCGTACTATGCTGTAGTCTCATGGTCGAGTTCTATTGCTGTTCGGC GGCAGGGCAATATTACCAACCCGTTTTGC
10008	Ecoli_ch9_fw	CTAAGATCGCATAACGTCTCCTAGCTTCTGCCATATCAGTCCGCGTATAGAATTGT CTGACATTACCGGCGTAGCCCCATG
11032	terHIS3_rv	ACGCAATATCGGCCATCGTGCGAGTGTCTCAAACTATCTGTATGCAAATTCGTGC GTGTGGCATCTGTGCGGTATTTCACAC
11365	prCCW12_m Ruby_tENO1 _fw	TGCCGCCGAACAGCAATAGAACTCGACCATGAGACTACAGCATAGTACGGAAGA TGTAGCAACGCACCCATGAACCACAC
11366	prCCW12_m Ruby_tENO1 _rv	CTCCACTGTACTGCATGTAGCATTCGCCGATCTGCATGATGTGTGACATTCTGCTA TCGGGGCAGCATACATGGGTGACCAAA
11508	ARS417_rv	AATCATGTGACCCAGGCTTGCGCATACATGATCCTTCTTGCGCTGCATGGGCGAC TATATCTTACGCTCAATTCCTTTATTTTTTTATATTTATGTAGCTTTTT
11509	BU-His3_fw	ATATAGTCGCCCATGCAGCGCAAGAAGGATCATGTATGCGCAAGCCTGGGTCAC ATGATTTGCGGCATCAGAGCAGATTG
11510	Chunk_4A_rv	CTAGGCTCTGCTGCATGTCAGTGATTTCTATTAGGCAGCGCTTACCCATGATTAGC GCAGCTACGGTCAGTTCGCCTTTC
11511	Chunk_4B_f w	CTGCGCTAATCATGGGTAAGCGCTGCCTAATAGAAATCACTGACATGCAGCAGAG CCTAGGTGTCAGCTTTCGTGGTGTG
11512	Chunk_4B_rv	AGTCACGCTGAGTCCATGCTGACCATGATTCACACTCAGTGCCGATAATTCCATAG TCTGCTCTCCGGCATTGACGGAAC
11513	Chunk_4C_f w	CAGACTATGGAATTATCGGCACTGAGTGTGAATCATGGTCAGCATGGACTCAGCG TGACTGCCAATTTCCGTGTTGTAGG

11514	Chunk_4C_rv	TCAATCATTCGTTCTCGCAGATCTACAATCGTCCTGAGCTCTGTGAGTGATGTACG CTCCCTAACGCGTCAATGCACTCC
11515	Chunk_4D_fw	GGAGCGTACATCACTCACAGAGCTCAGGACGATTGTAGATCTGCGAGAACGAAT GATTGATACCGTCACGCCACGTCCAC
11516	Chunk_9.2A_rv	GCGCGACGTGTCTCGTATATTAGTGAAGTTGGATCTGTCCATGAATCCTCGGCTCT GGTGTGCAAGCGGTATGAGGAAAG
11517	Chunk_9.2B_fw	CACCAGAGCCGAGGATTCATGGACAGATCCAACTTCACTAATATACGAGACACGT CGCGCTATACTGCGGGTAGGAAAGG
11518	Chunk_9.2B_rv	GTTCAGGATTCTGTCGATGCCACATCGAGTCAGTCGTAGTAACATGGAACGCAGT GCATCTCACGTTCTGGTATTGGGTGC
11519	Chunk_9.2C_fw	GATGCACTGCGTTCCATGTTACTACGACTGACTCGATGTGGCATCGACAGAATCC TGAACGCGTCGCTTTACGCCAGGTC
11520	Chunk_9.2C_rv	CAGATACTGGGCAGGCTCTATAGGAGCTTGTACCGCATTGGCTTTGCCACTCATTC GAGAGCTGCCGCCGATGAGATCGC
11521	Chunk_9.2D_fw	TCTCGAATGAGTGGCAAAGCCAATGCGGTACAAGCTCCTATAGAGCCTGCCCAGT ATCTGGACTATCTGCTGACTGAGTTGCTGTTG
11522	Chunk_9.2D_rv	ATAAGGAATGATGCACGCGCGACGCTGCTTCAATCAGATGTATAGCATTGCCTTC TGCGCGGTTGCATACTGTGGCAACTGAC
11523	Chunk_5A_rv	GAGGCTTCACAGTGCTTTATTAGTATGATTGCCTAGCTGGTATATGTGTTCCTGGA GCGCTGTGGATCTGGCGGTTACGG
11524	Chunk_5B_fw	GCGCTCCAGGAACACATATACCAGCTAGGCAATCATACTAATAAAGCACTGTGAA GCCTCGCTGATTACCGCAGCCTGAA
11525	Chunk_5B_rv	AGGATCGCTCGCGTACTCATGCATTCTCCCACATATTGAGGCCCTGATTCCATGCA ATGTGGAAAATCTCCGCCATTCCC
11526	Chunk_5C_fw	ACATTGCATGGAATCAGGGCCTCAATATGTGGGAGAATGCATGAGTACGCGAGC GATCCTATGGCTTACGGCAGCATTGG
11527	Chunk_5C_rv	TCTGTCAGTTGGTTAAGCGCCGCTACGATTACTACACATGCCACAGACTGATCTAC AATGGTACCGCTCTGCACCACAGG
11528	Chunk_5D_fw	CATTGTAGATCAGTCTGTGGCATGTGTAGTAATCGTAGCGGCGCTTAACCAACTG ACAGAACAAAGTACCGCCAGCCAGG
11535	Chunk_7A_rv	GGCGCACATGGTATATTATGATCGGAGATGCGGCAACATAGCTGGGTGTGATCC TCTCTACGCCATCCGTGGGTCTTTTC
11536	Chunk_7B_fw	TAGAGAGGATCACACCCAGCTATGTTGCCGCATCTCCGATCATAATATACCATGT GCGCCTGGGCGTCATTGTCCGGAGT
11537	Chunk_7B_rv	GAGCATACTGTCCTATCATGTCGACTCTTGTCACATCTGACGCCTCTCTGCGATAG GATTTCCGGCGGCAGCCATCAAAG
11538	Chunk_7C_fw	AATCCTATCGCAGAGAGGCGTCAGATGTGACAAGAGTCGACATGATAGGACAGT ATGCTCCCAGGCGGAAGAAGTCTTTGAAGAC
11539	Chunk_7C_rv	CAGCAAGTGCGTAGAGATCAGCATTATCTGACTGTGGATGATCCTACATCGTCAT CAGAGCGCCGCTTCATAAGCGCCAA
11540	Chunk_7D_fw	CTCTGATGACGATGTAGGATCATCCACAGTCAGATAATGCTGATCTCTACGCACTT GCTGAATGGCGATCCCCGAGCAAC
11541	Chunk_8A_rv	ACAATGAGAATCGAGCGCCGCTGCTTAATCTGTCAGTCGATCCTATGGTTGCTGC TGAGCACAGTGCAGCGCGTTTGGTC
11542	Chunk_8B_fw	GCTCAGCAGCAACCATAGGATCGACTGACAGATTAAGCAGCGGCGCTCGATTCTC ATTGTCTGGCGGGTTACTGGCTGTG
11543	Chunk_8B_rv	GGCCGCTGTGTAGTCTCTATGCATGTACTTAGATCCTAGCGCATCTTCGCCAGCTA TATTTGCCGACTTACGCCGTGGTT

11544	Chunk_8C_fw	AATATAGCTGGCGAAGATGCGCTAGGATCTAAGTACATGCATAGAGACTACACAGCGGCCTGATCGGACTGGGCGATCAC
11545	Chunk_8C_rv	GGCATTCGGCGTGATTCCATCATGCTATGCACTGATCTCGCACATAATCTCGGTCGGCTGCGGTATGACCCTGGCGGAAG
11546	Chunk_8D_fw	CAGCCGACCGAGATTATGTGCGAGATCAGTGCATAGCATGATGGAATCACGCCGAATGCCTTTCAGCGTGCTCTGTTTACCC
13395	Telomerator_l_fw	AGGGTAATCACCCACCACAC
13396	Telomerator_l_rv	TGACGCGCTTTCAACCATGTTGCTGGGATTTCAGATTAGCCACGTACCTGGCATCAATGACCAAAGCTGGAGCTCCACCG
13397	ARS1-AD_fw	CCGATAGCAGAATGTCACACATCATGCAGATCGGCGAATGCTACATGCAGTACAGTGGAGGGCCTTTTGAAAAGCAAGCATAAAAGATCTAAAC
13398	Chunk_18A_fw	TAAGATGTGTGAACTGCGTCATACTCGCGTCAGGGATGCTTTCAGGCGTGCATGATGTAT
13399	Chunk_18A_rv	GAGATGACTGGGTCCACTCTTTCGTGTATTTCGAGAGAGCGATACGCATGTCTCCATCGTGCTAACTGTCACCCAACATAC
13400	Chunk_18B_fw	ACGATGGAGACATGCGTATCGCTCTCTCGAAATACACGAAAGAGTGGACCCAGTCATCTCGATCCGCAAGTTCTTCATCG
13401	Chunk_18B_rv	CAGATCAGTGTCAATGAAGGTAGGCTGCTTGGCAATGCTTCTGGTGACTGGTAGATCATCGCGATGTGCAATGTTCTTTGTTAC
13406	Chunk_15A_fw	TCCTCGACGCGATGGCATATCCAGTGTGATAACGTATGAGAAGGTACTGGAAGCTACTGCTGCGAGCTGAATGCCATGAC
13407	Chunk_15A_rv	GATGAACGTGCCTTCGATTTATAGAAACTGCGCTGCCCTGTGATGAATTGTCTTAGCGCGAAAGCGGCAGGTTGAGGTCC
13408	Chunk_15B_fw	CGCGCTAAGACAATTCATCACAGGGCAGCGCAGTTTCTATAAATCGAAGGCACGTTCATCCTGCGACCACGCAGTTTGAG
13409	Chunk_15B_rv	CGTGCCGGTTAATGAGCTATGCGTGTCATGTATCCTTAGGCATATCCTTAACACGCAGTGCGCCTTTGGCATGATCGAACAG
13410	Chunk_15C_fw	CACTGCGTGTTAAGGATATGCCTAAGGATACATGACACGCATAGCTCATTAACCGGCACGAACCGGCAGGTTATAGCTGATG
13411	Chunk_15C_rv	CGGGTCATTAGAGATAGTCTCTCAGGATTCAACTAGATGGTGATCTATTGTCTACGCGGCATGGCCCATATACACTTCGAGCAC
13412	Chunk_15D_fw	GCCGCGTAGACAATAGATCACCATCTAGTTGAATCCTGAGAGACTATCTCTAATGACCCGATGCGTGAATGGCTGGCAGAG
13413	Chunk_15D_rv	CGCTGACCTGTCTAACGTATCAACAGAATGCACGTCAGTCGTATGCTTGACGTGTCTGCCCAGTATCAACCACCGGGTAAC
13414	CEN6_ARS4_DJ_fw	GGCAGACACGTCAAGCATACGACTGACGTGCATTCTGTTGATACGTTAGACAGGTCAGCGGGTCCTTTTCATCACGTGCTATAAAAATAATTATAATTTAAATTTTTTAATATAAATATA
13415	Chunk_16A_fw	ATGTTCGGAAGAGCTGTCTCTATCTGGTACGAGTGCTGCTGTCATCATCCTCATGCGTTGTGATGCGCGATGCTTATCAGG
13416	Chunk_16A_rv	CGCCGCTCTTAGAAGGCTATACGAGCTATGAGAGAGACTCGCTATCCATTCCGCTGAGTTCGTCAGCGATGAGACGTTAC
13417	Chunk_16B_fw	AACTCAGCGGAATGGATAGCGAGTCTCTCTCATAGCTCGTATAGCCTTCTAAGAGCGGCGGCATCGGTGAACAGGGTGCTAAG
13418	Chunk_16B_rv	CGCAAATGTCCCATCGTATTTCAGAACCTTGTCACTCATGCGAGCAAGTGTGACAGCTATATGGCGTTCTCCGCCCAGTATG

13419	Chunk_16C_fw	ATAGCTGTCACACTTGCTCGCATGAGTGACAAGGTTCTGAAATACGATGGGACATTTGCGCCGGCGCAGATCACTTTCATAG
13420	Chunk_16C_rv	CGACAAAGTGCTCGTCACGTGCGTCAGCTTCGAGGCATATCAAGCACCTGCCGGATGATTATGCCCGTGAATGGCAAAGCG
13421	Chunk_16D_fw	AATCATCCGGCAGGTGCTTGATATGCCTCGAAGCTGACGCACGTGACGAGCACTTTGTCGAACACCGGACGGCCTTTGCTAC
13422	Chunk_16D_rv	CCTCCGCTGCGTAGAGTAATCCTGGCTCTCGCGTGTATATTGATAGATTGTCTGTCAGGCTCGCGCAGGTATGGTTCAGG
13423	Chunk 17A_fw	GCCTGACAGACAATCTATCAATATACACGCGAGAGCCAGGATTACTCTACGCAGCGGAGGTACGCAGTTTATCGGCCAGTTG
13424	Chunk 17A_rv	CAACCACCTGACTAGAGTGTCAAAGCGTGCTCCTACATAGGTAGAGTTGCATAATCTGGCAAATCGCTGAAGCGTTCC
13425	Chunk 17B_fw	GCCAGATTATGCAACTCTACCTATGTAGGAGCACGCTTTGACACTCTAGTCAGGTGGTTGTTGATAATCGCGGATGGACG
13426	Chunk 17B_rv	ATTCAGCGGGTGATCCGACTTGACTACATTTAGGTGTGGCCTCCTTACTACTCTGAGATGCATCCGGTGAAAGCGTACCC
13427	Chunk 19A_fw	CATCTCAGAGTAGTAAGGAGGCCACACCTAAATGTAGTCAAGTCGGATCACCCGCTGAATGCGATGGTCATTATTTACGGTAG
13428	Chunk 19A_rv	ATGCCGGTGGCCGAATCTATGGTCCACATTATTTGCTGCACAAGATAGTGCAGTAGCGTTCCATTATTGGCAGGATACTTTGAG
13429	Chunk 17D_fw	AACGCTACTGCACTATCTTGTGCAGCAAATAATGTGGACCATAGATTCGGCCACCGGCATTGGGTGTTTATGCCCGGACTAGC
13430	Chunk 17D_rv	ATCGACGGTCCTCGCAAGATCTCAATGTGCAGTGGTATGCTGATAACTTGTGCCTGTGGCGGGATTTAGATCCCACATTAACG
13431	ARS417_DR_fw	GCCACAGGCACAAGTTATCAGCATACCACTGCACATTGAGATCTTGCGAGGACCGTCGATACAAGTCCTTAAGAATATACAAAAAGCTACATAAATATAA
13432	Turquoise_AL_rv	CTGACTCATTGCCATTGCATGTGCAGACTCAACACCATGACATCTTAATACAGAGGACGCCGTCTCATTGGCAGCATAAA
13433	Turquoise_DS_fw	ATCAAGACTGAGGAGTACGTCAGGTTGCAGAGGATCACTTGTAATGAATGTGTGCTCGCTGAACGTTGATAGGTCAAGATCAATG
13434	Telomerator_r_fw	AGCGAGCACACATTCATTACAAGTGATCCTCTGCAACCTGACGTACTCCTCAGTCTTGATCCGGGGGATCCGGTGATTG
13435	Telomerator_r_rv	GTTATCCCTACCCACACAC
13436	rm_Telomerator_r_rv	GTTATCCCTACCCACACACCCACACACCCCAACACACCCACACACCACACACTCGAGCAATTGGGACCGTGCAATTC
13509	Chunk 19C_fw	GATGATCTACCAGTCACCAGAAGCATTGCCAAGCAGCCTACCTTCATTGACACTGATCTGGGTGCTGCAGTTGACCAGAC
13510	Chunk 19C_rv	AGCTGGCGTCGCGCATAAATGCATGATCTGTCCTGGCTGACACGCATCTGCACTAATGATGCGGCAAGAGAATTGGTTAG
13511	Chunk 19D_fw	ATCATTAGTGCAGATGCGTGTCAGCCAGGACAGATCATGCATTTATGCGCGACGCCAGCTCTGTCGTCCATGCCGGATAC
13512	Chunk 19D_rv	GCAGTAGCTTCCAGTACCTTCTCATACGTTATCACACTGGATATGCCATCGCGTCGAGGAAATCACGCGGAAATAGCTGG

Supplementary Table 16 - Primers for of *amdSYM* deletion

Primer number	Primer name	Sequence (5' to 3')
gRNA construction		
6005	p426 CRISP rv	GATCATTTATCTTTCACTGCGGAGAAG
11588	targetAmdS FW	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATAAATGATCAT CACATCCGAACATAAACAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG GCTAGTCCGTTATCAAC
11589	targetAmdS RV	GTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACTGT TTATGTTCGGATGTGATGATCATTTATCTTTCACTGCGGAGAAGTTTCGAAC GCCGAAACATGCGCA
Primers to make repair fragment		
11590	Repair AmdS FW	AAGATAGTCGCCGAACTCGCAAGAGTCATTAACACCTCGCAATTGATGGGA AGTCCTCGCATATGACCTGAACCGACGGCAAATGCTCTTCAACTACGGCATA CTTGCGGAAGCTACGGC
11591	Repair AmdS RV	GCCGTAGCTTCCGCAAGTATGCCGTAGTTGAAGAGCATTTGCCGTCGGTTC AGGTCATATGCGAGGACTTCCCATCAATTGCGAGGTGTTAATGACTCTTGC GAGTTCGGCGACTATCTT
Diagnostic primers to check gene deletion		
2433	K glycolysis Fw	GACGCCATTTGGAACGAAAAAAAG
3366	L glycolysis Rv	AATGAGTGGTAATTAATGGTGACATGAC

Supplementary Table 17 - Primers for deletion of *GND2, NQM1, SOL4* and *TKL2*

Primer number	Primer name	Sequence (5' to 3')
Primers to make repair fragments		
7299	Gnd2_repair_FW_new	AAGAATTCGTAGGTGCAGGTGAGCATATTGCCGGATAAGTGT AGTTACGCAACTACAATTGTTACTAAGGCCCAATCCGGTTGGA GAAGAACTATTGCCCTTGCTGCTACTTACGGTATT
7300	Gnd2_repair_RV_new	AATACCGTAAGTAGCAGCAAGGGCAATAGTTCTTCTCCAACCG GATTGGGCCTTAGTAACAATTGTAGTTGCGTAACTACACTTATC CGGCAATATGCTCACCTGCACCTACGAATTCTT
9504	SOL4_repair oligo fw	CAGCAGTTTTCCAAACAAAGAATGCCATTCATCAAATAATCCAC AACCACCTCAAGAAAATTACACTCGTCTTTATACGAAACTGGCT CCGTTAATCACGACAGACAACCTTAATTACAT
9505	SOL4_repair oligo rv	ATGTAATTAAGGTTGTCTGTCGTGATTAACGGAGCCAGTTTCG TATAAAGACGAGTGTAATTTTCTTGAGGTGGTTGTGGATTATTT GATGAATGGCATTCTTTGTTTGGAAAACTGCTG
9509	TKL2_repair oligo fw	TTGTTGGGAGGAGTCCTGAATAAGGAGTGTCGAATATAGGGA GCTTCATTCGTTGTCAAGGAAGTAAACAGTTCTTTGCTATTTCA CACTTCCTGGTTGATGGTCACTTGCTGCCTGAAA
9510	TKL2_repair oligo rv	TTTCAGGCAGCAAGTGACCATCAACCAGGAAGTGTGAAATAG CAAAGAACTGTTTACTTCCTTGACAACGAATGAAGCTCCCTATA TTCGACACTCCTTATTCAGGACTCCTCCCAACAA

12570	NQM1_repair oligo fw	TTCTTGCTAGCGTAAGTCATAAAAAATAGGAAATAATCACATATATAC AAGAAATTAAATTCATTAAGAGTAGAGGTACCTACTTATATATATAAA TATATATATACCACTTTCCTTTTC
12571	NQM1_repair oligo rv	GAAAAGGAAAGTGGTATATATATATTTATATATATAAGTAGGTACCTC TACTCTTAATGAATTTAATTTCTTGTATATATGTGATTATTTCCTATTTT TTATGACTTACGCTAGCAAGAA
Diagnostic primers to check gene deletion		
7258	FW_gnd2KO_check	TCTGACAGGTGGCAGTTTCC
7259	RV_gnd2KO_check	ATCCGAAAGGCGGCAATAGG
9506	SOL4_dg fw	GGGTGGACGTTTAAGCATAC
9507	SOL4_dg rv	GTATCACCGGGTGAGCTATG
1360	TKL2 dis500 fw	TCTTAATGGTGGCTCGCTGTC
1361	TKL2 dis500 rv	TCAATGCAGCCCATACACTC
12572	NQM1_dg fw	CCTTGATCTGGCTCTGGCTC
12573	NQM1_dg rv	CGCAAGGTAATTACGCCACG

Supplementary Table 18 - Primers for deletion of *ura3, his3* and *SpHIS5*

Primer number	**Primer name**	**Sequence (5' to 3')**
Primers to make repair fragments		
10521	HIS3_repair oligo fw	AATGTGATTTCTTCGAAGAATATACTAAAAAATGAGCAGGCAAGATA AACGAAGGCAAAGTGACACCGATTATTTAAAGCTGCAGCATACGATA TATATACATGTGTATATATGTATACC
10522	HIS3_repair oligo rv	GGTATACATATATACACATGTATATATATCGTATGCTGCAGCTTTAAAT AATCGGTGTCACTTTGCCTTCGTTTATCTTGCCTGCTCATTTTTTAGTAT ATTCTTCGAAGAAATCACATT
12685	spHIS5 Repair oligo FW	GCCCAACTCAGCTTCCGTAAACCACAACACCACCACTAATACAACTCT ATCATACACAAGTCTTTTTACATTTTTTTGGTTTGTGTACGTATCCCACC GTACTTACCATCTTCTCTCCTT
12686	spHIS5 Repair oligo RV	AAGGAGAGAAGATGGTAAGTACGGTGGGATACGTACACAAACCAAA AAAATGTAAAAAGACTTGTGTATGATAGAGTTGTATTAGTGGTGGTG TTGTGGTTTACGGAAGCTGAGTTGGGC
13807	URA3repair_FW	CGGTTTCCTTGAAATTTTTTTGATTCGGTAATCTCCGAACAGAAGGAA GAACGAAGGAAGGGAATCTCGGTCGTAATGATTTCTATAATGACGAA AAAAAAAAAATTGGAAAGAAAAAGC
13808	URA3repair_RV	GCTTTTTCTTTCCAATTTTTTTTTTTTCGTCATTATAGAAATCATTACGA CCGAGATTCCCTTCCTTCGTTCTTCCTTCTGTTCGGAGATTACCGAATC AAAAAAATTTCAAGGAAACCG
Diagnostic primers to check gene deletion		
111	URA3 CTRL RV	TATACGCCAGTACACCTTATCGGC
1026	HIS3 outside fw	CGCTTTGTCTTCATTCAACGTTTCC
1024	HIS3 outside rv	CCACTTGCCACCTATCAC
1460	GLK1FW1	CGCGCCCATATAAATATCC
1461	GLK1RV1	CCCGTTTCCGATGATATTG
1521	URA3 Ctrl Fw2	GCTACTGCGCCAATTGATGAC

Supplementary Table 19 - Primers for deletion of *ARO10*

Primer number	Primer name	Sequence (5' to 3')
Primers to make repair fragments		
7247	ARO10 CRISPR repair upper	ACAAGTTGACGCGACTTCTGTAAAGTTTATTTACAAGATAACAAAGAA ACTCCCTTAAGCAAACTTGTGGGCGCAATTATAAAACACTGCTACCAA TTGTTCGTTTTCTGTTCATTAACA
7248	ARO10 CRISPR repair lower	TGTTAATGAACAGAAAACGAACAATTGGTAGCAGTGTTTTATAATTGC GCCCACAAGTTTGCTTAAGGGAGTTTCTTTGTTATCTTGTAAATAAACT TTACAGAAGTCGCGTCAACTTGT
Diagnostic primers to check gene deletion		
2359	Aro10 KO CHK for	TGCTTGTACACCTCATGTAG
2360	Aro 10 KO chk rev	GCAGACATTTAGCAGATGTAG

Supplementary Table 20 - List of NeoChr25 (linear) and NeoChr26 (circular) chromosome parts

[#]The +, - or 0 signifies the orientation of the part with respect to the neochromosome
* Size of the fragments does not include the SHR sequences.

SHR Fw	Component[#]	SHR Rv	Size*	Template	Primer Fw	Primer Rv
Unique to NeoChr25						
	Left TeSS (0)	BJ	813	pLM092	13395	16577
Common to Neochr25 and NeoChr26						
BJ	chunk 7BC (0)	BL	4909	*E.coli* (migula) Castellani and Chalmers (ATCC 47076)	11536	11539
BL	ARS1 (0)	AN	56	Annealing of complementary primers	16578	9989
AN	ZWF1 (+)	BP	2623	pUDC275	16526	16527
BP	TKL1 (+)	DE	3148	pUDC276	16528	16529
DE	GND1 (+)	BQ	2575	pUDC277	16530	16531
BQ	RKI1 (-)	BR	1882	pUDC278	16532	16533
BR	TAL1 (-)	AL	2113	pUDC279	16534	16535
AL	mTurqouise2 (-)	DS	1683	pUDC192	13432	13433
DS	RPE1 (-)	DF	1822	pUDC280	16536	16537
DF	SOL3 (-)	DI	1855	pUDC281	16538	16539
DI	ARS417 (0)	BE	64	Annealing of complementary primers	16579	16580
BE	aroG^{fbr} (+)	DK	2089	pUDC298	16540	16541
DK	aroB (+)	AC	2104	pUDC294	16542	16543
AC	mRuby2 (+)	AD	1667	pUDC191	11365	11366
AD	aroD (+)	DM	1931	pUDC296	16544	16545
DM	aroE (+)	DN	1860	pUDC297	16546	16547
DN	aroL (+)	DO	1534	pUDC299	16548	16549
DO	aroA (+)	DP	2718	pUDC293	16550	16551

DP	aroC (-)	DQ	1746	pUDC295	16552	16553
DQ	$tyrA^{fbr}$ (-)	DR	1874	pUDC301	16554	16555
DR	$pheA^{fbr}$ (-)	AJ	2171	pUDC300	16556	16557
AJ	tyrB (-)	DH	2499	pUDC302	16558	16559
DH	chunk 15CD (0)	DJ	4996	*E.coli* (migula) Castellani and Chalmers (ATCC 47076)	13410	13413
DJ	CEN6/ARS4 (0)	AE	519		13414	9991
AE	chunk 16AB (0)	DL	5019	*E.coli* (migula) Castellani and Chalmers (ATCC 47076)	13415	16560
DL	FBA1 (+)	H	2185	pUDC212	16561	16562
H	TPI1 (+)	P	1856	pUDC222	12957	12958
P	PGK1 (+)	Q	2356	pUDC219	16563	16564
Q	ADH1 (+)	N	2152	pUDC229	16565	16566
N	PYK1 (+)	O	2612	pUDC220	12963	12964
O	TDH3 (+)	A	2104	pUDC230	16567	16568
A	ENO2 (+)	B	2423	pUDC231	12967	13227
B	HXK2 (-)	C	2566	pUDC214	16569	16570
C	PGI1 (-)	D	2774	pUDC232	12972	12971
D	PFK1 (-)	J	4069	pUDC216	16571	16572
J	PFK2 (-)	BU	3989	pUDC217	12976	13900
BU	HIS3 (-)	L	1254	pLM092	16573	16574
L	GPM1 (-)	M	1853	pUDC213	12980	12979
M	PDC1 (-)	AR	2797	pUDC215	16575	16576
AR	ARS1211 (0)	BS	251	*S. cerevisiae* CEN.PK113-7D	12983	13901
BS	chunk 9CD (0)	AQ	4994	*E.coli* (migula) Castellani and Chalmers (ATCC 47076)	11519	11522
Unique to NeoChr25						
AQ	right TeSS (0)		807	pLM092	16581	13435
Unique to NeoChr26						
AQ	telomerator (+)	BJ	1620	pLM092	16581	16577

Supplementary Table 21 - List of primers for amplifying NeoChr25 and NeoChr26 chromosome parts

Primer Number	Primer Name	Sequence (5' to 3')
13395	Telomerator_l_fw	AGGGTAATCACCCACCACAC
16577	BJ_tel_le _RV	GGCGCACATGGTATATTATGATCGGAGATGCGGCAACATAGCTGGGTGTG ATCCTCTCTACCAAAGCTGGAGCTCCACCG
11536	Chunk_7B_fw	TAGAGAGGATCACACCCAGCTATGTTGCCGCATCTCCGATCATAATATACC ATGTGCGCCTGGGCGTCATTGTCCGGAGT
11539	Chunk_7C_rv	CAGCAAGTGCGTAGAGATCAGCATTATCTGACTGTGGATGATCCTACATC GTCATCAGAGCGCCGCTTCATAAGCGCCAA
16578	BL_ARS1_FW	CTCTGATGACGATGTAGGATCATCCACAGTCAGATAATGCTGATCTCTACG CACTTGCTGGGCCTTTTGAAAAGCAAGCATAAAAGATCTAAAC
9989	ARS1_rv	ATACATCATGCACGCCTGAAAGCATCCCTGACGCGAGTATGACGCAGTTC ACACATCTTACTTGTTATTTTACAGATTTTATGTTTAGATCTTTTATGCTTGC TTTTCAAAA
16526	AN_pZWF1_FW	TAAGATGTGTGAACTGCGTCATACTCGCGTCAGGGATGCTTTCAGGCGTG CATGATGTATGTCCGGGTGCATGCATGAA
16527	BP_tZWF1_RV	GAGATGACTGGGTCCACTCTTTCGTGTATTTCGAGAGAGCGATACGCATG TCTCCATCGTATATATTCAATTCATATTTTATCTCTTTTT
16528	BP_pTKL1_FW	ACGATGGAGACATGCGTATCGCTCTCTCGAAATACACGAAAGAGTGGACC CAGTCATCTCTAAGCGCTTTTTTTTTTTTT
16529	DE_tTKL1_RV	GCAGTAGCTTCCAGTACCTTCTCATACGTTATCACACTGGATATGCCATCGC GTCGAGGAATATTCTTTATTGGCTTTATACTTG
16530	DE_pGND1_FW	TCCTCGACGCGATGGCATATCCAGTGTGATAACGTATGAGAAGGTACTGG AAGCTACTGCTCACTTCCGCCGCAAACAATAC
16531	BQ_tGND1_RV	CAGATCAGTGTCAATGAAGGTAGGCTGCTTGGCAATGCTTCTGGTGACTG GTAGATCATCCTACTCTACTTCTATCATGATAATAGGCAC
16532	BQ_tRKI1_FW	GATGATCTACCAGTCACCAGAAGCATTGCCAAGCAGCCTACCTTCATTGAC ACTGATCTGCTTGGTGTGTCATCGGTAGTAACG
16533	BR_pRKI1_RV	AGCTGGCGTCGCGCATAAATGCATGATCTGTCCTGGCTGACACGCATCTGC ACTAATGATTTGCGAACGCATTAAGTTGAG
16534	BR_tTAL1_FW	ATCATTAGTGCAGATGCGTGTCAGCCAGGACAGATCATGCATTTATGCGC GACGCCAGCTGACGTTGATTTAAGGTGGTTC
16535	AL_pTAL1_RV	GCGTCCTCTGTATTAAGATGTCATGGTGTTGAGTCTGCACATGCAATGGCA ATGAGTCAGGAAAAGCTAGAAAAGGAATTAGAC
13433	Turquoise_DS_fw	ATCAAGACTGAGGAGTACGTCAGGTTGCAGAGGATCACTTGTAATGAATG TGTGCTCGCTGAACGTTGATAGGTCAAGATCAATG
13432	Turquoise_AL_rv	CTGACTCATTGCCATTGCATGTGCAGACTCAACACCATGACATCTTAATAC AGAGGACGCCGTCTCATTGGCAGCATAAA
16536	DS_tRPE1_FW	AGCGAGCACACATTCATTACAAGTGATCCTCTGCAACCTGACGTACTCCTC AGTCTTGATAAATGGATATTGATCTAGATGGCGG

16537	DF_pRPE1_RV	GATGAACGTGCCTTCGATTTATAGAAACTGCGCTGCCCTGTGATGAATTGT CTTAGCGCGACCACTTGACAACGGTCTTG
16538	DF_tSOL3_FW	CGCGCTAAGACAATTCATCACAGGGCAGCGCAGTTTCTATAAATCGAAGG CACGTTCATCAGGATGCACTCTACAAATAC
16539	DI_pSOL3_RV	CGGGTCATTAGAGATAGTCTCTCAGGATTCAACTAGATGGTGATCTATTGT CTACGCGGCCTGACTGCAATAGGAAACTG
16579	DI_ARS417_FW	GCCGCGTAGACAATAGATCACCATCTAGTTGAATCCTGAGAGACTATCTCT AATGACCCGACAAGTCCTTAAGAATATACAAAAAGCTACATAAATATAA
16580	BE_ARS417_RV	TCAATCATTCGTTCTCGCAGATCTACAATCGTCCTGAGCTCTGTGAGTGAT GTACGCTCCCTTACGCTCAATTCCTTTATTTTTTTATATTTATGTAGCTTTTT
16540	BE_pHHF1_FW	GGAGCGTACATCACTCACAGAGCTCAGGACGATTGTAGATCTGCGAGAAC GAATGATTGATCTTGGGGCCTTACCACC
16541	DK_tTEF1_RV	CGCCGCTCTTAGAAGGCTATACGAGCTATGAGAGAGACTCGCTATCCATTC CGCTGAGTTGGTATCACCATAGATTTCGAAAC
16542	DK_pHHF2_FW	AACTCAGCGGAATGGATAGCGAGTCTCTCTCATAGCTCGTATAGCCTTCTA AGAGCGGCGTGTGGAGTGTTTGCTTGG
16543	AC_tTEF2_RV	GCTACATCTTCCGTACTATGCTGTAGTCTCATGGTCGAGTTCTATTGCTGTT CGGCGGCAAGGAAACGTAAATTACAAGG
11365	prCCW12_mRuby _tENO1_fw	TGCCGCCGAACAGCAATAGAACTCGACCATGAGACTACAGCATAGTACGG AAGATGTAGCAACGCACCCATGAACCACAC
11366	prCCW12_mRuby _tENO1_rv	CTCCACTGTACTGCATGTAGCATTCGCCGATCTGCATGATGTGTGACATTC TGCTATCGGGGCAGCATACATGGGTGACCAAA
16544	AD_pRPL25_FW	CCGATAGCAGAATGTCACACATCATGCAGATCGGCGAATGCTACATGCAG TACAGTGGAGAGGTATGTTAGTGCTAAAAG
16545	DM_tGPH1_RV	CGACAAAGTGCTCGTCACGTGCGTCAGCTTCGAGGCATATCAAGCACCTG CCGGATGATTAAACGTCAGTACATCCTACC
16546	DM_pRPP0_FW	AATCATCCGGCAGGTGCTTGATATGCCTCGAAGCTGACGCACGTGACGAG CACTTTGTCGTTCAACAATTCGTTATATATATGGTAGGCT
16547	DN_tCYC1_RV	CCTCCGCTGCGTAGAGTAATCCTGGCTCTCGCGTGTATATTGATAGATTGT CTGTCAGGCAAGCTTGTCCCAAAACCTTCTC
16548	DN_pHTB2_FW	GCCTGACAGACAATCTATCAATATACACGCGAGAGCCAGGATTACTCTAC GCAGCGGAGGTATATATTAAATTTGCTCTTGTTCTG
16549	DO_tPGM2_RV	CAACCACCTGACTAGAGTGTCAAAGCGTGCTCCTACATAGGTAGAGTTGC ATAATCTGGCAACTCGGGGTAGGTAATC
16550	DO_pRPL3_FW	GCCAGATTATGCAACTCTACCTATGTAGGAGCACGCTTTGACACTCTAGTC AGGTGGTTGAGAGTCTTGGAGATTTTCGACCTG
16551	DP_tSOL4_RV	ATTCAGCGGGTGATCCGACTTGACTACATTTAGGTGTGGCCTCCTTACTAC TCTGAGATGAGTCATAGCATTAAGATTAACGCGTTG
16552	DP_tGPD2_FW	CATCTCAGAGTAGTAAGGAGGCCACACCTAAATGTAGTCAAGTCGGATCA CCCGCTGAATTTAAGGGCTATAGATAACAG

16553	DQ_pRPL8A_RV	ATGCCGGTGGCCGAATCTATGGTCCACATTATTTGCTGCACAAGATAGTGC AGTAGCGTTAACGACATAAATAATTTCTATTAAC
16554	DQ_tGDB1_FW	AACGCTACTGCACTATCTTGTGCAGCAAATAATGTGGACCATAGATTCGGC CACCGGCATCAAATACGTACGTGGCAACCCTTTC
16555	DR_pRPL10_RV	ATCGACGGTCCTCGCAAGATCTCAATGTGCAGTGGTATGCTGATAACTTGT GCCTGTGGCTCACTTGTCTGTGTGTTTAACTGCC
16556	DR_tGSY2_FW	GCCACAGGCACAAGTTATCAGCATACCACTGCACATTGAGATCTTGCGAG GACCGTCGATGTATGACTATATGTTGATAACTG
16557	AJ_pRPL18A_RV	ACGCAATATCGGCCATCGTGCGAGTGTCTCAAACTATCTGTATGCAAATTC GTGCGTGTGAAGAGGATGTCCAATATTTTTTT
16558	AJ_tGLC3_FW	CACACGCACGAATTTGCATACAGATAGTTTGAGACACTCGCACGATGGCC GATATTGCGTTAGGTTAAACCTTGGAAGAG
16559	DH_pCWP2_RV	CGTGCCGGTTAATGAGCTATGCGTGTCATGTATCCTTAGGCATATCCTTAA CACGCAGTGCTAATAGACAAGGTGCTATGAG
13410	Chunk_15C_fw	CACTGCGTGTTAAGGATATGCCTAAGGATACATGACACGCATAGCTCATTA ACCGGCACGAACCGGCAGGTTATAGCTGATG
13413	Chunk_15D_rv	CGCTGACCTGTCTAACGTATCAACAGAATGCACGTCAGTCGTATGCTTGAC GTGTCTGCCCAGTATCAACCACCGGGTAAC
13414	CEN6_ARS4_DJ_fw	GGCAGACACGTCAAGCATACGACTGACGTGCATTCTGTTGATACGTTAGA CAGGTCAGCGGGTCCTTTTCATCACGTGCTATAAAAATAATTATAATTTAA ATTTTTTAATATAAATATA
9991	CEN6_ARS4_rv	CAACGCATGAGGATGATGACAGCAGCACTCGTACCAGATAGAGACAGCTC TTCCGAACATGGACGGATCGCTTGCCTGTAAC
13415	Chunk_16A_fw	ATGTTCGGAAGAGCTGTCTCTATCTGGTACGAGTGCTGCTGTCATCATCCT CATGCGTTGTGATGCGCGATGCTTATCAGG
16560	DL_Chunk 16B_rv	CGCAAATGTCCCATCGTATTTCAGAACCTTGTCACTCATGCGAGCAAGTGT GACAGCTATATGGCGTTCTCCGCCCGGTATG
16561	DL_pFBA1_FW	ATAGCTGTCACACTTGCTCGCATGAGTGACAAGGTTCTGAAATACGATGG GACATTTGCGTGAACAACAATACCAGCCTTCC
16562	H_tFBA1_RV	GTCACGGGTTCTCAGCAATTCGAGCTATTACCGATGATGGCTGAGGCGTT AGAGTAATCTAATGAGCTATCAAAAACGATAGATC
12957	TPI FW + H	AGATTACTCTAACGCCTCAGCCATCATCGGTAATAGCTCGAATTGCTGAGA ACCCGTGACAACGAAGACCCAGAGATGTTGTTGT
12958	TPI Rv + P	CTGATAGTGCTGTAAGTCGCCTCCATCTTAGCAGAGCTGTCCCTGAATGCG TACTCGTGATGAGTAACCCATATAGAGATCGTAC
16563	P_pPGK1_FW	TCACGAGTACGCATTCAGGGACAGCTCTGCTAAGATGGAGGCGACTTACA GCACTATCAGTCTTTTTATTAACCTTAATTTTTAT
16564	Q_tPGK1_RV	GAGCTGAATGTATATGCTGCGGGATCATTGCACAGCTCTGAGAGCCCTGC AACGCGATATAAATAATATCCTTCTCGAAAGC

16565	Q_pADH1_FW	ATATCGCGTTGCAGGGCTCTCAGAGCTGTGCAATGATCCCGCAGCATATAC ATTCAGCTCAAGTCCAATGCTAGTAGAGAAG
16566	N_tADH1_RV	TTCTAGGCTTTGATGCAAGGTCCACATATCTTCGTTAGGACTCAATCGTGG CTGCTGATCTTGTCCTCTGAGGACATAAAATAC
12963	PYK1 Fw + N	GATCAGCAGCCACGATTGAGTCCTAACGAAGATATGTGGACCTTGCATCA AAGCCTAGAAAACGTGGTCAAACTTCAGAACTAAG
12964	PYK1 Rv + O	ATACTCCCTGCACAGATGAGTCAAGCTATTGAACACCGAGAACGCGCTGA ACGATCATTCATAATCATGATAACCTTGAGGGAAG
16567	O_pTDH3_FW	GAATGATCGTTCAGCGCGTTCTCGGTGTTCAATAGCTTGACTCATCTGTGC AGGGAGTATATACTAGCGTTGAATGTTAGCGTC
16568	A_tTDH3_RV	GTGCCTATTGATGATCTGGCGGAATGTCTGCCGTGCCATAGCCATGCCTTC ACATATAGTATCCTGGCGGAAAAAATTCATTTG
12967	ENO2 Fw + A	ACTATATGTGAAGGCATGGCTATGGCACGGCAGACATTCCGCCAGATCAT CAATAGGCACAACGGATGATGAAAACACTAAACGA
13227	ENO2 Rv + B	GTTGAACATTCTTAGGCTGGTCGAATCATTTAGACACGGGCATCGTCCTCT CGAAAGGTGTAACGAAGACGTTACCAGCTGATTG
16569	B_tHXK2_FW	CACCTTTCGAGAGGACGATGCCCGTGTCTAAATGATTCGACCAGCCTAAG AATGTTCAACACTTGAACAATAAATACGAAATCC
16570	C_pHXK2_RV	CTAGCGTGTCCTCGCATAGTTCTTAGATTGTCGCTACGGCATATACGATCC GTGAGACGTACGCTGGTAAAGTACAGCTA
12971	PGI1 Fw + D	AATCACTCTCCATACAGGGTTTCATACATTTCTCCACGGGACCCACAGTCGT AGATGCGTAACGTATTCTTAGTGGATAACATGC
12972	PGI1 Rv + C	ACGTCTCACGGATCGTATATGCCGTAGCGACAATCTAAGAACTATGCGAG GACACGCTAGTTTTAAACAGTTGATGAGAACCTTT
16571	D_tPFK1_FW	ACGCATCTACGACTGTGGGTCCCGTGGAGAAATGTATGAAACCCTGTATG GAGAGTGATTATTCCATAGCTTAGTTTAATCAAGG
16572	J_pPFK1_RV	CGACGAGATGCTCAGACTATGTGTTCTACCTGCTTGGACATCTTCGCGTAT ATGACGGCCCGGCTAGTAAAAAAGAAAATTAATA
12976	PFK2 Rv + J	GGCCGTCATATACGCGAAGATGTCCAAGCAGGTAGAACACATAGTCTGAG CATCTCGTCGAAATCGTCTATATCACATATTCCAG
13900	PFK2_fw + BU	AATCATGTGACCCAGGCTTGCGCATACATGATCCTTCTTGCGCTGCATGGG CGACTATATAACGATTCTCTGCTGCTTTGTTGCA
16573	BU_tHIS3_FW	ATATAGTCGCCCATGCAGCGCAAGAAGGATCATGTATGCGCAAGCCTGGG TCACATGATTGCATCTGTGCGGTATTTCACAC
16574	L_pHIS3_RV	GCCGTAGCTTCCGCAAGTATGCCGTAGTTGAAGAGCATTTGCCGTCGGTTC AGGTCATATTGCGGCATCAGAGCAGATTG
12980	GPM1 Rv + L	ATATGACCTGAACCGACGGCAAATGCTCTTCAACTACGGCATACTTGCGGA AGCTACGGCTATTGCTATAACATGTCATGTCACC
12979	GPM1 Fw + M	ACGAGAGATGAAGGCTCACCGATGGACTTAGTATGATGCCATGCTGGAAG CTCCGGTCATAACGGTGATACTTTGACAGGAGCTA

16575	M_tPDC1_FW	ATGACCGGAGCTTCCAGCATGGCATCATACTAAGTCCATCGGTGAGCCTTC ATCTCTCGTACAGTGTTCCTTAATCAAGGATAC
16576	AR_pPDC1_RV	TGACGAGATTTGAGAAGTCCCCAATATCGACTCGTGATGTGCCATGCGTG CTGTCAGTATCATGCGACTGGGTGAGCATA
12983	ARS1211_fw +AR	ATACTGACAGCACGCATGGCACATCACGAGTCGATATTGGGGACTTCTCA AATCTCGTCAGACATAGTATTTCGCAACCTTTCAG
13901	ARS1211_rv + BS	GTTCAGGATTCTGTCGATGCCACATCGAGTCAGTCGTAGTAACATGGAAC GCAGTGCATCGACAGGCGTTTCTGTACCGCTGTTA
11519	Chunk_9.2C_fw	GATGCACTGCGTTCCATGTTACTACGACTGACTCGATGTGGCATCGACAGA ATCCTGAACGCGTCGCTTTACGCCAGGTC
11522	Chunk_9.2D_rv	ATAAGGAATGATGCACGCGCGACGCTGCTTCAATCAGATGTATAGCATTG CCTTCTGCGCGGTTGCATACTGTGGCAACTGAC
16581	AQ_tel_right_FW	GCGCAGAAGGCAATGCTATACATCTGATTGAAGCAGCGTCGCGCGTGCAT CATTCCTTATCCGGGGGATCCGGTGATTG
13435	Telomerator_r_rv	GTTATCCCTACCCACACAC

Supplementary Table 225 - Primers for glycolysis deletion

Primer number	Primer name	Sequence (5' to 3')
Primers for repair fragment IMF27 transformation		
13273	URA3 repair SGA1 Fw	TTTTCTCATCTCTTGGCTCTGGATCCGTTATCTGTTCTGTTACACAA GAAATCGTACATAACTGTCATCCTGCGTGAAGATTAA
13274	URA3 repair SGA1 Rv	TCTCGCTTTTCTTTTATTTTTTTTTGTCTACAAACTCTGTAAAACTTC TTGTCTTATTTGAGTGTTGCACCGTGCCAATGCAGGT
Primers to make repair fragment IMF29 transformation		
6075	COUNTER SELECT oligo fw	TTTTTCTCATCTCTTGGCTCTGGATCCGTTATCTGTTCTGTTACACA AGAAATCGTACATACTAGAGCAAGATTTCAAATAAGTAACAGCA GCCATACGTTGAAACTACGGCAAAGGATT
6076	COUNTER SELECT oligo rv	AATCCTTTGCCGTAGTTTCAACGTATGGCTGCTGTTACTTATTTGA AATCTTGCTCTAGTATGTACGATTTCTTGTGTAACAGAACAGATA ACGGATCCAGAGCCAAGAGATGAGAAAAA
Diagnostic primers		
3751	sequence primer right - fw	GGTCAGCAGTACAGAACCGTCG
4229	Sequence SGA1 2 rv	TGGTCGACAGATACAATCCTGG
4880	c I-SceI inside rv	GCCAATCAAACCCTTCTTCTC
7298	FW_sga1u_check	TTGTTCAATGGATGCGGTTC

Supplementary Table 23 - Primers to repair *RKI1* mutation in IMF32

Primer number	Primer name	Sequence (5' to 3')
Primers to make repair fragment		
17614	RKI1_repair_SNP1_fw	GAGAATTTACAATTAATTAAAGGTGGTGGTGCTTGTCTATTTCAAGAA AAATTGGTTAGCACTAGCGCTAAAACATTCATTGTCGTTGCTGATTCA AGAAAAAAGTCCCCAAAACATCTA
17615	RKI1_repair_SNP1_Rv	TAGATGTTTTGGGGACTTTTTTCTTGAATCAGCAACGACAATGAATGT TTTAGCGCTAGTGCTAACCAATTTTTCTTGAAATAGACAAGCACCACC ACCTTTAATTAATTGTAAATTCTC
Diagnostic primers to check gene deletion		
17623	RKI1_SNPT_dg_fw	GGTGGTGCTTGTCTATTTCAAT
17624	RKI1_SNPG_dg_fw	GGTGGTGCTTGTCTATTTCAAG
17625	RKI1_SNP_dg_rv	GGTTTCCACCACTCCCACTAAA

Supplementary Table 24 - Primers for deletion of native *ZWF1, GND1, SOL3, RKI1, TAL1, TKL1* and *RPE1* ORFs

Primer number	Primer name	Sequence (5' to 3')
Primers to make repair fragment		
7363	FW_Gnd1_repair	TAAACCTGTATTGTTGCCATTACAGAAAAAAGCCACTTTCTATACAAA AACTACAATAAATTCAGAGTGTTGCCAGAATGTGCTTCTGACAACTTG CCAGTAGACAAGGATATCCATATC
7364	RV_Gnd1_repair	GATATGGATATCCTTGTCTACTGGCAAGTTGTCAGAAGCACATTCTGG CAACACTCTGAATTTATTGTAGTTTTTGTATAGAAAGTGGCTTTTTTCT GTAATGGCAACAATACAGGTTTA
8868	FW_zwf1_repair	CAATTGGCTGTATAGACAGAAAGAGTAAATCCAATAGAATAGAAAAC CACATAAGGCAAGAGATACGAAGGATAATTAGAAAAATGCAAGCAC ATTCATTTATCGGCTAAGTCACTGAAA
8869	RV_zwf1_repair	TTTCAGTGACTTAGCCGATAAATGAATGTGCTTGCATTTTTCTAATTAT CCTTCGTATCTCTTGCCTTATGTGGTTTTCTATTCTATTGGATTTACTCT TTCTGTCTATACAGCCAATTG
9281	RPE1_repair oligo fw	CAATTTCATGCAAGAAGGCCATTTGCTAATTCCAAGAGCGAGGTAAA CACACAAGAAAAATTGTACATATGCGGCATTTCTTATATTTATACTCTC TATACTATACGATATGGTATTTTT
9282	RPE1_repair oligo rv	AAAAATACCATATCGTATAGTATAGAGAGTATAAATATAAGAAATGCC GCATATGTACAATTTTTCTTGTGTGTTTACCTCGCTCTTGGAATTAGCA AATGGCCTTCTTGCATGAAATTG
16891	SOL3_repair oligo fw	GCCTCGAGGATAATAGAAGGCAATGCACCATCAATTGCTTTACCCCTG GTCCGCGACCAAAAAAGACACACATGCGAGCTTTCGAACCTCAGATG CTAATATTACGTGTTATATATACCA
16892	SOL3_repair oligo rv	TGGTATATATAACACGTAATATTAGCATCTGAGGTTCGAAAGCTCGCA TGTGTGTCTTTTTTGGTCGCGGACCAGGGGTAAAGCAATTGATGGTG CATTGCCTTCTATTATCCTCGAGGC
16899	RKI1_repair oligo fw	TGTTACATAAACTTGGTTACCGCATACTGCAACCTCATATAAATACAAC ATAGGAAAGAAGCAGATCAAAGGCAAAGACAGAAACCGTAGTAAAG GTTGACTTTTCACAACAGTGTCTCC
16900	RKI1_repair oligo rv	GGAGACACTGTTGTGAAAAGTCAACCTTTACTACGGTTTCTGTCTTTG CCTTTGATCTGCTTCTTTCCTATGTTGTATTTATATGAGGTTGCAGTAT GCGGTAACCAAGTTTATGTAACA
16901	TKL1_repair oligo fw	ACAACAGAGAAGGAAGCTCATCCCAAGCAACTCTACATAGTTACCTCT TTAGCAAACAAAATTCTGATCGTAGATCATCAGATTTGATATGATATT ATTTGTGAAAAAATGAAATAAAAC
16902	TKL1_repair oligo rv	GTTTTATTTCATTTTTTCACAAATAATATCATATCAAATCTGATGATCTA CGATCAGAATTTTGTTTGCTAAAGAGGTAACTATGTAGAGTTGCTTGG GATGAGCTTCCTTCTCTGTTGT
16907	TKL1_repair oligo fw	ACAACAGAGAAGGAAGCTCATCCCAAGCAACTCTACATAGTTACCTCT TTAGCAAACAAAATTCTGATCGTAGATCATCAGATTTGATATGATATT ATTTGTGAAAAAATGAAATAAAAC

16908	TKL1_repair oligo rv	GTTTTATTTCATTTTTTCACAAATAATATCATATCAAATCTGATGATCTA CGATCAGAATTTTGTTTGCTAAAGAGGTAACTATGTAGAGTTGCTTGG GATGAGCTTCCTTCTCTGTTGT
16911	TAL1_repair oligo fw	AGGTAAAATTTAGTACGATAGTAAAATACTTCTCGAACTCGTCACATA TACGTGTACATAGGAAGTATCTCGGAAATATTAATTTAGGCCATGTCC TTATGCACGTTTCTTTTGATACTT
16912	TAL1_repair oligo rv	AAGTATCAAAAGAAACGTGCATAAGGACATGGCCTAAATTAATATTTC CGAGATACTTCCTATGTACACGTATATGTGACGAGTTCGAGAAGTATT TTACTATCGTACTAAATTTTACCT
Diagnostic primers to check gene deletion		
1046	TAL1Rv1	AAGAACACCGAGCGGCTTTG
1047	TAL1Fw1	CTGTACACTAGGAAGCCCTGTT
2122	BG26-DF	GCTGCAGTATTGTTCCTGAG
2123	BG26-DR	CCTGTTTGCCTTTCCTTACG
4494	RPE1 DG fw	TATCCAAGTCGAGCTGGGAAAG
4495	RPE1 DG rv	CCCATGAGTTAGGCACTTACG
5598	TKL1 DG fw	CGTTCCGTTCGCAATCTC
5599	TKL1 DG rv	GGTGTGATTCTCTCGAAGG
8566	FW_zwf1_outside	GGGTGGCGAATTCTTCAATG
8567	RV_zwf1_outside	ATTGCGTACGATGCGGTATG
16893	SOL3_dg fw	TGTCGCTGCTATCTACTGCG
16894	SOL3_dg rv	GATGAGGCACGCAAAGGTTG
16901	RKI1_dg fw	CATGGCCCAGATTGCTTGTG
16902	RKI1_dg rv	ATCCGGACAGGGTCTTGTTG

Supplementary Table 25 - Parts of the "basic design" of the anthocyanin pathway

* Size of the fragments does not include the SHR sequences.

SHR Fw	Component	SHR Rv	Size*	Template	Primer Fw	Primer Rv
Chunk 16AB	*pRPS3-coAtCPR1-tIDH2*	F	3559	pUDC348	17908	17909
F	*pSePDC1-AtPAL1-tLAT1*	DW	3282	pUDC349	14612	17910
DW	*pSeGPM1-coRcTAL1-tCIT1*	DX	2403	pUDC350	17911	17912
DX	*pSeTPI1-At4CL3-tSDH2*	DY	2785	pUDC351	17913	17914
DY	*pTEF1-coAtCHS3-tMDH1*	AM	2292	pUDC352	17915	17916
AM	*tSDH4-AtCHI1-pSkADH1*	AB	1849	pUDC353	17917	15587
AB	*tADH3-coAtC4H-pSeFBA1*	DC	2603	pUDC354	15168	14460
DC	*tSDH3-coAtF3H-pSkTDH3*	EA	2186	pUDC355	17918	17919
EA	*tACO1-coGhDFR-pSePGK1*	EB	2202	pUDC356	17920	17921
EB	*tFUM1-coAtANS-pSeENO2*	EC	2180	pUDC357	17922	17923
EC	*tDIC1-coAt3GT-pSePYK1*	CJ	2498	pUDC358	17924	17925
CJ	ARS106	DL	236	CEN.PK113-7D genomic DNA	13183	17926

Supplementary Table 26 - List of primers for amplifying the fragments of the "basic design" of the anthocyanin pathway and diagnosing integration

Primer number	Primer name	Sequence (5' to 3')
13183	ARS106 + Tag CJ Fw	TCGACCCATGTTTATCGCTAGCAGTCGCTTCAGCTAGATTCACAGAGT GGCCGTGACAATCAATGTTTTATCTACGTTGGAGTAA
14460	DC - pFBA1 - FW NEW	TGAGCCAGTGCATTCCATCGATGCAGATTCGCGTCCACGTAACGTATC GGAAGCATAGGCCTTTTCCCATGTTTCCAATG
14612	flank F - pPDC1 (Se) - FW	CATACGTTGAAACTACGGCAAAGGATTGGTCAGATCGCTTCATACAGG GAAAGTTCGGCAGATGAAGTGACGCGCGCCCGGA
15168	AB-tADH3-rv	TCAGCGTGTTGTAATGATGCGCCATGAATTAGAATGCGTGATGATGTG CAAAGTGCCGTCTCTCTTCGGCCCTTTTATCGTG
15587	AB-SkADH1p_fwd	GACGGCACTTTGCACATCATCACGCATTCTAATTCATGGCGCATCATT ACAACACGCTGAACTCCCAAATAATCAAGGG
17908	Chunk16AB_pRPS3_fw	CGTAAGAACCGACTAACGTCCCCATATTGATGTTTACCGCCGAAGTGG GATCGGCCATCTTCTGCTACTTTCCATTATCTGGTC
17909	F_tIDH2_rv	TGCCGAACTTTCCCTGTATGAAGCGATCTGACCAATCCTTTGCCGTAG TTTCAACGTATGTCCACTGAGGGACATTTTGAG
17910	DW_tLAT1_rv	ACCCACAGTCGTAGATGCGTTGTCAGAATTTCCAGGTGTGGCTACATC TTCCGTACTATGAAACTTTATGCGTTATATCCTATATCCCACTCC
17911	DW_pSeGPM1_fw	CATAGTACGGAAGATGTAGCCACACCTGGAAATTCTGACAACGCATCT ACGACTGTGGGTTAAACCTGATCTTTCACCTCAGTAAC
17912	DX_tCIT1_rv	ACAGGTCCTCAGGGCGATATTAATGGGATTGATGTCTGCCCTCCACTG TACTGCATGTAGCTTGACGTAGTATATCGACTACAGGC
17913	DX_pSeTPI1_fw	CTACATGCAGTACAGTGGAGGGCAGACATCAATCCCATTAATATCGCC CTGAGGACCTGTGGATGTCGTTGTTCTTGTTACAC
17914	DY_tSDH2_rv	ACACAGTCTAAGGAGAGTCTGCAATCCCTTATGAGTCAGTCAACGCAT GAGGATGATGACAAGCCAAAAGGCCCTTCAA
17915	DY_pTEF1_fw	GTCATCATCCTCATGCGTTGACTGACTCATAAGGGATTGCAGACTCTC CTTAGACTGTGTCCTTGCCAACAGGGAGTTC
17916	AM_tMDH1_rv	CAGTGACATGCCGCTCAGTACTCGTATCTTACATGACGTGGGCATGGG TTCCGCTCATATGTTTATTCATCATTATCATCATCATCATC
17917	AM_tSDH4_fw	ATATGAGCGGAACCCATGCCCACGTCATGTAAGATACGAGTACTGAGC GGCATGTCACTGAATTGAAAATCCGCGAGTG
17918	DC(i)_tSDH3_fw	GCCTATGCTTCCGATACGTTACGTGGACGCGAATCTGCATCGATGGAA TGCACTGGCTCAGCAGAAATTATCTTGATATCTGT
17919	EA_pSkTDH3_rv	AAGTAGGTCAGAGTAGCACTGGCTATGATTCGCAATGCTTGGTGAATT GAGAGCTATCCTAACGGCGAATTTTTACTAACC
17920	EA_tACO1_fw	AGGATAGCTCTCAATTCACCAAGCATTGCGAATCATAGCCAGTGCTAC TCTGACCTACTTGCTCAGCCTTATTACTTAATTT
17921	EB_pSePGK1_rv	AAGCCTCGGACTCGAAGCATGAATCATGTATCATAGGCGGCTCAGCCT TAGCCAATATGAGCTTCAATTCAAGATACACAG
17922	EB_tFUM1_fw	TCATATTGGCTAAGGCTGAGCCGCCTATGATACATGATTCATGCTTCG AGTCCGAGGCTTTGCGGGTAATACTAGGTCC
17923	EC_pSeENO2_rv	TGAAATTATTCTGTGCCGGGCAGCGAAATGGCAGTATGCTCAGTGACG TGAGTGCCATCTAACGCCAAGAAGATGCCG
17924	EC_tDIC1_fw	AGATGGCACTCACGTCACTGAGCATACTGCCATTTCGCTGCCCGGCAC AGAATAATTTCAGCCCAGCAAAATTCGAAA

17925	CJ(i)_pSePYK1_rv	ATTGTCACGGCCACTCTGTGAATCTAGCTGAAGCGACTGCTAGCGATA AACATGGGTCGAAACGTGTAAATACCGGTTTTAGC
17926	DL_ARS106_rv	CGCAAATGTCCCATCGTATTTCAGAACCTTGTCACTCATGCGAGCAAG TGTGACAGCTATGCCGAAAAGGAGGTTTTCTTCTTATTC
Diagnostic primers		
18079	Chunk 16AB fw	GCCTCGACATACTGTTCATC
18080	pRPS3 rv	CTTACATCAGCGCAGCAC
18081	ARS106 fw	GGGTCTGTCCAGCGAATAAG
18082	pFBA1 rv	TAACGTGGGCGAAGAAGAAG
18083	*CoAtF3H* fw	CGTCGATACCAGCCAAAGAG
18084	tACO1 rv	GTTCGGCTGGAGAAGTCAAG
18085	pSkADH1 fw	GCGGGTATGGTGAGGTAAC
18086	coAtC4H rv	GCTAACGGTAACGACTTCAG
18087	tCIT1 fw	AGACCCTCCAGCCTAAATCC
18088	pSeTPI1 rv	AACTGGATGCCGAAACAGAG
18089	tLAT11 fw	CAAACGGTGCGTCAACATC
18090	coRcTAL1 rv	TCTAGCTTCGGCCCAAGAC

Supplementary Table 27 - List of primers for amplifying the fragments of the "elaborate design" of the anthocyanin pathway with several copies of the chalcone synthase and diagnostic PCR.

Primer number	Primer name	Sequence (5' to 3')
14823	YPRCtau3_pTEF1_fw	ACAGTTTTGACAACTGGTTACTTCCCTAAGACTGTTTATATTAGGATT GTCAAGACACTCCCTTGCCAACAGGGAGTTC
18003	can1_pTEF1_fw	GATGAGAAAAGTAAAGAATTGTATCCATTGCGCTCTTTCCCGACGAGA GTAAATGGCGAGCCTTGCCAACAGGGAGTTC
18004	can1_tMDH1_rv	GGTGTATGACTTATGAGGGTGAGAATGCGAAATGGCGTGGGAATGTGA TTAAAGGTAATAGTTTATTCATCATTATCATCATCATCATC
18005	X2_pTEF1_fw	TCACAGAGGGATCCCGTTACCCATCTATGCTGAAGATTTATCATACTA TTCCTCCGCTCGCCTTGCCAACAGGGAGTTC
18006	X2_tMDH1_rv	GTCATAACTCAATTTGCCTATTTCTTACGGCTTCTCATAAAACGTCCC ACACTATTCAGGGTTTATTCATCATTATCATCATCATCATC
18007	YPRCtau3_tMDH1_rv	ATAATTATAATATCCTGGACACTTTACTTATCTAGCGTATGTTATTAC TCGATAAGTGCTGTTTATTCATCATTATCATCATCATCATC
18008	SPR3_pTEF1_fw	AGAAATAAATAAATAAATAATAAAAACCTAAAATTCCTTTTGCGTCAT TGAATTTTTATTCCTTGCCAACAGGGAGTTC
18009	SPR3_tMDH1_rv	TTTATTATGTAGAGCAAAGCTTGCGCGAAATTATTGGCTTTTTTTTTT TTTTAATTAATAGTTTATTCATCATTATCATCATCATCATC
18225	CAN1_pAgTEF1_fw	GATGAGAAAAGTAAAGAATTGTATCCATTGCGCTCTTTCCCGACGAGA GTAAATGGCGAGGACATGGAGGCCCAGAATACC
18234	chunk7BC_pTEF1_fw	CCGCTGCCACCACGCCAAGCGCGGTTAAACCGACGGTATCGGCGGTGG AATGAGTACCCACCTTGCCAACAGGGAGTTC
18235	chunk7BC_tMHD1_rv	TCACGGCTGCCTGGGCTGTGCGGAAAATGGTTTCTGGGATCGCGGTTC GTTCTACAGCCGGCGTTTATTCATCATTATCATCATCATC
18236	chunk15CD_pTEF1_fw	AGATATACCAGACAAATCAATGTCAGAATCCAAATCAGATATTCCCGG CGTATTTATCCGCCTTGCCAACAGGGAGTTC
18237	chunk15CD_tMHD1_rv	ATATTGAAAACATTAATGCGTGTGATGATGTTTTTTCTGAGTATTGTT TTGATGATGAAAGCGTTTATTCATCATTATCATCATCATC
18238	tADH1/SHR-N_pTEF1_fw	CAATGAGTTGATGAATCTCGGTGTGTATTTTATGTCCTCAGAGGACAA GATCAGCAGCCACCTTGCCAACAGGGAGTTC
18239	pPYK1/SHR-N_tMHD1_rv	TATTTTTCTTAGTTCTGAAGTTTGACCACGTTTTCTAGGCTTTGATGC AAGGTCCACATAGCGTTTATTCATCATTATCATCATCATC
18240	chunk9CD_pTEF1_fw	AACCAGCGACGGATATTGCTGTGCCAGTTGTCGGGCAAGCGTAATGCC GTCGATATCCGGCCTTGCCAACAGGGAGTTC
18241	chunk9CD_tMHD1_rv	CTGTTGGCAATGCCGCGCAGGCTTTAGAGACACTGCAAAATAGCGAAC CGTTTGCTGCCGGCGTTTATTCATCATTATCATCATCATC
Diagnostic primers		
92	YGR059w CTRL RV	ATGATGTCGCGCATTTGATGCCTTAAATAC
2496	FW-conf-upstrm	CGGGAGCAAGATTGTTGTG
2497	RV-conf-dwnstrm	GGTTGCGAACAGAGTAAACC
2820	Probe AmdS fw	AGCTTCTGCTGCTGACTTGG
2908	D_FW PDH construct ctrl	GGATTGGGTGTGATGTAAGGATTCGC
3853	Fus GF cassette fw	GCTGCATCCTTCCCATGCAAAGTG
6028	AmdS ORF rv (DT37)	TGTCAGCAGCCAATTCTTC

7376	FW_x-2_outside	GGTCTAGGCCTGCATAATCG
7377	RV_X-2_outside	TGCGGCATCATGTCTACTTG
13261	YPRCtau3 dg FW	AATACGAGGCGAATGTCTAGG
13262	YPRCtau3 dg RV	GCCTCCCCTAGCTGAACAAC
13336	TEF1p seq	GCTCATTAGAAAGAAAGCATAGCAATC
17360	CHS_Citrus_reticulat_fw	CGGTTTCGGTCCAGGTTTGAC
17950	AtCHI1 dg - FW	CCCGTTCTTCCGTGAAATAG
18156	AM_dg_fw	AACATATGAGCGGAAGAC
18157	DZ_dg_rv	GAATCACAGTCGCCCTTG
18158	DZ_dg_fw	CAAGGGCGACTGTGATTC
18159	ED_dg_rv	ATATCGGCCATCGTGCCTTG
18160	ED_dg_fw	ATGGCCGATATTGCGTTGAG
18161	EE_dg_rv	CGATATTGCCAGTCAGGTCAG
18162	EE_dg_fw	CCTGACTGGCAATATCGTTAC
18163	EF_dg_rv	ATGGTCGTGGACTCTATCTG
18164	EF_dg_fw	GATAGAGTCCACGACCATCC
18165	tSDH4_dg_rv	CGCCGGTATATTCCTTTGC
18378	diag_7BC fw	GAACAAACCGCGCATTCC
18379	diag_15CD fw	GGCAGAGCTTCAGAGTCTATC
18380	diag_15CD rv	GACGTGTCGGTATCTAAAGC
18381	diag 9CD fw	TCCCGCGGAATAATGAACTG
18382	diag 9CD rv	CGCTAACCCAGCGAATTAC

Supplementary Table 28 List of primers for amplifying the correct *CoAtANS* transcriptional unit and the diagnostic primers used to confirm correct integration.

Primer number	**Primer name**	**Sequence (5' to 3')**
18740	SHR AL_tFUM1_fw	CTGACTCATTGCCATTGCATGTGCAGACTCAACACCATGACATCTTAA TACAGAGGACGCTGCGGGTAATACTAGGTCC
18741	SHR DS_pSeENO2_rv	ATCAAGACTGAGGAGTACGTCAGGTTGCAGAGGATCACTTGTAATG AATGTGTGCTCGCTAACGCCAAGAAGATGCCG
3537	ilv5 flanking rv	AATCGTAGCTGTCCCGATGAGG
17730	17730_RPE1_dg_rv	GTGGTTTGGGCAAGGAGACAATC
17973	*coAtANS* diag rv	GGTTCCAAACCCAAACCAACAG
17974	*coAtANS* diag fw	GGCCAAAGACTCCATCTGAC

Supplementary Methods 1: Strains, growth medium and maintenance.

For liquid cultures yeast was cultivated in 50-/100-/500 mL shakeflasks containing, respectively 10-/20-/100 mL media in an Innova 44 Incubator shaker (New Brunswick Scientific, Edisan, NJ, USA) at 30 °C and 200 rpm. Cultures on solid media were incubated at 30°C until single colonies were visible. For non-selective growth, yeast strains were cultivated on Yeast extract Peptone Dextrose (YPD) medium containing: 10 g L^{-1} Bacto yeast extract, 20 g L^{-1} Bacto peptone and 20 g L^{-1} glucose. For selective growth to maintain plasmids or NeoChrs, Synthetic Medium (SM) was used, consisting of: 3 g L^{-1} KH_2PO_4, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 5 g L^{-1} $(NH_4)_2SO_4$ and 1 mL L^{-1} of a trace element solution [15]. Alternatively, synthetic medium with urea as sole nitrogen source was used, consisting of 3 g L^{-1} KH_2PO_4, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 5 g L^{-1} K_2SO_4, 2.3 g L^{-1} urea and 1 mL L^{-1} of a trace element solution [16]. Media were set to pH 6 by 1M KOH addition and for solid media, 20 g L^{-1} Bacto agar was added. Autoclaving was performed for 20 min at 110°C and 120°C for YPD and SM medium, respectively. Thereafter SM medium was supplemented with 1 mL L^{-1} of a filter sterilized vitamin solution and 20 g L^{-1} of glucose separately autoclaved for 20 min at 110°C. For auxotrophic strains, SM was supplemented with 125 mg L^{-1} histidine and/or 150 mg L^{-1} uracil. Disruption of the *URA3* marker was verified by growth on SMD with 150 mg L^{-1} uracil and 1 g L^{-1} 5-FluoroOrotic Acid (SMD–5-FOA). For the selection based on the markers *hphNT1, KanMX* and *amdS,* SM medium without nitrogen source was prepared by replacing $(NH_4)_2SO_4$ with 6.6 g L^{-1} K_2SO_4. For *hphNT1* and *KanMX,* 2.3 g L^{-1} urea was used as nitrogen source and 200 mg L^{-1} hygromycin (Hyg) and 200 mg L^{-1} G418 were added to the medium, respectively. For *amdS,* 1.8 g L^{-1} filter sterilized acetamide was employed as nitrogen source.

All *E. coli* strains were cultivated in Lysogeny Broth (LB) medium containing: 10 g L^{-1} tryptone, 5.0 g L^{-1} yeast extract and 5 g L^{-1} NaCl. For plasmid selection 100 mg mL^{-1} ampicillin (ampR), 50 mg mL^{-1} kanamycin (kanR), or 25 mg mL^{-1} chloramphenicol (camR), was supplemented to the medium. Liquid cultivation was performed in 5 mL medium in a 15 ml Greiner Tubes at 37°C and 200 rpm in an Innova 4000 shaker (New Brunswick Scientific). Cultures on solid media were incubated at 37°C until single colonies were visible.

S. cerevisiae and *E.coli* strains were stored at -80°C in 1 mL vials containing cultures mixed with glycerol (30% v/v).

Supplementary Methods 2: Molecular biology techniques

Genomic DNA from *E.coli* (migula) Castellani and Chalmers (ATCC 47076) or *S. cerevisiae* used for strain construction purposes was isolated using the QIAGEN Blood & Cell Culture Kit with 100/G Genomic-tips (Qiagen, Hilden, Germany) or alternatively for yeast with the YeaStar genomic DNA kit (Zymo Research, Irvine, CA). *E.coli* DNA from a mixed population of *E.coli* XL1-Blue and *E.coli* BL21 used for construction of the test NeoChrs was isolated as described by Postma *et al.* [8]. Plasmids were isolated from *E.coli* using the GenElute Plasmid Miniprep Kit (Sigma-Aldrich, St. Louis, MO) or the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's instructions.

All PCRs for strain construction purposes were performed with Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific) using either desalted or PAGE purified (in case of ORFs) primers (Sigma-Aldrich). PCR products were verified by separation on 1% (w/v) or 2% (w/v) agarose (TopVision Agarose, Thermo Fisher Scientific) gels in 1x Tris-acetate-EDTA (TAE) buffer (Thermo Fisher Scientific) or 1x Tris-Borate-EDTA (TBE) (Thermo Fisher Scientific) buffer. For size determination GeneRuler DNA Ladder mix (Sigma-Aldrich) or GeneRuler DNA Ladder 50bp (Sigma-Aldrich) were used. For DNA staining 10 µL L^{-1} SERVA (SERVA Electrophoresis GmbH, Heidelberg, Germany) was added to the agarose gel solution. DNA was purified using either the Zymoclean Gel DNA Recovery kit (Zymo Research), the GenElute PCR Clean-Up kit (Sigma-Aldrich), the GeneJET PCR Purification Kit (Thermo Fisher Scientific) or using AMPure XP beads (Beckman Coulter, Brea, CA) according to the suppliers' protocols. Purity of DNA was checked using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) and the concentration was measured either by the NanoDrop 2000 (Thermo Fisher Scientific) or by the Qubit dsDNA BR Assay kit (Thermo Fisher Scientific) using the Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA). Gibson assembly used to construct gRNA plasmids and some expression plasmids was performed with the NEBuilder® HiFi DNA Assembly Master Mix (New England Biolabs, Ipswich, MA) in a final volume of 5 µL according to the supplier's instruction.

Chemical *E.coli* XL1-Blue transformation was performed as described by Inoue *et al.* [17] and correct assembly of plasmids was verified by diagnostic PCR or restriction analysis. *S. cerevisiae* was transformed using the lithium acetate/polyethylene glycol method [18]. For diagnostic PCR, DNA was isolated by resuspending some culture in 0.2 M NaOH or by using the method described by Looke *et al.* [19]. All diagnostic PCRs were performed using DreamTaq PCR Master Mix (Thermo Fisher Scientific) according to the manufacturer's instruction. For yeast, single colony isolates were obtained by three consecutive re-streaks on solid selective medium.

Supplementary Methods 3: Detailed construction of the host strain IMX2770

Before assembly of coding NeoChrs a suitable starting strain was engineered by several rounds of CRISPR/Cas9 gene deletions as described by Mans *et al.* [9] . As parental strain, the SwYG strain IMX589 from Kuijpers *et al.* [6] was used. In this strain the minor paralogs of glycolysis are deleted and the major paralogs are centralized at the *sga1* locus on chromosome IX. From this strain, the *amdSYM* marker located between the major paralogs of glycolysis was deleted using *in vivo* assembly of a pMEL10 gRNA plasmid backbone (amplified with primer 6005, Supplementary Table 16) and a gRNA insert made from annealing primers (11588 & 11589, Suppl. Table S15). The DSB was repaired with a 120 bp repair fragment homologous to the flanking SHRs K and L, made by annealing of complementary primers (11590 & 11591, Supplementary Table 16), resulting in strain IMX1433 and IMX1769 before and after plasmid recycling, respectively.

Subsequently the minor paralogs of the pentose phosphate pathway, *GND2, NQM1, SOL4* and *TKL2*, were deleted by transformation of two gRNA plasmids (pUDR286 & pUDR590, Supplementary Table 8A) and 120 bp repair fragments (Supplementary Table 17) homologous to the 60 bp upstream and downstream of the ORF. The strains were stocked before and after discarding the gRNA plasmids, resulting in respectively IMX2154 and IMX2204

Next as much as possible of the promoter, gene and terminator of the *ura3* and *his3* as well as of the functional *SpHIS5* gene were removed using gRNA plasmids pUDR426 and pUDR546 and repair fragments (Supplementary Tables 8A and 18) obtaining strain IMX2234 after plasmid recycling.

Finally, in the last round of deletion, the *ARO10* gene was removed with gRNA plasmid pUDR406 and a 120 bp repair fragment (Supplementary Tables 8A and 19). Again, the plasmid was removed and the strain was stocked as IMX2270.

Supplementary Methods 4: MinION long-read sequencing

Average DNA size and integrity were verified with the TapeStation 2200 (Agilent Technologies, Santa Clara, CA). Before sequencing, flow cell quality was assessed by running the MinKNOW platform QC. All samples were sequenced in-house on a MinION (Oxford Nanopore). Samples NeoChr10.10, NeoChr10.13 (IMF22), Neochr10.47, NeoChr10.54, NeoChr10.16, NeoChr10.62, NeoChr10.67, NeoChr10.69, NeoChr11.19, NeoChr11.22, NeoChr25.25, NeoChr25.47, NeoChr25.53, NeoChr25.56 (IMF27), NeoChr26.2, NeoChr26.4 (IMF29), NeoChr26.6, Neochr26.9 and NeoChr26.1 were sequenced on a FLO-MIN106 flowcell with sequencing kit SQK-LSK108.

Samples NeoChr12 (IMF23), NeoChr30 (IMF41), NeoChr31 (IMF42), NeoChr33 (IMF47) and NeoChr34 (IMF48) were sequenced on a FLO-MIN111 with sequencing kit SQK-LSK109. Basecalling was performed for samples with NeoChr10 and Neochr11 by using Albacore (version 2.3.1, Oxford Nanopore). Demultiplexing of the fastq files of the NeoChr10 and NeoChr11 samples was performed with Porechop (https://github.com/rrwick/Porechop). Basecalling and demultiplexing was performed with Guppy (Oxford Nanopore) for samples with NeoChr25 and NeoChr26 with version 3.1.5, samples IMF41, IMF42, IMF47 with version 4.4.2 and IMF48 with version 4.5.4. All resulting fastq files were filtered on length (> 1kb) followed by *de novo* assembly by Canu version 2.0 [20].

Supplementary Methods 5: Analysis of aromatics

A. HPLC analysis of aromatic compounds up until naringenin

For extracellular aromatic compounds, a sample containing broth was mixed 1:1 with 96% ethanol, vortexed thoroughly, spun down for 5 minutes at 14800 rpm and the supernatant was used for further analysis. The aromatic compounds up until naringenin (2-phenylethanol (2PE), *p*-hydroxyphenylethanol (*p*OH2PE), phenylacetic acid (PAA), *p*-hydroxyphenylacetic acid (*p*OHPAA), phenylpyruvic acid (PPY), coumaric acid (COUM), cinnamic acid (CIN), phloretic acid (PHLOR) and naringenin (NAR) were measured using an Agilent Zorbax Eclipse plus C18 column (4.6 x 100mm, 3.5 µm) (Agilent). As mobile phase, 0.020 M KH_2PO_4 set at pH 2.0 containing 1% acetonitrile was used at a flow rate of 0.8 mL min^{-1} at an operating temperature of 40°C. The amount of acetonitrile was gradually increased to 10% within 6 minutes, then to 40% after 23 minutes, followed by a decrease in amount to 1% after 30 minutes. The compounds were detected using a diode array and a multiple wavelength detector (Agilent G1315C) at different wavelengths: 200 nm for PAA, 210 nm for PPY, 214 nm for 2PE, *p*OH2PE, *p*OHPAA and PHLOR, 270 nm for CIN and finally 280 nm for NAR and COUM.

The extracellular concentrations in the supernatant of the aromatic compounds kaempferol (KEA), dihydrokaempferol (DHK), kaempferol 3-O-glucoside (K3G), pelargonidin (PEL) and pelargonidin 3-O-glucoside (P3G) were detected using LS-MS/MS, as described in the next section. Additionally, since P3G has never been measured extracellular before, the intracellular concentrations of P3G, and its precursors kaempferol, dihydrokaempferol, K3G and pelargonidin were also measured. A certain amount of cell culture was spun down for 5 minutes at 5000 rpm, washed once with dH_2O, resuspended in 0.5-1 ml methanol (0.75% HCL) and the samples were stored overnight at -80°C. Next, the samples were lyophilized for 24 h using a Mini Lyotrap freeze-dryer (LTE Scientific TLD, UK) operated at -80 °C, connected to a Pirani 501 manometer (Edwards Vacuum, UK) using a RV8 pump (Edwards Vacuum, UK). Finally, the pellet was resuspended in 1 mL methanol (2.0% HCL) and stored overnight at -80 °C.

B. Mass spectrometric analysis of anthocyanin pathway compounds

Identification and quantification of compounds from the anthocyanin pathway downstream of naringenin was performed using an ACQUITY UPLC chromatography system (Waters, UK) coupled online to a high-resolution Orbitrap mass spectrometer (Q-Exactive Focus, Thermo Fisher Scientific, Germany). For chromatographic separation, a reverse phase separation column (ACQUITY UPLC BEH C18, 1.0 mm × 100 mm, 3 µm particle size, part No 186002346, Waters UK) was operated at room temperature using H_2O plus 0.1% formic as mobile phase A, and acetonitrile plus 0.1% formic acid as mobile phase B. A gradient

was maintained at 50 µL/min at 7.5% B over 5 minutes. Solvent B was then increased to 80% over 4 minutes, and kept constant for additional 3 minutes before equilibrating back to the starting conditions. The metabolite extracts were taken from -80°C immediately before injection, brought to room temperature, vortexed and 15 µL crude extract were mixed with 85 µL 1 mM HCl. The mixture was carefully vortexed and centrifuged using a bench top centrifuge for 1 minute to remove insoluble materials. 5 µL were subsequently injected onto the UPLC reverse phase separation system. The mass spectrometer was operated alternating in full scan and PRM mode. Full scan was acquired from 250–700 m/z in ESI positive mode (+ 3.25 kV), at a resolution of 70 K. Parallel reaction monitoring was performed for the precursor masses for dihydrokaempferol (DHK, Cas No. 104486-98-8) 289.07 m/z $[M+H]^+$ using a NCE of 26, kaempferol (KEA, Cas No. 520-18-3) 287.05 m/z $[M+H]^+$ using a NCE of 30, kaempferol 3-O-glucoside (K3G, Cas No. 480-10-4) 449.10 m/z $[M+H]^+$ using a NCE of 24, pelargonidin (PEL, Cas No. 134-04-3) 271.06 m/z $[M]^+$ using a NCE of 30 and pelargonidin 3-O-glucoside (Cas No. 18466-51-8) m/z 433.10 $[M]^+$ using a NCE of 24. Fragment ions were measured at fixed first mass of 75 m/z, a resolution of 35K, a max IT of 100 ms and an AGC target of 1e5, by acquiring 2 microscans. Raw data were analyzed using XCalibur 4.1 (Thermo) where retention and unique fragments for each individual compound were compared to commercial standards. For quantification, peak intensities of identified compounds from the samples were summed using Matlab 2020b, and compared against an external calibration curve established using commercial standards. The standards were purchased from Sigma Aldrich (dihydrokaempferol Cat No. 91216, kaempferol Cat No. 60010, kaempferol 3-O-glucoside Cat No. PHL89237, pelargonidin chloride Cat No. PHL80084, pelargonidin 3-O-glucoside chloride Cat No. PHL89753). The mass spectrometer was calibrated using the Pierce™ LTQ ESI positive ion calibration solution (Thermo Fisher Scientific, Germany).

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