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Protocol status: In development
We are still developing and optimizing this protocol. The protocol is a part of the project regarding the preparation of synthetic precursor of Pichia pastoris nanochromosomes.

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77324

nanochromosome arrays combinatory assembly

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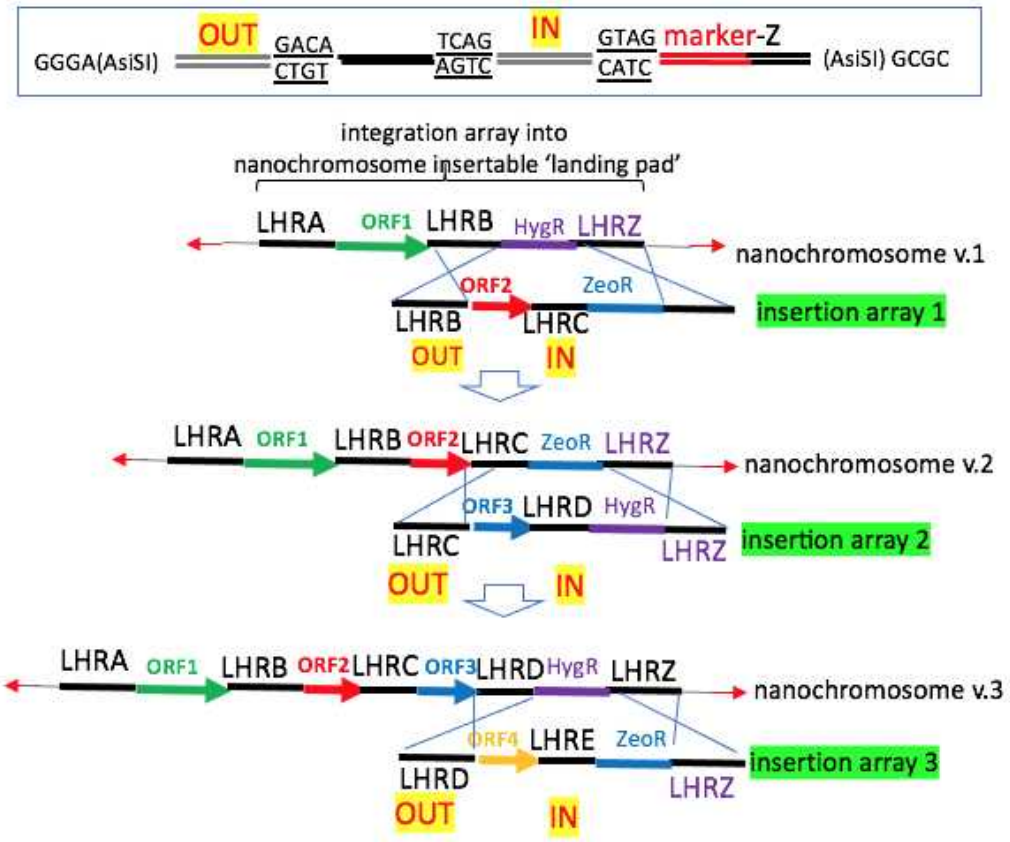
Dariusz Abramczyk

ABSTRACT

The yeast Pichia pastoris is used widely to biomanufacture high-value recombinant proteins. Its cells can secrete copious quantities of post-translationally modified proteins. Currently, however, heterologous genes must first be integrated into the genome in order to achieve expression.
We produced a synthetic 'chromosome-like' construct called here nanochromosome, an autonomously replicating and mitotically stable synthetic construct expressing heterologous genes. The nanochromosome contains essential scaffolding features as telomeres, centromere and yeast replication origins along with a versatile platform for genetic engineering. This platform could be used for the accurate and controlled insertion of multiple expression cassettes placed on 'landing pad', in which an array of genes of interest alternate with ~1kp non-coding DNA sequences (LHR) chosen to facilitate simultaneous double cross-overhomologous recombination and serve as spacers. The landing zone translates along the nanochromosome in an inchworming mode of sequential gene integrations that recycles a pair of antibiotic-resistance markers



Module with expression cassettes and LHR spacers as a pre-prepared in-vitro assembled DNA parts (insertion or integration arrays) is delivered to Pichia by transformation

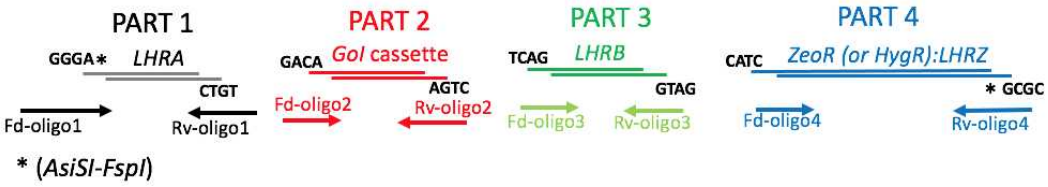
Both the "integration" (receptor) and "insertion" (donor) arrays referred to herein consist of long, HR-ready, regions (LHRs) alternating with gene-expression cassettes. We constructed array parts containing either two or three LHRs e.g.LHR^{n}-GoI^{n}-$AMR^{H/Z}$-LHR^{Z} or LHR^{n}-GoI^{n}-LHR^{m}-$AMR^{H/Z}$-LHR^{Z}, respectively, wherein: LHR^{n}, LHR^{m} and LHR^{Z} are unique sequences of ~1000 bp from a library of $LHRs^{A-Z}$, with LHR^{Z} reserved as the last LHR in the array; GoI^{n} is a cassette consisting of a gene-of-interest with promoter and terminator regions; and $AMR^{Z/H}$ is a zeocin-resistance gene or hygromycin-resistance gene also with promoter and terminator regions.

Preparation of ZeoR-LHRZ and HygR-LHRZ pair tandems in separate protocol https://www.protocols.io/view/preparation-of-parts-hygr-lhrz-and-zeor-lhrz-cqujvwun

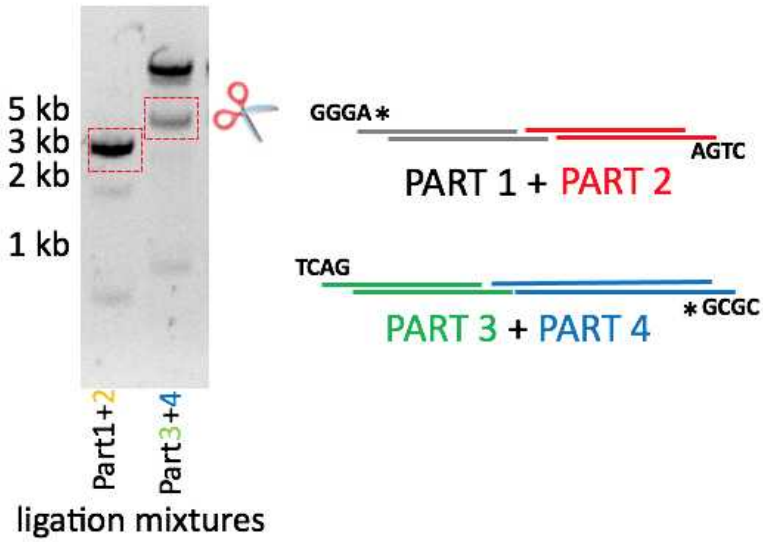
The protocol for multi-DNA parts arrays preparation is based on combinatory assembly and molecular biology techniques customised to create a DNA parts (arrays) library in pUC19 plasmid.

Major 3 experimental steps:

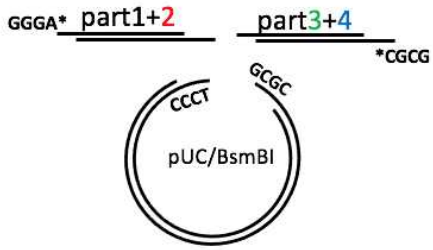
Step 1 - DNA parts generated by PCR with a complementary overhangs designed to create a combined linear dsDNA suitable for insertion into BsmBI-sites of pUC19 (DNA arrays library)
- a part generating by PCR, oligos deliver a complementary overhangs for digestion with BsmBI (Esp3I) or BsaI,
- PCR product digestion with BsmBI or BsaI
- checking digested PCR products on agarose gel
- PCR clean up OR gel extraction of DNA and clean up (only in the case if a PCR product is not homogenous
- estimation of DNA concentration (molar)
Note: (*) stands for AsiSI-FspI RE recognition site provides in Fd-oligo1 and RV-olig4



Step 2 - preparation of semi-products before the the final ligation with linearlized pUC adaptor plasmid
- ligation part1+2 with T7 DNA ligase
- ligation part 3+4 with T7 DNA ligase
- loading ligation mixtures and separation on agarose gel,
- gel extraction of estimated DNA band
- agarose gel verification, DNA concentration (molar)



Step3 - the final ligation of pre-assembled parts with BsmBI-linearized pUC19
- part1+2 and part 3+4 and linearized pUC19 ligation (DNA T7 ligase)
- transformation into E.coli and selection on LB ampicillin plates
- verification by bacterial colony PCR
- plasmid isolation and Sanger (Plasmidsaurus) sequencing
- positively verified clone storage in glycerol stock



The assembled part can be retained by:
- PCR amplification using flanking oligonucleotides
- Digested by AsiSI or FspI (need to be checked for the absence RE site in internal array) and digestion mixture can be used for Pichia transformation

Note: LHR part (e.g. LHRA) in flanking region (as a part 1) sometimes called 'OUT' while the LHRA in internal site (as a part 3) sometimes called 'IN'

Preparation of triple-LHR integration array LHRA-PAOXPDI-... 30m

1 DNA parts preparation by PCR reactions

List of DNA parts

	DNA part	Forward oligo	Revers oligo	product	template
	LHRA (OUT) BsmBI	F315-CCGTCTCCggaGCGATCGCCTGTGTTAAACCGTCTTTAAGTCAACCC	R301-GCGTCTCCtgtcCAAACTAAGATTGGACTATTGCTATC	883 bp	eDA8
	PAOX1PDIhis(BsmBI)	F302-CCGTCTCCgacaAACATCCAAAGACGAAAGGTTG	R303-GCGTCTCCctgaTCTCACTTAATCTTCTGTACTCTG	2905 bp	eDA226*
	LHRE (IN) (BsaI)	F336-CGGTCTCCtcagGACTTACCTCGTTTTAACTTAGTCGG	R350-GGGTCTCCctacACAAGTTTAACTAAGCGTCAGCC	892 bp	eDA9

	DNA part	Forward oligo	Revers oligo	product	template
	HygR-LHRZ (BsaI)	F306-CCTGTCGACGGTCTCAgtagGACATGGAGGCCCAGAATACCC	R317-GGGGTACCGGTCTCAcgcgGCGATCGCCCTGCAGGTTGAGTTGGCGAAGGTGCG	2583 bp	eDA115**

* see 1.1 for prepartion (below)

**link https://www.protocols.io/view/preparation-of-parts-hygr-lhrz-and-zeor-lhrz-cqujvwun

1.1 Prestep - Generation of pPICZA with P_{OAX1}PDIhis - (eDA226)

30m

Plasmid pPICZalphaPDI eDA143 (reference citation)

CITATION

Kerr H, Herbert AP, Makou E, Abramczyk D, Malik TH, Lomax-Browne H, Yang Y, Pappworth IY, Denton H, Richards A, Marchbank KJ, Pickering MC, Barlow PN (2021). Murine Factor H Co-Produced in Yeast With Protein Disulfide Isomerase Ameliorated C3 Dysregulation in Factor H-Deficient Mice.. Frontiers in immunology.

LINK

https://doi.org/10.3389/fimmu.2021.681098

Parts amplified by PCR (using ProFlex PCR system, Applied Biosystems). Thermal Cycler

Q5 High-Fidelity DNA Polymerase - 500 units **New England Biolabs Catalog #M0491L**

Betain solution 5M **Merck MilliporeSigma (Sigma-Aldrich) Catalog #B0300**

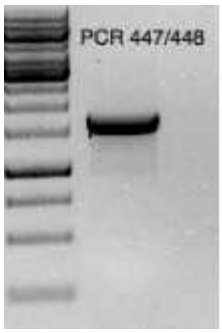
volumes in microliter

	reagents	PDIhis
	water	up to 50
	Q5 HF enzyme	0.7
	eDA143 100pg/uL	1
	betaine 5M	10
	oligo447/ oligo448	2.5/2.5
	10mM dNTP (TAKARA)	4

	reagents	PDIhis
	5xQ5 buffer	10

	program Q5	B	C	D
	Td-initial	98oC	30sec	
	Td	98oC	10sec	33 cycles
	Ta	**55oC**	20sec	
	Te	72oC	1 min 40sec	
	Final extension	72oC	2 min	
	hold	4oC	hold	

	A	B	C
	F447	*PDI* part (MfeI)	CCCCAATTGACAAGCTTTTG ATTTTAACG
	R448	*PDI* part (NotI) introducing HIS-tag-TAA stop codon (*italics*)	TTTTTGCGGCCGC*TTAATGA TGATGGTGGTGATG*CAACTC ATCGTGAGCATCAGCTTC



PDI PCR product 1.7kb

PCR clean-up QIAquick PCR Purification Kit **Qiagen Catalog #28104**

RE digested with MfeI/NotI MfeI-HF - 500 units **New England Biolabs Catalog #R3589S**

NotI-HF - 2,500 units **New England Biolabs Catalog #R3189L**

pPICZA Thermo Fisher vectors

	reagents	PCR Paox1PDI	pPICZA
	1	2	
	pPICZA 100ng/uL		13
	PCR product of 447/448	48	
	MfeI-Hf (20u/ul)	1.5	1
	NotI-Hf (20u/ul)	1.5	1
	rcutsmart	8	6
	water	up to 80	up to 60
			after digestion additional dephosorylation reation
			+ 7ul AP buffer + 2uL Antarctic Phosphatase

60 uL of pPICZA MfeI/NotI digested mixed with 7 ul AP buffer and 2 uL

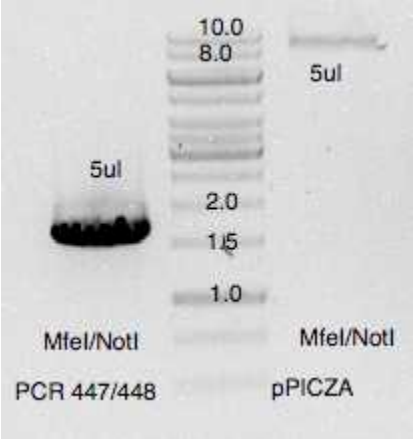
Antarctic Phosphatase - 1,000 units **New England Biolabs Catalog #M0289S** and

incubation 00:30:00

37 °C

PCR product and pPICZA (MfeI/NotI) cleanup

QIAquick PCR Purification Kit **Qiagen Catalog #28104**



Ligation reactions T4 DNA ligase (NEB)

T4 DNA Ligase - 20,000 units **New England Biolabs Catalog #M0202S**

	A	1
	pUC B/K BamHI/KpnI	1
	T4 DNA ligase	1
	Paox1PDI Mfe/NotI	1
	10 X T4 buffer	6
	water	up to 10 ->
	pPICZA MfeI/NotI	1

Overnight 16oC

E.coli transformation (DH5alpha chemically LiAc competent cells)[E.coli chemical competent cells]

Selection on LB +100ug/mL

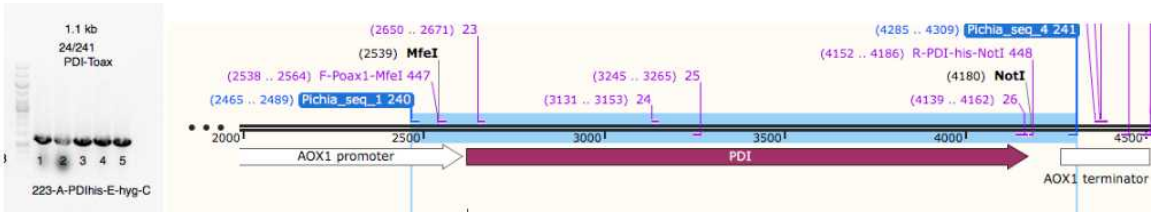
Carbenicillin, disodium salt **Bio Basic Inc. Catalog #CDJ469.SIZE.1g** and growth at

Overnight 37 °C

Several colonies obtained on both agar plates. Single colonies transferred on new LB +100ug/mL Carbenicillin, disodium salt **Bio Basic Inc. Catalog #CDJ469.SIZE.1g** plate, growth overnight and a single colonies submitted for bacterial colony PCR.

see protocol [colony PCR]

Material loaded on agarose gel for verification. 5 colonies verified positively using oligo 24/241.



Positively verified clones re-cultured Overnight in LB + 100 ug/ml carbenicillin, plasmid eDA226 was extracted by miniprep (plasmid preparation)

Qiagen Plasmid Plus Midi Kit **Qiagen Catalog #12945**

1.2 **STEP 1** - PCR amplification of parts and digestion with suitable IIS RE

Parts amplified by PCR (using ProFlex PCR system, Applied Biosystems). Thermal Cycler

Q5 High-Fidelity DNA Polymerase - 500 units**New England Biolabs Catalog #M0491L**

Betain solution 5M **Merck MilliporeSigma (Sigma-Aldrich) Catalog #B0300**

volumes in microliter

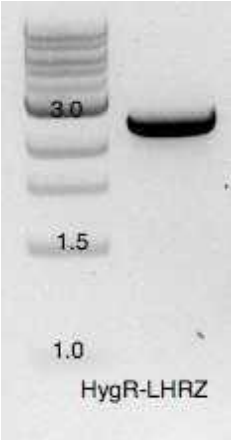
see oligo tables step 1 (above)

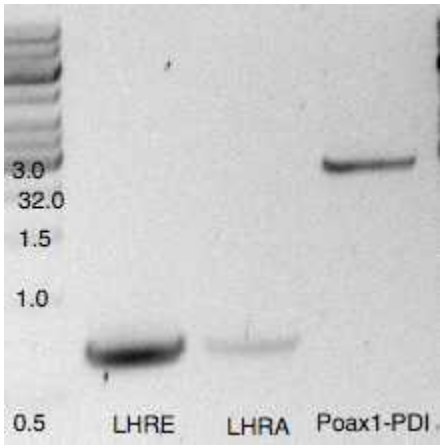
reagents	LHRA	Paox1PDIhis	LHRE	HygR-LHRZ
5xQ5 buffer	10	10	10	10
betaine 5M	10	10	10	10
10mM dNTP (TAKARA)	4	4	4	4
oligo 301/ oligo 315	2.5/2.5			
oligo 302/ oligo 303		2.5/2.5		
oligo 336/ oligo 350			2.5/2.5	
oligo 306/ oligo 317				2.5/2.5

	reagents	LHRA	Paox1PDIhis	LHRE	HygR-LHRZ
	eDA8 100ng/ul	1			
	eDA226 (see 1.1) 100ng/ul		1		
	eDA9 100ng/ul			1	
	eDa115 100ng/ul				1
	Q5 HF enzyme	0.7	0.7	0.7	0.7
	water	up to 50 uL	up to 50	up to 50	up to 50

	program Q5	B	C	D
	Td-initial	98oC	30sec	
	Td	98oC	10sec	33 cycles
	Ta	58oC	20sec	
	Te	72oC	2min 30sec	
	Final extension	72oC	1 min	
	hold	4oC	hold	

All PCR products purified by QIAquick PCR Purification Kit **Qiagen Catalog #28104** and analysed on 1% agarose gel (with Thiazole Orange Dye powder **Merck MilliporeSigma (Sigma-Aldrich)**)





	size (bp)	concentration (ng/ul)	Molar concentration (nM)
LHRA	883	28	48
Poax1PDI	2905	64	34
LHRE	892	82	142
HygR-LHRZ	2583	101	60

Digestion with BsaI-HF - 1,000 units **New England Biolabs Catalog #R3535S** or Esp3I - 300 units **New England Biolabs Catalog #R0734S**

reagents	LHRA	Paox1PDIhis	LHRE	HygR-LHRZ
LHRA 315/301	46			
Paox1PDI 302/303		46		
LHRE 336/335			46	
HygR-LHRZ 306/317				46
BsaI HF (20U/ul)	2		2	2
Esp3I (10U/ul))		3		
buf10x cutsmart	6	6	6	6

	reagents	LHRA	Paox1PDIhis	LHRE	HygR-LHRZ
	water	up to 60uL	up to 60uL	up to 60uL	up to 60uL

All PCR products purified by QIAquick PCR Purification Kit **Qiagen Catalog #28104** and DNA concentration checked on DeNovix DS-11

DNA molar concentration calculation before ligation

	A	B	C	D
		size (bp)	concentration (ng/ul)	Molar concentration (nM)
	LHRA	883	28	48
	Poax1PDI	2905	64	34
	LHRE	892	82	142
	HygR-LHRZ	2583	101	60

1.3 **STEP 2** - preparation of semi-products, a partial ligated arrays

1h

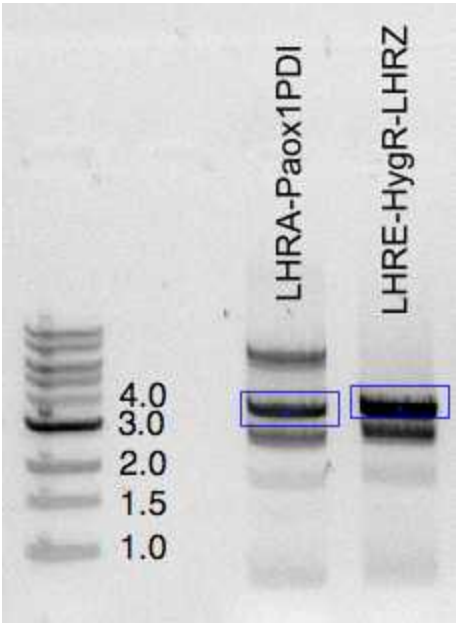
	reagents	LHRA-Paox1PDI	LHRE-HygR-LHRZ
		expected size ~3.7kb	expected size ~3.47kb
	2x T7 ligase buffer	15	15
	HygR-LHRZ	-	10
	LHRa	5.7	-
	LHRE	-	4
	Poax1PDI	8.3	-
	T7 DNA ligase	1	1
	water	-	-
		ul	ul

Ligation reaction

01:00:00 25 °C

All ligation mixtures loaded on 1% agarose gel and separated to excise a corresponding size

band



DNA bands representing expected size were excised and purified using PureLink™ Quick Gel Extraction Kit **Thermo Fisher Catalog #K210012**

DNA concentration calculated by DeNovix DS-11

	A	B	C
		ng/uL	Molar concentration (nM)
	LHRA-Paox1PDI	10	4
	LHRE-HygR-LHRZ	16	7

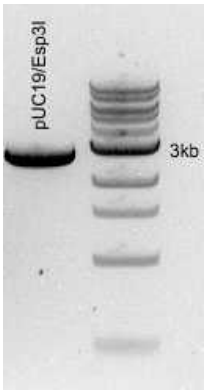
1.4 pUC19 digestion with Esp3I

Digestion with Esp3I - 300 units **New England Biolabs Catalog #R0734S**

	reagents	vector

	reagents	vector
	pUC19 500ng/uL	5 uL
	Esp3I	2 ul
	rCutsmart 10X	4 ul
	water	up to 40 uL

Digestion mixture cleanup QIAquick PCR Purification Kit **Qiagen Catalog #28104** and DNA concentration checked on DeNovix DS-11 and 1%agarose gel



	ng/uL	Molar concentration (nM)
pUC19/Esp3I	35	19

1.5 STEP 3 - the final ligation of pre-assembled parts with Esp3I-linearized pUC19, E.coli transformation and colony PCR verification

17h

T4 DNA Ligase - 20,000 units **New England Biolabs Catalog #M0202S**

	nM	ml
T4 DNA ligase		1
10x T4 ligase buffer		1.5
LHRA-Paox1PDI (4nM)	4	7
LHRE-HygR-LHRZ (7nM)	7	4

pUC19/Esp3I (19nM)	19	1.5
water		-

Overnight 16 °C

E.coli transformation (DH5alpha chemically LiAc competent cells)E.coli chemical competent cells

Selection on LB +100ug/mL +

Carbenicillin, disodium salt **Bio Basic Inc. Catalog #CDJ469.SIZE.1g** and growth at

Overnight 37 °C

Several colonies obtained on both agar plates. Single colonies transferred on new LB agar plates +100 ug/mL carbanicillin + 150 ug/mL hygromycin B

Carbenicillin, disodium salt **Bio Basic Inc. Catalog #CDJ469.SIZE.1g**

Hygromycin B, 50mg/ml solution (in PBS), sterile **Bio Basic Inc. Catalog #BS725.SIZE.2ml**

Growth overnight and a single colonies (8 colonies) submitted for bacterial colony PCR.
see protocol colony PCR
Material loaded on agarose gel for verification. 2 of 8 colonies verified positively using oligo 24/241. (expected 1.1kb amplicon) and 42/394 (0.91kb amplicon).

Colony 1 and 2 inoculated into LB + carbanicillin + hygromycin and growth Overnight
Plasmids isolated by plasmid isolation (miniprep) Qiagen miniprep 01:00:00
Sanger sequencing confirmed the sequence of eDA227

eda227 LHRA-PDI-LHRE-HYGR_LHRZ.dna

Preparation of double-LHR integration array LHRE-PtefGFP-.. 30m

2 DNA parts preparation by PCR reactions LHRE+PtefGFP (1st pre-assembly ligation mixture)

List of DNA parts

	DNA part	Forward oligo	Revers oligo	product	template
	LHRE (OUT) (BsaI)	F335-CGGTCTCCggaGCGATCGCGACTTACCTCGTTTTAACTTAGTCGG	R338-GGGTCTCCtgtcACAAGTTTAACTAAGCGTCAGCC	900 bp	eDA9
	PtefGFP (BsmBI)	F302a-CCGTCTCCgacaAACATCCAAAGACGAAAGGTTG	R304a-CCGTCTCCctacGCCTTCGAGCGTCCC	1442 bp	eDA189*
	ZeoR-LHRZ (BsmBI)	F308-CCTGTCGACGGTCTCAgtagGACATGGAGGCCCAGAATACCC	R318-GGGGTACCGGTCTCAcgcgGCGATCGCCCTGCAGGTTGAGTTGGCGAAGGTGCG	2171 bp	eDA105**

* eDA189 map .dna file eda189.dna

**link https://www.protocols.io/view/preparation-of-parts-hygr-lhrz-and-zeor-lhrz-cqujvwun

2.1 **STEP 1** - PCR amplification of parts LHRE(out)+PtefGFP and digestion with suitable IIS RE

6h

Parts amplified by PCR (using ProFlex PCR system, Applied Biosystems). Thermal Cycler

Q5 High-Fidelity DNA Polymerase - 500 units **New England Biolabs Catalog #M0491L**

Betain solution 5M **Merck MilliporeSigma (Sigma-Aldrich) Catalog #B0300**

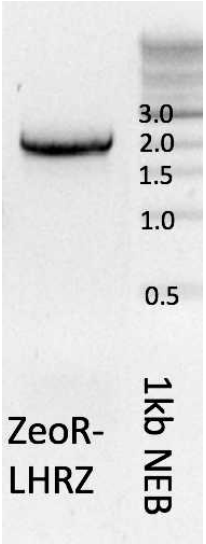
volumes in microliter

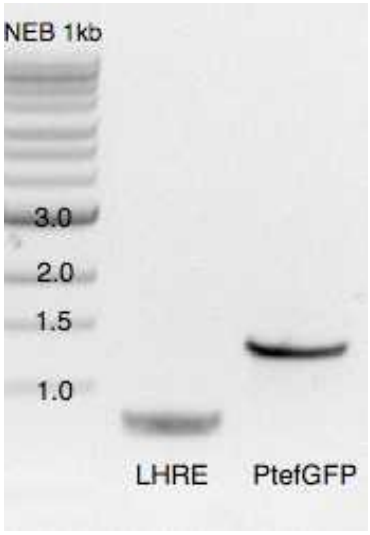
	reagents	LHRE (out)	PtefGFP	ZeoR-LHRZ
	Q5 HF enzyme	0.7	0.7	0.7
	10mM dNTP (TAKARA)	4	4	4
	water	up to 50 uL	up to 50	up to 50
	oligo 302a/ oligo 304a		2.5/2.5	
	oligo 335/ oligo 338	2.5/2.5		

	reagents	LHRE (out)	PtefGFP	ZeoR-LHRZ
	oligo 308/ oligo 318			2.5/2.5
	eDA189 100ng/ul		1	
	eDA9 100ng/ul	1		
	eDa105 100ng/ul			1

	program Q5	B	C	D
	Td-initial	98oC	30sec	
	Td	98oC	10sec	33 cycles
	Ta	58oC	20sec	
	Te	72oC	2min 10sec	
	Final extension	72oC	1 min	
	hold	4oC	hold	

All PCR products purified by QIAquick PCR Purification Kit **Qiagen Catalog #28104** and analysed on 1% agarose gel (with Thiazole Orange Dye powder **Merck MilliporeSigma (Sigma-Aldrich)**)





Digestion with BsaI-HF - 1,000 units **New England Biolabs Catalog #R3535S** or Esp3I - 300 units **New England Biolabs Catalog #R0734S**

	reagents	LHRE	PtefGFP	ZeoR-LHRZ
	LHRE 335/338	46		
	PtefGFP 302a/304a		46	
	ZeoR-LHRZ 308/318			46
	BsaI HF (20U/ul)	2		
	Esp3I (10U/ul))		3	3
	buf10x cutsmart	6	6	6
	water	up to 60uL	up to 60uL	up to 60uL

Reaction 37 °C 06:00:00

All PCR products purified by QIAquick PCR Purification Kit **Qiagen Catalog #28104** and DNA concentration checked on DeNovix DS-11

DNA molar concentration calculation before ligation

	A	B	C	D
		size (bp)	concentration (ng/ul)	Molar concentration (nM)
	LHRE out	900	66	113
	PtefGFP	1442	92	98
	ZeoR-LHRZ	2171	111	78

2.2 **STEP 2** - preparation of semi-product, a partial ligated array LHRE-PtefGFP

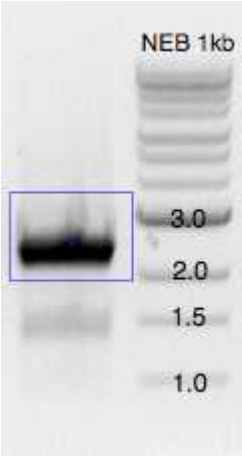
1h

	reagents	LHRE-PtefGFP
		expected size ~2.3kb
	2x T7 ligase buffer	15
	LHRE (out)	6.5
	PtefGFP	7.5
	T7 DNA ligase	1
	water	-
		ul

Ligation reaction

01:00:00 25 °C

Ligation mixture loaded on 1% agarose gel and separated to excise a corresponding size band



DNA band representing expected size was excised and purified using

PureLink™ Quick Gel Extraction Kit **Thermo Fisher Catalog #K210012**

DNA concentration calculated by DeNovix DS-11

	A	B	C	D	E
		size kbp	ng/uL	Molar concentration (nM)	
	LHRA-PtefGDP	~2.3	55	37	
	ZeoR-LHRZ*	2.17	101	72	

2.3 STEP 3 - the final ligation of pre-assembled parts with Esp3I-linearized pUC19, E.coli transformation and colony PCR verification

17h

	A	B	C
		uL	nM
	T4 DNA ligase	1	
	10x T4 ligase buffer	1	
	LHRE-PtefGFP	2.3	37
	ZeoR-LHRZ	1.2	78
	pUC19/Esp3I	4.5	19

Overnight 16 °C

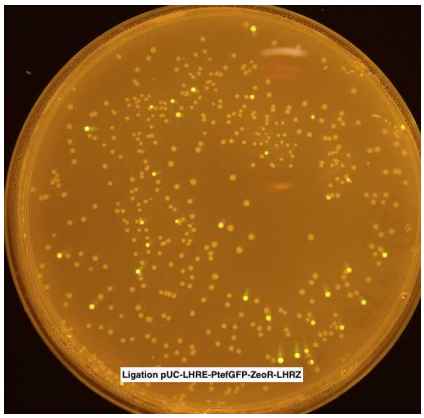
E.coli transformation (DH5alpha chemically LiAc competent cells)E.coli chemical competent cells

Selection on LB +100ug/mL carbanicillin + 50ug/mL zeocin

Carbenicillin, disodium salt **Bio Basic Inc. Catalog #CDJ469.SIZE.1g**

Zeocin **Bio Basic Inc. Catalog #Z706211.SIZE.100mg** growth at Overnight

37 °C



Several fluorescent colonies obtained on both agar plates. Single colonies transferred on new LB agar plates +100 ug/mL carbanicillin + 50 ug/mL zeocin

Single fluorescent colonies inoculated on fresh LB + carbanicillin/zeocin and growth

Overnight

Next day a pasmid isolation (miniprep) Qiagen miniprep 01:00:00

Sanger sequencing confirmed the sequence of eDA197

eda197 assembly.dna

Preparation of double-LHR insertion array LHRE-PtefMCH-H... 7h

3 DNA parts preparation by PCR reactions

List of DNA parts

	A	B	C	D	E

	A	B	C	D	E
	DNA part	Forward oligo	Revers oligo	product	template
	LHRE (OUT) (BsaI)	F335-CGGTCTCCggaGCGATCGCGACTTACCTCGTTTTAACTTAGTCGG	R338-GGGTCTCCtgtcACAAGTTTAACTAAGCGTCAGCC	900 bp	eDA9
	PtefMCh (BsmBI)	F302a-CCGTCTCCgacaAACATCCAAAGACGAAAGGTTG	R304a-CCGTCTCCctacGCCTTCGAGCGTCCC	1442 bp	eDA191*
	HygR-LHRZ (BsaI)	F306-CCTGTCGACGGTCTCAgtagGACATGGAGGCCCAGAATACCC	R317-GGGGTACCGGTCTCAcgcgGCGATCGCCCTGCAGGTTGAGTTGGCGAAGGTGCG	2583 bp	eDA115**

* eDA191 map .dna file eda191.dna

**link https://www.protocols.io/view/preparation-of-parts-hygr-lhrz-and-zeor-lhrz-cqujvwun

3.1

STEP 1 - PCR amplification of parts

6h

LHRE(out) (done in section 2, step 2.1) with oligos 335/338

HygR-LHRZ (done in section 1, step 1.2) with oligos 306/317

PtefMCh amplification and digestion with suitable IIS RE

Parts amplified by PCR (using ProFlex PCR system, Applied Biosystems). Thermal Cycler

Q5 High-Fidelity DNA Polymerase - 500 units **New England Biolabs Catalog #M0491L**

Betain solution 5M **Merck MilliporeSigma (Sigma-Aldrich) Catalog #B0300**

volumes in microliter

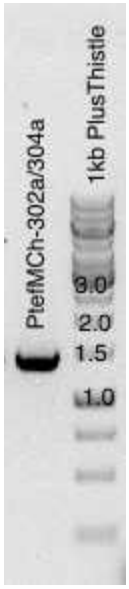
	reagents	PtefMCh
	Q5 HF enzyme	0.7
	10mM dNTP (TAKARA)	4
	water	up to 50

	reagents	PtefMCh
	oligo 302a/ oligo 304a	2.5/2.5
	eDA191 100ng/ul	1

volume in microliters

	program Q5	B	C	D
	Td-initial	98oC	30sec	
	Td	98oC	10sec	33 cycles
	Ta	58oC	20sec	
	Te	72oC	2min 10sec	
	Final extension	72oC	1 min	
	hold	4oC	hold	

PCR product purified by QIAquick PCR Purification Kit **Qiagen Catalog #28104** and analysed on 1% agarose gel (with Thiazole Orange Dye powder **Merck MilliporeSigma (Sigma-Aldrich)**)



Digestion with BsaI-HF - 1,000 units **New England Biolabs Catalog #R3535S** or Esp3I - 300 units **New England Biolabs Catalog #R0734S**

	reagents	TtefMCh
	Esp3I (10U/ul))	3
	PtefMCh 302a/304a	46
	buf10x cutsmart	6
	water	up to 60uL

Reaction 37 °C 06:00:00

All PCR products purified by QIAquick PCR Purification Kit **Qiagen Catalog #28104** and

DNA concentration checked on DeNovix DS-11

DNA molar concentration calculation before ligation

	A	B	C	D
		size (bp)	concentration (ng/ul)	Molar concentration (nM)
	LHRE out	900	66	113
	PtefMCh	1442	92	98
	HygR-LHRZ	2583	101	60

3.2 **STEP 2** - preparation of semi-product, a partial ligated array LHRE-PtefMCh 1h

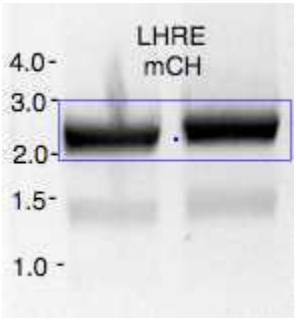
	reagents	LHRE-PtefMCh

	reagents	LHRE-PtefMCh
		ul
		expected size ~2.3kb
	PtefGFP	7.5
	LHRE (out)	6.5
	2x T7 ligase buffer	15
	T7 DNA ligase	1
	water	-

Ligation reaction (in duplicate)

01:00:00 25 °C

Ligation mixture loaded on 1% agarose gel and separated to excise a corresponding size band



DNA band representing expected size was excised and purified using

PureLink™ Quick Gel Extraction Kit **Thermo Fisher Catalog #K210012**

DNA concentration calculated by DeNovix DS-11

	A	B	C	D
		size kbp	ng/uL	Molar concentration (nM)
	LHRE-PtefMCH	~2.3	96	64
	HygR-LHRZ*	2.5	101	72

* HygR-LHRZ from step 1.2

3.3 STEP 3 - the final ligation of pre-assembled parts with Esp3I-linearized pUC19, E.coli transformation and colony PCR verification

17h

	uL	nM
T4 DNA ligase	1	
10x T4 ligase buffer	1	
LHRE-PtefMCH	1.6	64
ZeoR-LHRZ	1.4	72
pUC19/Esp3I	5	19

Overnight 16 °C

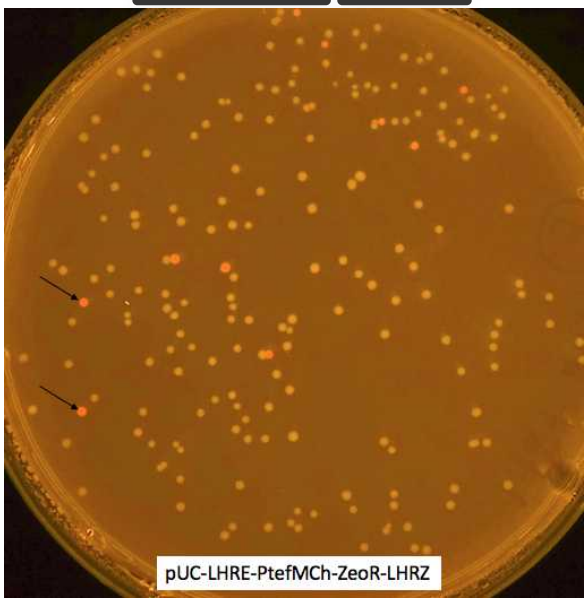
E.coli transformation (DH5alpha chemically LiAc competent cells)[E.coli chemical competent cells]

Selection on LB +100ug/mL carbanicillin + 150ug/mL hygromycin B

Carbenicillin, disodium salt **Bio Basic Inc. Catalog #CDJ469.SIZE.1g**

Hygromycin B, 50mg/ml solution (in PBS), sterile **Bio Basic Inc. Catalog #BS725.SIZE.2ml**

growth at Overnight 37 °C



Several fluorescent colonies obtained on both LB agar plates. Single colonies transferred on new LB agar plates +100 ug/mL carbanicillin + 50 ug/mL zeocin

Single fluorescent colonies inoculated on fresh LB + carbanicillin/hygromycin and growth

Overnight

Next day a pasmid isolation (miniprep) Qiagen miniprep 01:00:00

Sanger sequencing confirmed the sequence of eDA199

eda199 assembly.dna

Preparation of triple-LHR insertion array LHRA-GFP:CFH-LH...

1h

4 DNA parts preparation by PCR reactions

List of DNA parts

	DNA part	Forward oligo	Revers oligo	product	template
	LHRE (OUT) (BsaI)	F335-CGGTCTCCggaGCGATCGCGACTTACCTCGTTTTAACTTAGTCGG	R338-GGGTCTCCtgtcACAAGTTTAACTAAGCGTCAGCC	900 bp	eDA9
	PAOX1GFP:CFH (BsmBI)	F302-CCGTCTCCgacaAACATCCAAAGACGAAAGGTTG	R303-GCGTCTCCctgaTCTCACTTAATCTTCTGTACTCTG	5991bp	eDA89*
	LHRD (IN) (BsmBI)	F304-CCGTCTCCtcagGACAGCAACCTAACCGAC	R305-GCGTCTCCctacCAGTCCTCGTGAAAGACGAG	1105 bp	eDA9
	ZeoR-LHRZ (BsmBI)	F308-CCTGTCGACGGTCTCAgtagGACATGGAGGCCCAGAATACCC	R318-GGGGTACCGGTCTCAcgcgGCGATCGCCCTGCAGGTTGAGTTGGCGAAGGTGCG	2171 bp	eDA105**

* map eDA89.dna plasmid prepared as described in the paper (DOI.....PBarlow,DAbramczyk)

**link https://www.protocols.io/view/preparation-of-parts-hygr-lhrz-and-zeor-lhrz-cqujvwun

4.1 **STEP 1** - PCR amplification of parts and digestion with suitable IIS RE

Parts amplified by PCR (using ProFlex PCR system, Applied Biosystems). Thermal Cycler

Q5 High-Fidelity DNA Polymerase - 500 units **New England Biolabs Catalog #M0491L**

Betain solution 5M **Merck MilliporeSigma (Sigma-Aldrich) Catalog #B0300**

volumes in microliters

	reagents	LHRE (out)	Paox1GFP:CFH	LHRD (in)	ZeoR-LHRZ
	5xQ5 buffer	10	10	10	10
	betaine 5M	10	10	10	10
	10mM dNTP (TAKARA)	4	4	4	4
	oligo 335/ oligo 338	2.5/2.5			
	oligo 302/ oligo 303		2.5/2.5		
	oligo 304/ oligo 305			2.5/2.5	
	oligo 308/ oligo 318				2.5/2.5
	eDA89 100ng/uL		1		
	eDA9 100ng/ul	1		1	
	eDa105 100ng/ul				1
	Q5 HF enzyme	0.7	0.7	0.7	0.7
	water	up to 50 uL	up to 50	up to 50	up to 50

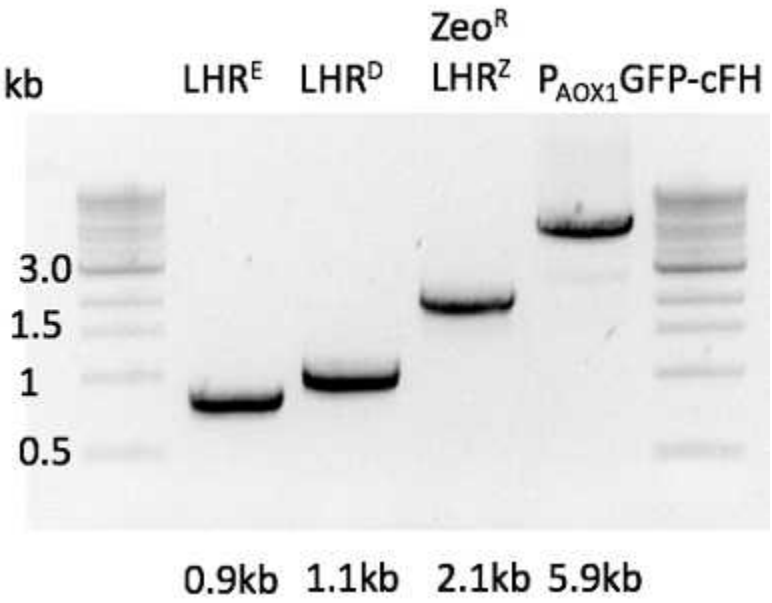
Part LHRE (out) generated as described above in protocol 2.1

Part ZeoR-LHRZ generated as described above in protocol 2.1

	program Q5	B	C	D
	Td-initial	98oC	30sec	
	Td	98oC	10sec	33 cycles
	Ta	58oC	20sec	
	Te	72oC	*1min 10 sec (for LHRD) ** 5min 30 sec for Poax1GFP:CFH	

	program Q5	B	C	D
	Final extension	72oC	1 min	
	hold	4oC	hold	

All PCR products purified by QIAquick PCR Purification Kit **Qiagen Catalog #28104** and analysed on 1% agarose gel (with Thiazole Orange Dye powder **Merck MilliporeSigma (Sigma-Aldrich)**)



Digestion with BsaI-HF - 1,000 units **New England Biolabs Catalog #R3535S** or Esp3I - 300 units **New England Biolabs Catalog #R0734S**

	reagents	LHRE	PaoxGFP:CFH	LHRD	ZeoR-LHRZ
	LHRE 335/338	46			

	reagents	LHRE	PaoxGFP:CFH	LHRD	ZeoR-LHRZ
	PaoxGFP:CFH 302/303		46		
	LHRD 304/305			46	
	ZeoR-LHRZ 308/318				46
	BsaI HF (20U/ul)	2			
	Esp3I (10U/ul))		3	3	3
	buf10x cutsmart	6	6	6	6
	water	up to 60uL	up to 60uL	up to 60uL	up to 60uL

All PCR products purified by QIAquick PCR Purification Kit **Qiagen Catalog #28104** and DNA concentration checked on DeNovix DS-11

DNA molar concentration calculation before ligati

	A	B	C	D
		size (bp)	concentration (ng/ul)	Molar concentration (nM)
	LHRE out	900	66	48
	PaoxGFPCFH	5991	123	34
	LHRD	1105	120	142
	ZeoR-LHRZ	2171	111	78

4.2 **STEP 2** - preparation of semi-products, a partial ligated arrays

1h

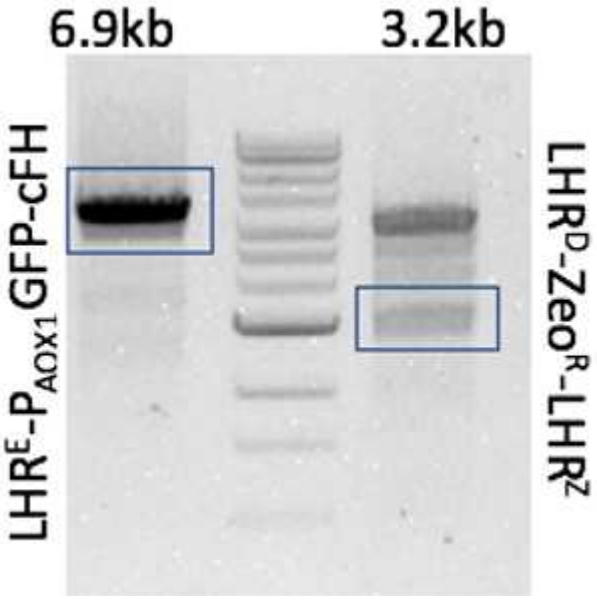
	reagents	LHRE-Paox1GFP:CFH	LHRD-ZeoR-LHRZ
	ZeoR-LHRZ	-	9
	LHRD	-	5
	water	-	-
	T7 DNA ligase	1	1

	reagents	LHRE-Paox1GFP:CFH	LHRD-ZeoR-LHRZ
	2x T7 ligase buffer	15	15
	LHRE out	5.8	-
	PoaxGFP:CFH	8.2	-
		expected size ~6.9kb	expected size ~3.2kb
		ul	ul

Ligation reaction

01:00:00 25 °C

All ligation mixtures loaded on 1% agarose gel and separated to excise a corresponding size band



DNA band representing expected size was excised and purified using

PureLink™ Quick Gel Extraction Kit **Thermo Fisher Catalog #K210012**

DNA concentration calculated by DeNovix DS-11

	ng/uL	Molar concentration (nM)
LHRE-PaoxGFPCFH	22	5
LHRD-ZeoR-LHRZ	9	4

4.3 STEP 3 - the final ligation of pre-assembled parts with Esp3I-linearized pUC19, E.coli transformation and colony PCR verification

17h

A	B	C
	nM	ml
T4 DNA ligase		1
10x T4 ligase buffer		1.5
LHRE-PaoxGFPCFH	5	5.0
LHRD-ZeoR-LHRZ	4	6.2
pUC19/Esp3I (19nM)	19	1.3
water		

Overnight 16 °C

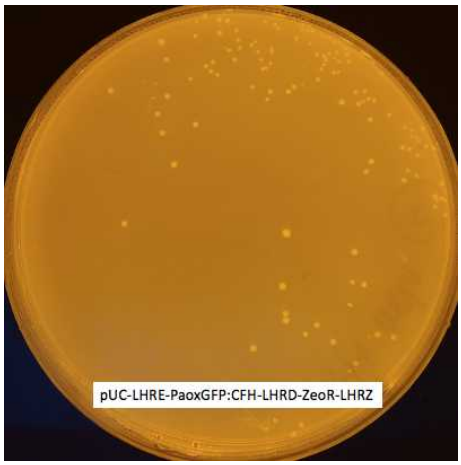
E.coli transformation (DH5alpha chemically LiAc competent cells)E.coli chemical competent cells

Selection on LB +100ug/mL + 50ug/mL

Carbenicillin, disodium salt **Bio Basic Inc. Catalog #CDJ469.SIZE.1g**

Zeocin **Bio Basic Inc. Catalog #Z706211.SIZE.100mg**

and growth at Overnight 37 °C

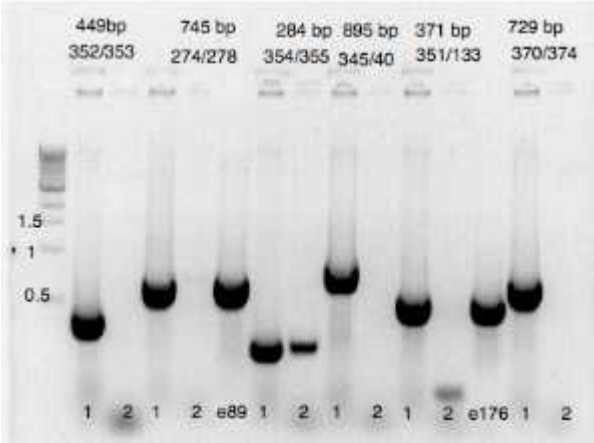


Note

No fluorescence observed, possibly not GFP leaking on yeast Paox1 promoter in E.coli

Several colonies obtained on both agar plates. Single colonies transferred on new LB agar plates +100 ug/mL carbanicillin + 50 ug/mL zeocin B.
Two of them submitted for bacterial colony PCR.
see protocol colony PCR

	A	B	C	D	E	F	G	H
		352/353	274/278	355/354	345/40	351/133	374/370	
			puc-E 0.45kb	GFP-FH 0.75kb	Aox-D 0.29kb	D-zeo 0.9kb	zeoc-C 0.6kb	0.7kb contr+
	1	clone1 (eDA250)	OK	OK	OK	OK	OK	OK
	2	clone2	none	none	poor	none	none	none



oligos positions on plasmid map in the file

Material loaded on agarose gel for verification. 1 of 2 colonies verified positively verified

The colony inoculated on fresh LB + carbanicillin/zeocin and growth Overnight

Next day a pasmid isolation (miniprep) Qiagen miniprep 01:00:00

Sanger sequencing confirmed the sequence of eDA250

puc19-E-PaoxGFPFH-D-ZeoC.dna

Plasmidsaurus sequencing confirms the correct sequence (added to .dna file)