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## nanochromosome arrays combinatory assembly

DOI

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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.



**Created:** February 20, 2023

**Last Modified:** July 16, 2023

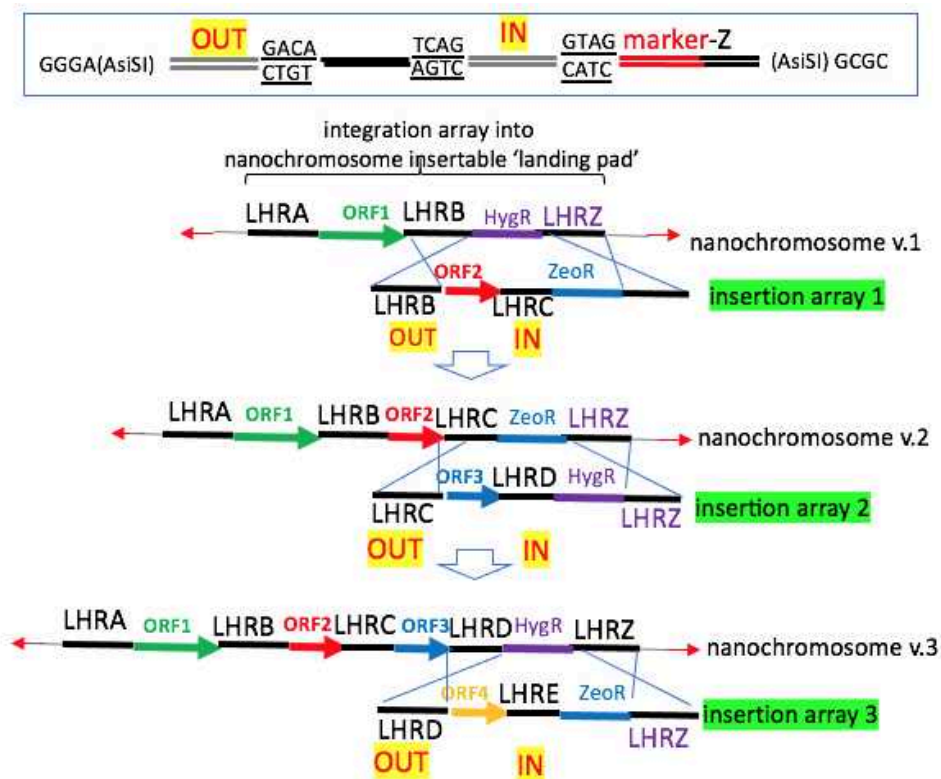
**Protocol Integer ID:** 77324

**Keywords:** nanochromosome array, nanochromosome, assembly the yeast pichia pastori, recombinant protein, yeast replication origin, assembled dna part, genetic engineering, expressing heterologous gene, mode of sequential gene integration, essential scaffolding features as telomere, dna arrays library, sequential gene integration, genome, value recombinant protein, genome in order, array of gene, heterologous gene, combinatory assembly, gene, dna sequence, yeast pichia pastori, modified protein, resistance gene, flanking oligonucleotide, dna part, molecular biology technique, chromosome, controlled insertion, dna band, insertion of multiple expression cassette, dna, replicating, t7 dna ligase, final ligation with linearized puc adaptor plasmid, stable synthetic construct, linear dsdna suitable for insertion

## 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-over/homologous 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;  $Gol^n$  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>

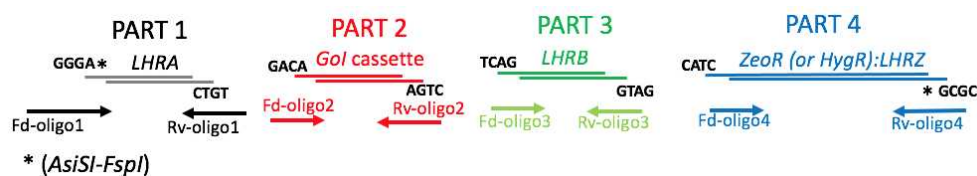
The protocol for multi-DNA parts arrays preparation is based on combinatory assembly and molecular biology techniques customised to create a DNA parts (arrays) library in pUC19 plasmid.

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)

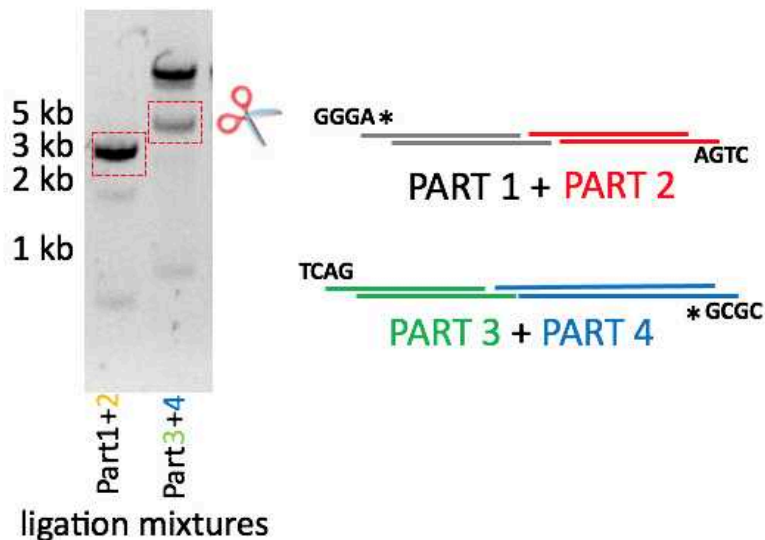
- a part generating by PCR, oligos deliver a complementary overhangs for digestion with BsmBI (Esp3I) or BsaI,
- PCR product digestion with BsmBI or BsaI
- checking digested PCR products on agarose gel
- PCR clean up OR gel extraction of DNA and clean up (only in the case if a PCR product is not homogenous)
- estimation of DNA concentration (molar)

Note: (\*) stands for AsiSI-FspI RE recognition site provides in Fd-oligo1 and RV-oligo4

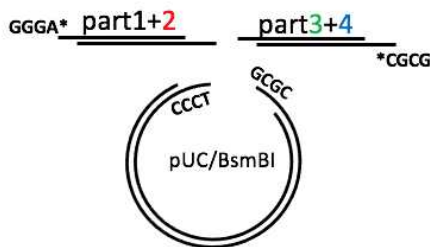


**Step 2** - preparation of semi-products before the the final ligation with linearized pUC adaptor plasmid

- 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'*



## Protocol materials

☒ Thiazole Orange Dye powder **Merck MilliporeSigma (Sigma-Aldrich)**

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

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

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

☒ Bsal-HF - 1,000 units **New England Biolabs Catalog #R3535S**

☒ Esp3I - 300 units **New England Biolabs Catalog #R0734S**

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

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

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

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

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

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

☒ MfeI-HF - 500 units **New England Biolabs Catalog #R3589S**

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

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

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

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

☒ PureLink<sup>®</sup> Quick Gel Extraction Kit **Thermo Fisher Catalog #K210012**

☒ Esp3I - 300 units **New England Biolabs Catalog #R0734S**

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

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

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

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

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

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

☒ Esp3I - 300 units **New England Biolabs Catalog #R0734S**

☒ Bsal-HF - 1,000 units **New England Biolabs Catalog #R3535S**

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



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

⊗ Thiazole Orange Dye powder **Merck MilliporeSigma (Sigma-Aldrich)**

⊗ PureLink<sup>®</sup>; Quick Gel Extraction Kit **Thermo Fisher Catalog #K210012**

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

⊗ Esp3I - 300 units **New England Biolabs Catalog #R0734S**

⊗ Thiazole Orange Dye powder **Merck MilliporeSigma (Sigma-Aldrich)**

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

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

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

⊗ BsaI-HF - 1,000 units **New England Biolabs Catalog #R3535S**

⊗ 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**

⊗ PureLink<sup>®</sup>; Quick Gel Extraction Kit **Thermo Fisher Catalog #K210012**

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

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

⊗ Thiazole Orange Dye powder **Merck MilliporeSigma (Sigma-Aldrich)**

⊗ Esp3I - 300 units **New England Biolabs Catalog #R0734S**

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

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

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

⊗ BsaI-HF - 1,000 units **New England Biolabs Catalog #R3535S**

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

⊗ PureLink<sup>®</sup>; Quick Gel Extraction Kit **Thermo Fisher Catalog #K210012**

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

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

## Troubleshooting



## Preparation of triple-LHR integration array **LHRA-PAOXPDI-LHRE-HygR-LHRZ** 30m

### 1 DNA parts preparation by PCR reactions

List of DNA parts

| DNA part               | Forward oligo                                                                              | Revers oligo                                                                            | product       | template    |
|------------------------|--------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------|---------------|-------------|
| LHRA (OUT)<br>BsmBI    | <b>F315-<br/>CCGTCTCCg<br/>ggaGCGATC<br/>GCCTGTGTT<br/>AAACCGTC<br/>TTTAAGTCA<br/>ACCC</b> | <b>R301-<br/>GCGTCTCC<br/>tgtcCAAAC<br/>TAAGATTG<br/>GACTATTG<br/>CTATC</b>             | <b>883 bp</b> | <b>eDA8</b> |
| PAOX1PDIhi<br>s(BsmBI) | F302-<br>CCGTCTCCg<br>acaAACATCC<br>AAAGACGAA<br>AGGTTG                                    | R303-<br>GCGTCTCC<br>ctgaTCTCA<br>CTTAATCTT<br>CTGTACTC<br>TG                           | 2905 bp       | eDA226*     |
| LHRE (IN)<br>(BsaI)    | F336-<br>CGGTCTCct<br>cagGACTTAC<br>CTCGTTTTA<br>ACTTAGTCG<br>G                            | R350-<br>GGGTCTCC<br>ctacACAAG<br>TTTAACTAA<br>GCGTCAGC<br>C                            | 892 bp        | eDA9        |
| HygR-LHRZ<br>(BsaI)    | F306-<br>CCTGTCGAC<br>GGTCTCAgta<br>gGACATGGA<br>GGCCCAGAA<br>TACCC                        | R317-<br>GGGGTACC<br>GGTCTCAc<br>gcgGCGAT<br>CGCCCTGC<br>AGGTTGAG<br>TTGGCGAA<br>GGTGCG | 2583 bp       | eDA115**    |

\* see 1.1 for preparation (below)

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

### 1.1 Prestep - Generation of pPICZA with P<sub>OA</sub>X<sub>1</sub>PDIhis - (eDA226) Plasmid pPICZalphaPDI eDA143 (reference citation)

30m





## 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.

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

LINK

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

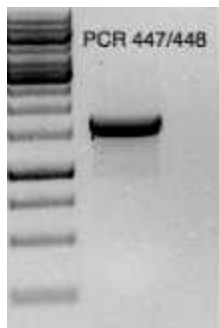
volumes in microliter

|  | reagents              | PDIhis   |
|--|-----------------------|----------|
|  | water                 | up to 50 |
|  | Q5 HF enzyme          | 0.7      |
|  | eDA143 100pg/uL       | 1        |
|  | betaine 5M            | 10       |
|  | oligo447/<br>oligo448 | 2.5/2.5  |
|  | 10mM dNTP (TAKARA)    | 4        |
|  | 5xQ5 buffer           | 10       |

|  | program Q5 | B    | C     | D |
|--|------------|------|-------|---|
|  |            |      |       |   |
|  |            |      |       |   |
|  | Td-initial | 98oC | 30sec |   |

|  | program Q5      | B           | C              | D         |
|--|-----------------|-------------|----------------|-----------|
|  | Td              | 98oC        | 10sec          | 33 cycles |
|  | Ta              | <b>55oC</b> | 20sec          |           |
|  | Te              | 72oC        | 1 min<br>40sec |           |
|  | Final extension | 72oC        | 2 min          |           |
|  | hold            | 4oC         | hold           |           |

|  | A    | B                                                                                  | C                                                                      |
|--|------|------------------------------------------------------------------------------------|------------------------------------------------------------------------|
|  | F447 | <i>PDI</i> part (MfeI)                                                             | CCCCAATTGACAAGCTTTT<br>GATTTTAACG                                      |
|  | R448 | <i>PDI</i> part (NotI)<br>introducing HIS-tag-TAA<br>stop codon ( <i>italics</i> ) | TTTTTGCGGCCGC TTAATG<br>ATGATGGTGGTGAACAAC<br>TCATCGTGAGCATCAGCTT<br>C |



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<br>100ng/uL           |              | 13                                                                |
| PCR<br>product of<br>447/448 | 48           |                                                                   |
| MfeI-<br>Hf (20u/uL)         | 1.5          | 1                                                                 |
| NotI-Hf<br>(20u/uL)          | 1.5          | 1                                                                 |
| rcutsmart                    | 8            | 6                                                                 |
| water                        | up to 80     | up to 60                                                          |
|                              |              | after<br>digestion<br>additional<br>dephosphorylation<br>reaction |
|                              |              | + 7uL AP<br>buffer + 2uL<br>Antarctic<br>Phosphatase              |

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

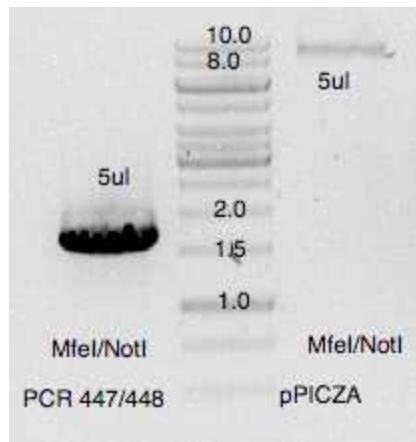
⊗ Antarctic Phosphatase - 1,000 units **New England Biolabs Catalog #M0289S** and

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<br>BamHI/KpnI | 1          |
| T4 DNA<br>ligase      | 1          |
| Paox1PDI<br>Mfe/NotI  | 1          |
| 10 X T4<br>buffer     | 6          |
| water                 | up to 10 → |
| pPICZA<br>Mfe/NotI    | 1          |

🕒 Overnight 16oC

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

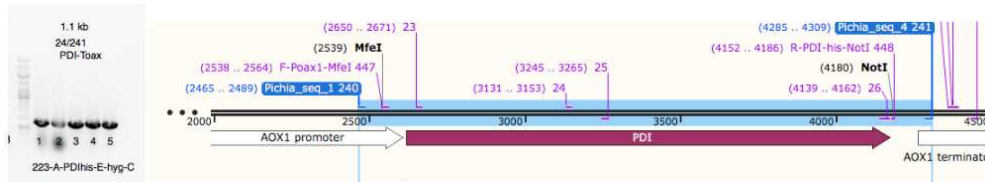
Selection on LB +100ug/mL

⊗ 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/<br>oligo 315 | 2.5/2.5 |             |      |           |

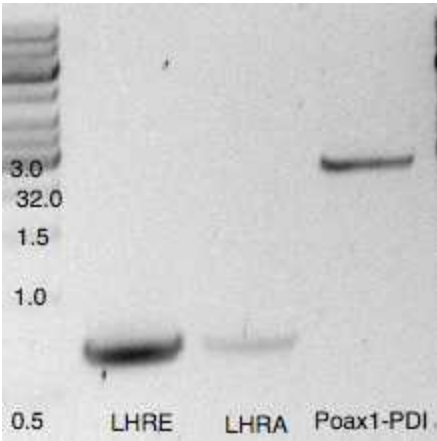
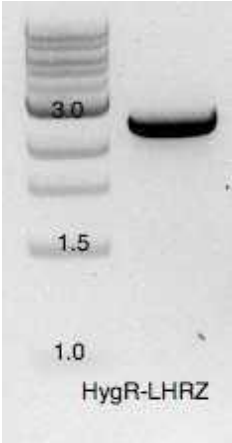
| reagents                        | LHRA        | Paox1PDIhis | LHRE     | HygR-LHRZ |
|---------------------------------|-------------|-------------|----------|-----------|
| oligo 302/<br>oligo 303         |             | 2.5/2.5     |          |           |
| oligo 336/<br>oligo 350         |             |             | 2.5/2.5  |           |
| oligo 306/<br>oligo 317         |             |             |          | 2.5/2.5   |
| eDA8<br>100ng/ul                | 1           |             |          |           |
| eDA226<br>(see 1.1)<br>100ng/ul |             | 1           |          |           |
| eDA9<br>100ng/ul                |             |             | 1        |           |
| eDa115<br>100ng/ul              |             |             |          | 1         |
| Q5 HF<br>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<br>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) | concentratio<br>n (ng/ul) | Molar<br>concentration<br>(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<br>315/301      | 46         |             |            |            |
| Paox1PDI<br>302/303  |            | 46          |            |            |
| LHRE<br>336/335      |            |             | 46         |            |
| HygR-LHRZ<br>306/317 |            |             |            | 46         |
| Bsal HF<br>(20U/ul)  | 2          |             | 2          | 2          |
| Esp3I<br>(10U/ul))   |            | 3           |            |            |
| buf10x<br>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 ligation

| A             | B            | C                        | D                              |
|---------------|--------------|--------------------------|--------------------------------|
|               | size<br>(bp) | concentration<br>(ng/ul) | Molar<br>concentration<br>(nM) |
| LHRA          | 883          | 28                       | 48                             |
| Paox1P<br>DI  | 2905         | 64                       | 34                             |
| LHRE          | 892          | 82                       | 142                            |
| HygR-<br>LHRZ | 2583         | 101                      | 60                             |

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

1h

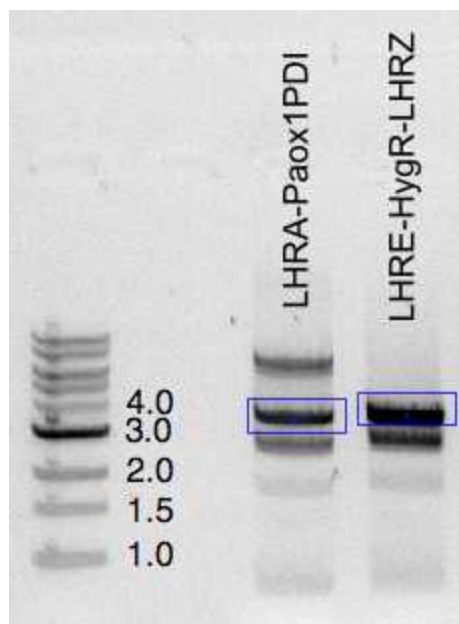


| reagents               | LHRA-Paox1PDI           | LHRE-HygR-LHRZ           |
|------------------------|-------------------------|--------------------------|
|                        | expected size<br>~3.7kb | expected size<br>~3.47kb |
| 2x T7 ligase<br>buffer | 15                      | 15                       |
| HygR-LHRZ              | -                       | 10                       |
| LHRa                   | 5.7                     | -                        |
| LHRE                   | -                       | 4                        |
| Poax1PDI               | 8.3                     | -                        |
| T7 DNA<br>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<sup>®</sup> 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                        |

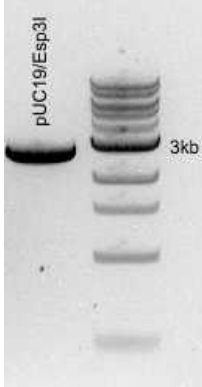
#### 1.4 pUC19 digestion with Esp3I

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

|  | reagents          | vector      |
|--|-------------------|-------------|
|  | pUC19<br>500ng/uL | 5 uL        |
|  | Esp3I             | 2 ul        |
|  | rCutsmart<br>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<br>conce<br>ntratio<br>n (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 carbenicillin + 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\_LH...

## Preparation of double-LHR integration array **LHRE-PtefGFP-ZeoR-LHRZ**

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)<br>(BsaI) | <b>F335-<br/>CGGTCTCCg<br/>ggaGCGATC<br/>GCGACTTAC<br/>CTCGTTTAA<br/>ACTTAGTCG<br/>G</b> | <b>R338-<br/>GGGTCTCC<br/>tgtcACAAG<br/>TTTAACTA<br/>AGCGTCAG<br/>CC</b>                | <b>900 bp</b> | <b>eDA9</b> |
| PtefGFP<br>(BsmBI)   | F302a-<br>CCGTCTCCg<br>acaAACATCC<br>AAAGACGAA<br>AGGTTG                                 | R304a-<br>CCGTCTCC<br>ctacGCCTT<br>CGAGCGTC<br>CC                                       | 1442 bp       | eDA189*     |
| ZeoR-LHRZ<br>(BsmBI) | F308-<br>CCTGTCGAC<br>GGTCTCAgta<br>gGACATGGA<br>GGCCCAGAA<br>TACCC                      | R318-<br>GGGGTACC<br>GGTCTCAc<br>gcgGCGAT<br>CGCCCTGC<br>AGGTTGAG<br>TTGGCGAA<br>GGTGCG | 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  |



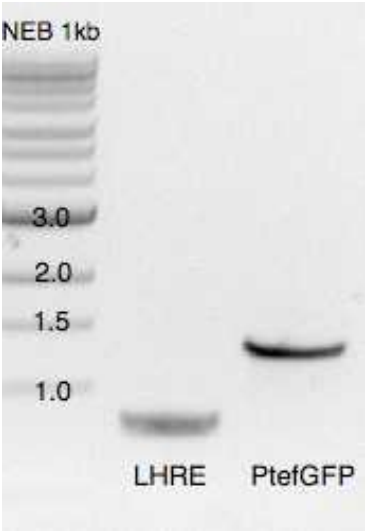
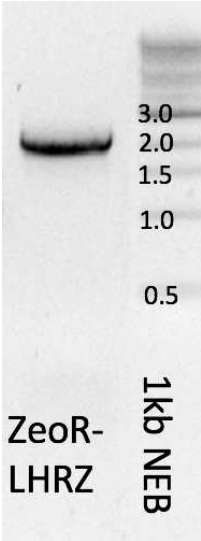
| reagents                  | LHRE (out) | PtefGFP | ZeoR-LHRZ |
|---------------------------|------------|---------|-----------|
| oligo 302a/<br>oligo 304a |            | 2.5/2.5 |           |
| oligo 335/<br>oligo 338   | 2.5/2.5    |         |           |
| oligo 308/<br>oligo 318   |            |         | 2.5/2.5   |
| eDA189<br>100ng/ul        |            | 1       |           |
| eDA9<br>100ng/ul          | 1          |         |           |
| eDa105<br>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<br>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<br>335/338      | 46   |         |           |
|  | PtefGFP<br>302a/304a |      | 46      |           |

| reagents          | LHRE       | PtefGFP    | ZeoR-LHRZ  |
|-------------------|------------|------------|------------|
| 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 |

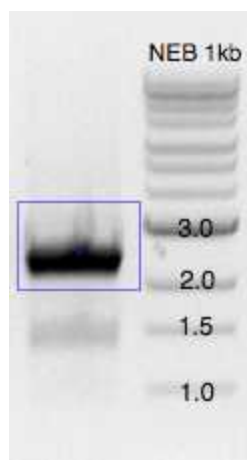


|  |                     |              |
|--|---------------------|--------------|
|  | reagents            | LHRE-PtefGFP |
|  | 2x T7 ligase buffer | 15           |
|  | LHRE (out)          | 6.5          |
|  | PtefGFP             | 7.5          |
|  | T7 DNA ligase       | 1            |
|  | water               | -            |
|  |                     | 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) |   |



|  | A            | B    | C   | D  | E |
|--|--------------|------|-----|----|---|
|  | 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/Esp 3I         | 4.5 | 19 |

Overnight

16 °C

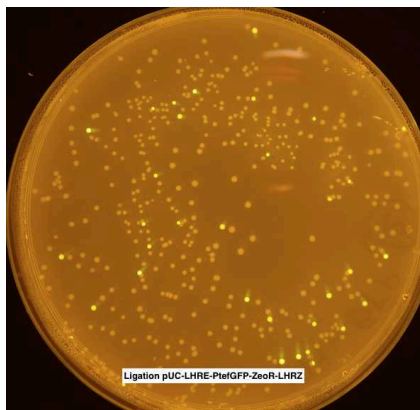
E.coli transformation (DH5alpha chemically LiAc competent cells)**E.coli chemical competent cells**

Selection on LB +100ug/mL carbenicillin + 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-HygR-LHRZ**

7h

### 3 DNA parts preparation by PCR reactions

List of DNA parts

| A                    | B                                                                           | C                                                            | D       | E        |
|----------------------|-----------------------------------------------------------------------------|--------------------------------------------------------------|---------|----------|
| DNA part             | Forward oligo                                                               | Revers oligo                                                 | product | template |
| LHRE (OUT)<br>(BsaI) | F335-<br>CGGTCTCC<br>gggaGCGAT<br>CGCGACTT<br>ACCTCGTT<br>TTAACTTAG<br>TCGG | R338-<br>GGGTCTCC<br>tgtcACAAGT<br>TTAACTAA<br>GCGTCAGC<br>C | 900 bp  | eDA9     |
| PtefMCh<br>(BsmBI)   | F302a-<br>CCGTCTCC<br>gacaAACAT<br>CCAAAGAC<br>GAAAGGTT<br>G                | R304a-<br>CCGTCTCC<br>ctacGCCTT<br>CGAGCGTC<br>CC            | 1442 bp | eDA191*  |

|  | A                | B                                                                       | C                                                                                       | D       | E        |
|--|------------------|-------------------------------------------------------------------------|-----------------------------------------------------------------------------------------|---------|----------|
|  | HygR-LHRZ (BsaI) | F306-<br>CCTGTCGA<br>CGGTCTCA<br>gtagGACAT<br>GGAGGCCC<br>AGAATACC<br>C | R317-<br>GGGGTACC<br>GGTCTCAc<br>gcgGCGAT<br>CGCCCTGC<br>AGGTTGAG<br>TTGGCGAA<br>GGTGCG | 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 Cyclers**



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 |
| oligo 302a/<br>oligo 304a | 2.5/2.5  |
| eDA191<br>100ng/ul        | 1        |

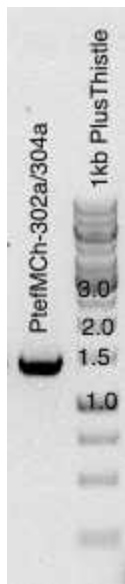
volume in microliters

|  | 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<br>(10U/ul))   | 3          |
| PtefMCh<br>302a/304a | 46         |
| buf10x<br>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<br>(bp) | conce<br>ntratio<br>n<br>(ng/ul) | Molar<br>concentration<br>(nM) |
|  |               |              |                                  |                                |
|  | LHRE<br>out   | 900          | 66                               | 113                            |
|  | PtefM<br>Ch   | 1442         | 92                               | 98                             |
|  | HygR-<br>LHRZ | 2583         | 101                              | 60                             |

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

1h

| reagents | LHRE-PtefMCh |
|----------|--------------|
|          | ul           |



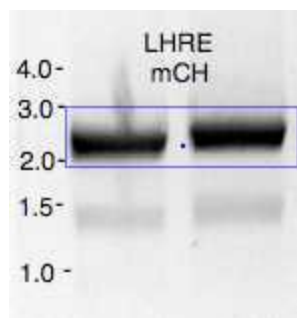
|  |                        |                         |
|--|------------------------|-------------------------|
|  | reagents               | LHRE-PtefMCh            |
|  |                        | expected size<br>~2.3kb |
|  | PtefGFP                | 7.5                     |
|  | LHRE (out)             | 6.5                     |
|  | 2x T7 ligase<br>buffer | 15                      |
|  | T7 DNA<br>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<br>concentration<br>(nM) |



|  | A            | B    | C   | D  |
|--|--------------|------|-----|----|
|  | 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**

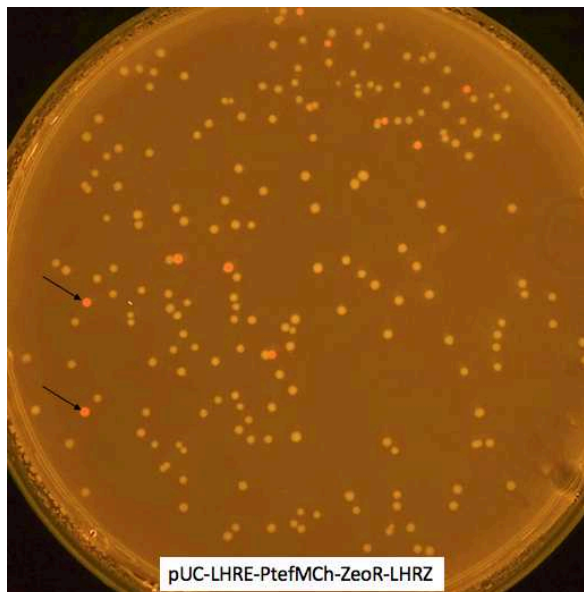
Selection on LB +100ug/mL carbenicillin + 150ug/mL hygromycin B

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-LHRE-ZeoR-LHRZ

1h

### 4 DNA parts preparation by PCR reactions

List of DNA parts

|  | DNA part             | Forward oligo                                                                            | Revers oligo                                                             | product       | template    |
|--|----------------------|------------------------------------------------------------------------------------------|--------------------------------------------------------------------------|---------------|-------------|
|  | LHRE (OUT)<br>(Bsal) | <b>F335-<br/>CGGTCTCCg<br/>ggaGCGATC<br/>GCGACTTAC<br/>CTCGTTTTA<br/>ACTTAGTCG<br/>G</b> | <b>R338-<br/>GGGTCTCC<br/>tgtcACAAG<br/>TTTAACTA<br/>AGCGTCAG<br/>CC</b> | <b>900 bp</b> | <b>eDA9</b> |

| DNA part                    | Forward oligo                                                       | Revers oligo                                                                            | product | template |
|-----------------------------|---------------------------------------------------------------------|-----------------------------------------------------------------------------------------|---------|----------|
| PAOX1GFP:<br>CFH<br>(BsmBI) | F302-<br>CCGTCTCCg<br>acaAACATCC<br>AAAGACGAA<br>AGGTTG             | R303-<br>GCGTCTCC<br>ctgaTCTCA<br>CTTAATCTT<br>CTGTACTC<br>TG                           | 5991bp  | eDA89*   |
| LHRD (IN)<br>(BsmBI)        | F304-<br>CCGTCTCct<br>cagGACAGC<br>AACCTAACC<br>GAC                 | R305-<br>GCGTCTCC<br>ctacCAGTC<br>CTCGTGAA<br>AGACGAG                                   | 1105 bp | eDA9     |
| ZeoR-LHRZ<br>(BsmBI)        | F308-<br>CCTGTCGAC<br>GGTCTCAgta<br>gGACATGGA<br>GGCCCAGAA<br>TACCC | R318-<br>GGGGTACC<br>GGTCTCAc<br>gcgGCGAT<br>CGCCCTGC<br>AGGTTGAG<br>TTGGCGAA<br>GGTGCG | 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         |



| reagents                | LHRE (out)  | Paox1GFP:CFH | LHRD (in) | ZeoR-LHRZ |
|-------------------------|-------------|--------------|-----------|-----------|
| oligo 335/<br>oligo 338 | 2.5/2.5     |              |           |           |
| oligo 302/<br>oligo 303 |             | 2.5/2.5      |           |           |
| oligo 304/<br>oligo 305 |             |              | 2.5/2.5   |           |
| oligo 308/<br>oligo 318 |             |              |           | 2.5/2.5   |
| eDA89<br>100ng/uL       |             | 1            |           |           |
| eDA9<br>100ng/ul        | 1           |              | 1         |           |
| eDa105<br>100ng/ul      |             |              |           | 1         |
| Q5 HF<br>enzyme         | 0.7         | 0.7          | 0.7       | 0.7       |
| water                   | up to 50 uL | up to 50     | up to 50  | up to 50  |

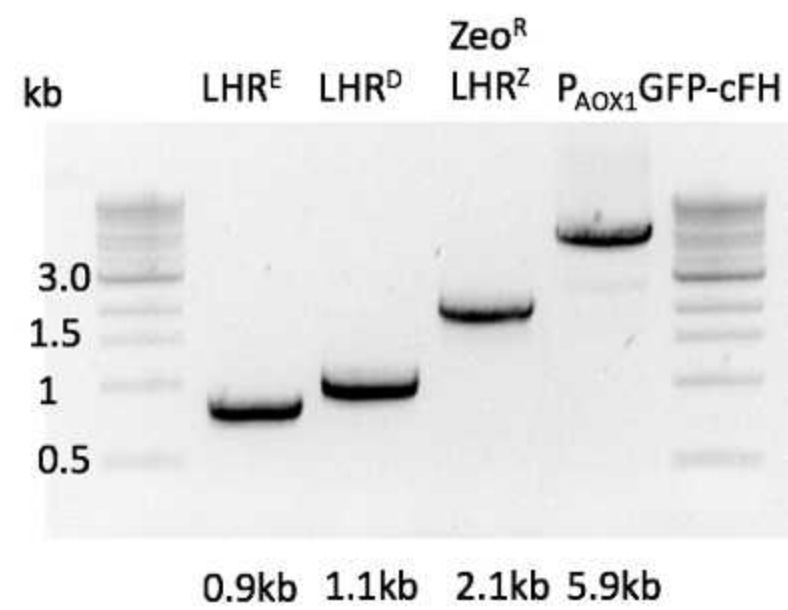
Part LHRE (out) generated as described above in protocol 2.1

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<br>sec (for<br>LHRD) **<br>5min 30 sec<br>for<br>Poax1GFP:C<br>FH |           |
| Final<br>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<br>335/338         | 46   |             |      |           |
| PaoxGFP:CF<br>H 302/303 |      | 46          |      |           |
| LHRD<br>304/305         |      |             | 46   |           |
| ZeoR-LHRZ<br>308/318    |      |             |      | 46        |
| BsaI HF<br>(20U/ul)     | 2    |             |      |           |



| reagents        | LHRE       | PaoxGFP:CFH | LHRD       | ZeoR-LHRZ  |
|-----------------|------------|-------------|------------|------------|
| 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) | concentratio<br>n (ng/ul) | Molar<br>concentration<br>(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

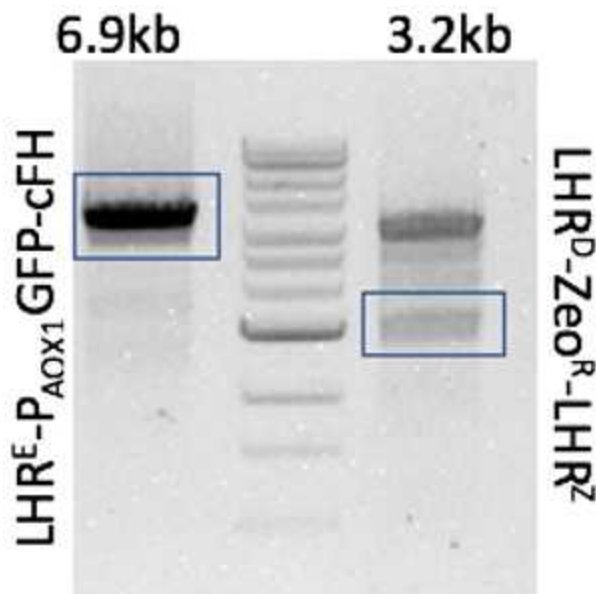
| reagents            | LHRE-Paox1GFP:CFH | LHRD-ZeoR-LHRZ |
|---------------------|-------------------|----------------|
| ZeoR-LHRZ           | -                 | 9              |
| LHRD                | -                 | 5              |
| water               | -                 | -              |
| T7 DNA ligase       | 1                 | 1              |
| 2x T7 ligase buffer | 15                | 15             |
| LHRE out            | 5.8               | -              |

| reagents    | LHRE-Paox1GFP:CFH       | LHRD-ZeoR-LHRZ          |
|-------------|-------------------------|-------------------------|
| PoaxGFP:CFH | 8.2                     | -                       |
|             | expected size<br>~6.9kb | expected size<br>~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

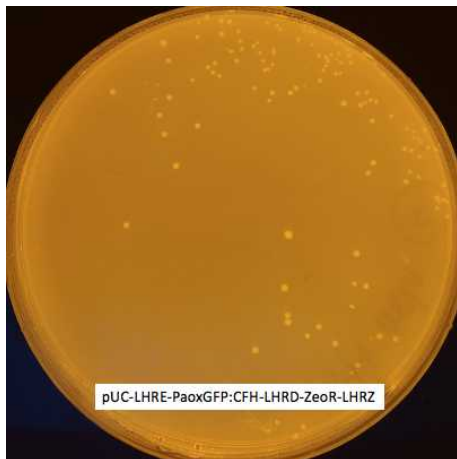
⊗ Carbenicillin, disodium salt **Bio Basic Inc. Catalog #CDJ469.SIZE.1g**

⊗ 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

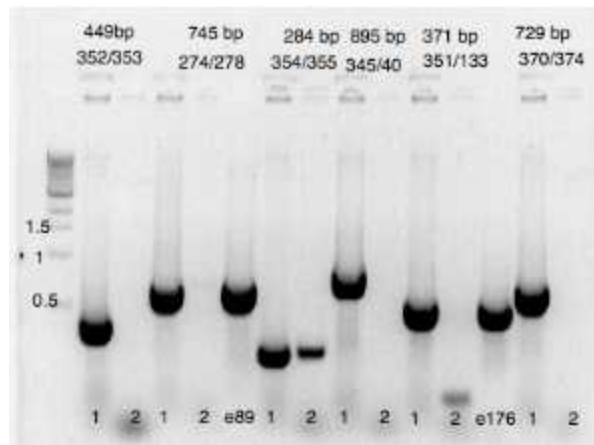
Several colonies obtained on both agar plates. Single colonies transferred on new LB agar plates +100 ug/mL carbanicillin + 50 ug/mL zeocin B.

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<br>0.45kb | GFP-FH<br>0.75kb | Aox-D<br>0.29kb | D-zeo<br>0.9kb | zeoc-C<br>0.6kb | 0.7kb<br>contr+ |
| 1 |   | clone1<br>(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)