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Preparation of parts HygR-LHRZ and ZeoR-LHRZ

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Protocol status: In development
We are still developing and optimizing this protocol. This is a protocol for preparation of a double-feature part (antibiotic selection marker- LHRZ)

Created: March 09, 2023

Last Modified: July 16, 2023

Protocol Integer ID: 78443

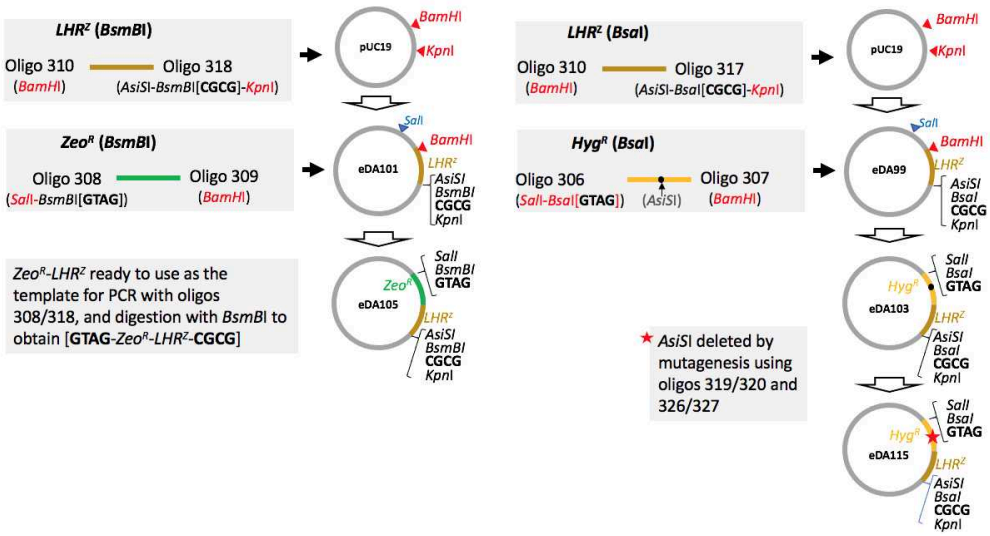
Keywords: lhrz this cloning protocol, preparation tandem pair hygr, insertion array before pichia transformation, combinatory assembly system for preparation, lhrz tandem pair, combinatory assembly system, homology recombination region, recombination process, cloning protocol, antibiotic selection marker, insertion array, pichia transformation, swapping hr, lhrz, hr event while the lhrz, preparation of parts hygr

Abstract

This cloning protocol is for preparation tandem pair HygR-LHRZ and ZeoR-LHRZ as a part of combinatory assembly system for preparation of integration/insertion arrays.
https://www.protocols.io/edit/nanochromosome-arrays-combinatory-assembly-cprkvm4w

Either the Hyg^R-LHR^Z or the Zeo^R-LHR^Z tandem pairs are designed to be use for all arrays and are displayed on 3'-end each of arrays.
During inchworming/swapping HR double cross-over recombination process, the antibiotic selection marker is replaced in every HR event while the LHRZ works as a homology recombination region used during all inchworming/swapping rounds.
(see https://www.protocols.io/edit/nanochromosome-arrays-combinatory-assembly-cprkvm4w)

Both parts are AsiSI free (this is for an optional excise site the cassette from the plasmid to isolate an insertion array before Pichia transformation)



General scheme for preparation library of AntR-LHRZ

Protocol materials

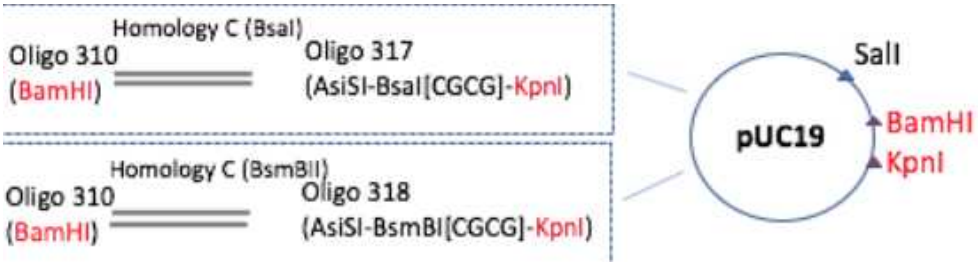
- BamHI-HF - 50,000 units **New England Biolabs** **Catalog #**R3136L
- KpnI-HF - 20,000 units **New England Biolabs** **Catalog #**R3142L
- T4 DNA Ligase - 20,000 units **New England Biolabs** **Catalog #**M0202S
- Carbenicillin
- Carbenicillin, disodium salt **Bio Basic Inc.** **Catalog #**CDJ469.SIZE.1g
- PrimeSTAR GXL Premix **Catalog #**R051B
- PrimeSTAR GXL Premix **Catalog #**R051B
- BamHI-HF - 10,000 units **New England Biolabs** **Catalog #**R3136S
- SalI-HF - 2,000 units **New England Biolabs** **Catalog #**R3138S
- T4 DNA Ligase - 20,000 units **New England Biolabs** **Catalog #**M0202S
- Carbenicillin, disodium salt **Bio Basic Inc.** **Catalog #**CDJ469.SIZE.1g
- PrimeSTAR GXL Premix **Catalog #**R051B
- Gibson Assembly Cloning Kit - 10 rxns **New England Biolabs** **Catalog #**E5510S
- AsiSI - 2,500 units **New England Biolabs** **Catalog #**R0630L
- 1 g Carbenoxolone Disodium **biorbyt** **Catalog #**orb320280

Troubleshooting

Cloning and preparation of HygR-LHRZ and ZeoR-LHRZ, integrative/insertion array lasting 3'-part

4h

1 Preparation of LHRZ - the first part of sequential cloning into pUC19



0.2 PCR preparation part LHRZ for cloning with KpnI/BamHI (3'-end of LHRZ with BsaI or BsmBI RE site delivered by an oligo)

2h

02:00:00

PrimeSTAR GXL Premix **Catalog** #R051B

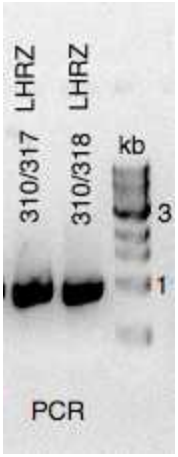
	A	expected size 998bp	expected size 998bp
	components	LHRZ for (BsmBI)	LHRZ for (BsaI)
	eDA8 (125pg/uL)	1	1
	oligo 310	1.25	1.25
	oligo317		1.25
	oligo 318	1.25	
	PrimeStar Premix 2x	25	
	water	up to 50	

LHR(non-coding DNA) delivered from the DNA library design by dr Valentin Zulkover and synthesised by prof Adele Marston **Adele Marston lab**

	A	B	C
	310 (F)	LHRZ (BamHI	CCGGATCC CCGTGTAG

	A	B	C
			GTCTACAA ACTGG
	318 (R)	LHRZ (KpnI-BsmBI-AsiSI)	GCGGTACC CGTCTCAc gcgGCGAT CGCCCTGC AGGTTGAG TTGGCGAA GGTGCG
	317 (R)	LHRZ (KpnI-BsaI-AsiSI)	GGGGTACC GGTCTCAc gcgGCGAT CGCCCTGC AGGTTGAG TTGGCGAA GGTGCG

	Program	time	number of cycles
	98oC	10 sec	33 x
	55oC	10sec	
	68oC	1min 30 sec	
	4oC	hold	



0.3 00:30:00 30m

PCR clean-up QIAquick PCR Purification Kit (Qiagen)**QIAquick PCR Purification Kit**

Elution with water 50uL

0.4 4h

KpnI and BamHI digestion of PCR product and pUC19 (all enzymes NEB) **BamHI**/ KpnI

KpnI-HF - 20,000 units **New England Biolabs Catalog #**R3142L

BamHI-HF - 50,000 units **New England Biolabs Catalog #**R3136L

	A	B	C	D
	10 x CutSmart	8	8	8
	BamHI-HF (20u/uL)	1.5	1.5	1
	KpnI-HF (20u/uL)	1.5	1.5	1
	PCR LHRZ 310/317	50		
	PCR LHRZ 310/318		50	
	pUC19 298 ng/uL			10
	water	up to 80 →		

RE digestion reactions

37 °C 04:00:00

0.5 00:30:00 30m

PCR clean-up QIAquick PCR Purification Kit (Qiagen)**QIAquick PCR Purification Kit**

Elution with water 50uL

Checking DNA concentration

LHRZ 310/317 (BamHI/KpnI) - ~100ng/ul

LHRZ 310/318 (BamHI/KpnI) - ~100ng/ul

0.6 Ligation reactions T4 DNA ligase (NEB)

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

	A	1	2
	pUC B/K ~100ng/uL) BamHI/KpnI	1	1

	A	1	2
	T4 DNA ligase	1	1
	LHRZ (100ng/ul) B/K(310/317)	5.5	
	10 X buffer	1	1
	water	up to 10 →	
	LHRZ (100ng/ul) (310/318) B/K		5.5

Overnight 16oC

0.7 02:00:00 2h

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

Selection on LB +100ug/mL Carbenicillin and growth at Overnight 37 °C

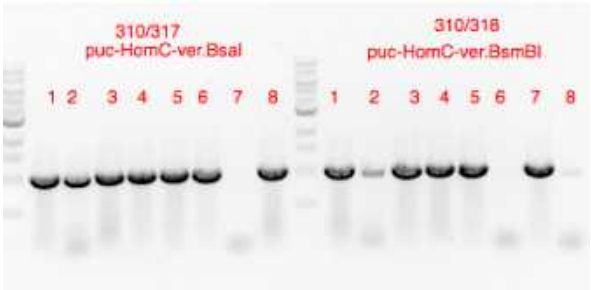
0.8 1h

01:00:00

Several colonies obtained on both agar plates. Single colonies transferred on new LB +100ug/mL ampicillin plate, growth overnight and a single colonies submitted for bacterial colony PCR.

see protocol **colony PCR**

Material loaded on agarose gel for verification



E.coli colony PCR

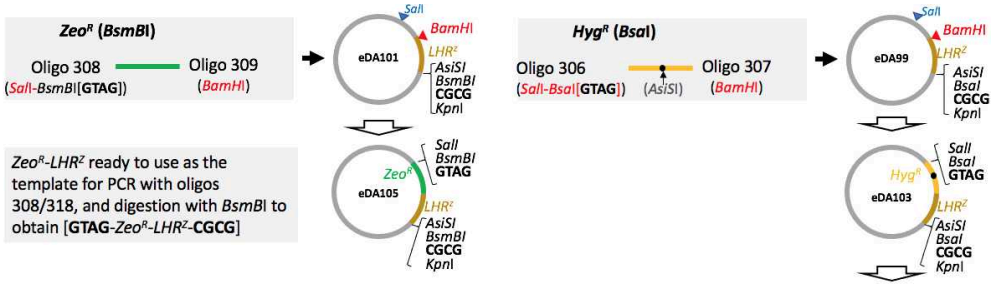
Positively verified clones re-cultured in LB + 100 ug/ml
Carbenicillin, disodium salt **Bio Basic Inc. Catalog** #CDJ469.SIZE.1g for miniprep (plasmid preparation) Overnight

0.9 Plasmid isolation (miniprep) Qiagen **miniprep** 01:00:00 1h

PCR verified and Sanger sequencing intermediate plasmid LHRZ(BsmBI)-pUC19 called eDA101 eda101.dna

PCR verified and Sanger sequencing intermediate plasmid LHRZ(BsaI)-pUC19 called eDA99 eDA99.dna

1 Preparation of ZeoR-LHRZ and HygR-LHRZ



The final step - insertion of ZeoR or HygR into plasmid eDA101 and eDA99 correspondingly

1.1 PCR amplification of Zeocin expression cassette (BsaI-free) for cloning with BsmBI
Zeocin and hygromycin cassettes originally derived from plasmids pGS-GnTI and pGS-GnTII (Glycoswitch) **glycoswitch plasmids**

PrimeSTAR GXL Premix **Catalog** #R051B

	components	ZeoR	HygR
	eDA48 (175 (ng/uL)		1
	eDA78 (125pg/uL)	1	
	oligo 308	1.25	
	oligo309	1.25	
	oligo 306		1.25
	oligo 307		1.25
	PrimeStar Premix 2x	25	25

	components	ZeoR	HygR
	water	up to 50	up to 50uL

	A	B	C
	308 (F)	Zeocin cassette (SalI-BsmBI) overhang GTAG	CTGTCGAC CGTCTCAgt agCCCACA CACCATAG CTTCAAAA TG
	309(R)	Zeocin cassette (BamHI)	GGGATCCG CAAATTAA AGCCTTCG AGCG
	306 (F)	HygR (SalI-BsaI)	CCTGTCGA CGGTCTCA gtagGACAT GGAGGCCC AGAATACC C
	307 (R)	HygR (BamHI)	GCGGATCC CAGTATAG CGACCAGC ATTCACATA C

1.2 00:30:00 30m

PCR clean-up QIAquick PCR Purification Kit (Qiagen)**QIAquick PCR Purification Kit**
Elution with water 50uL

1.3 SalI and BamHI digestion of PCR product and pUC19 (all enzymes NEB)**BamHI** 4h

BamHI-HF - 10,000 units **New England Biolabs Catalog #**R3136S

SalI-HF - 2,000 units **New England Biolabs Catalog #**R3138S

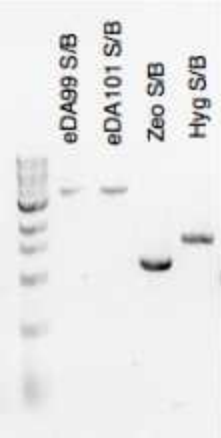
	components	B	C	D	E
	10x cutsmart buffer NEB	8	8	8	8
	BamHI-HF (20u/uL)	1.5	1.5	1.5	1.5

	components	B	C	D	E
	eDA99 70 ng/uL			30	
	eDA101 81 ng/uL				30
	PCR hygR 306/307		50		
	PCR zeo 308/309	50			
	SalI-HF (20u/uL)	1.5	1.5	1.5	1.5
	water	up to 80 →			

37 °C 04:00:00

1.4 00:30:00 30m

PCR clean-up QIAquick PCR Purification Kit (Qiagen)**QIAquick PCR Purification Kit**
Elution with water 50uL



loaded 2 ul of each fraction

1.5 Ligation reactions T4 DNA ligase (NEB)

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

	T4 DNA ligase	1	1
	10 X buffer	1	1

	eDA101 (S/B)		3
	zeo 308/309 (S/B)		5
	eDA99 (S/B)	3	
	hygR 306/307 (S/B)	5	
	water	up to 10 →	

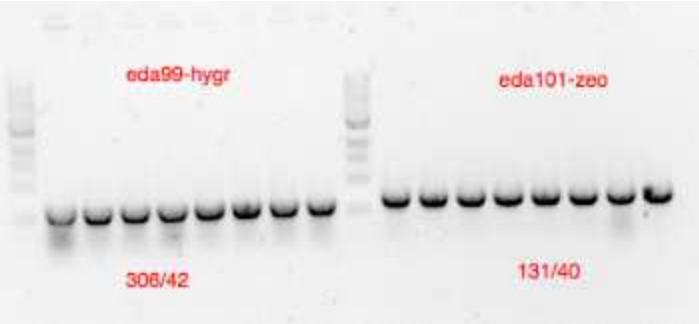
Overnight 16oC

1.6 02:00:00 2h

E.coli transformation (DH5alpha chemically LiAc competent cells)**E.coli chemical competent cells**
Selection on LB + 100ug/mL carbenicillin and growth at Overnight 37 °C
Carbenicillin, disodium salt **Bio Basic Inc. Catalog #**CDJ469.SIZE.1g

1.7 01:00:00 1h

.Several colonies obtained on both agar plates. Single colonies transferred on new LB +100ug/mL ampicillin plate, growth overnight and a single colonies submitted for bacterial colony PCR
see protocol **colony PCR**
(oligo 131/40 for verification ZeoR-LHRC-puC see plasmid map eda105.dna
(oligo 306/42 for verification HygR-LHRC-puC see plasmid map eda103.dna
Material loaded on agarose gel for verification



All colonies verified positively

Positively verified clones re-cultured in LB ampicillin for miniprep (plasmid preparation)
Overnight

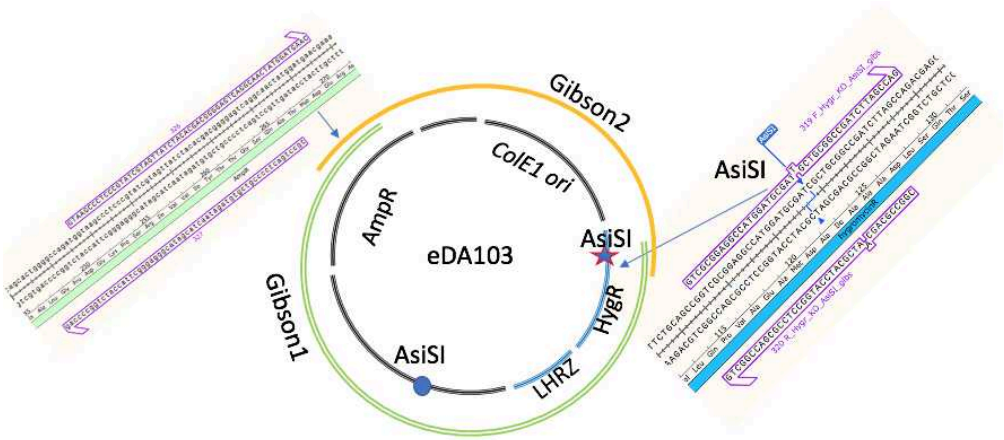
1.8 Plasmid isolation (miniprep) Qiagen **miniprep** 01:00:00 1h

PCR verified and Sanger sequencing intermediate plasmid ZeoR-LHRC-puC see plasmid eda105.dna

Tandem paired ZeoR-LHRZ part (BsmBI-free, AsiSI-free,) ready for cloning with BsmBI as a PCR template for a use in the protocol https://www.protocols.io/edit/nanochromosome-arrays-combinatory-assembly-cprkvm4w

PCR verified and Sanger sequencing intermediate plasmid HygR-LHRC-puC see plasmid map eda103.dna The part is BsaI-free but contains internal AsiSI in hph gene , requires mutagenesis

2 Mutagenesis of AsiSI in eDA103 plasmid by Gibson assembly



Schematics of mutagenesis of AsiSI site in HygR by Gibson assembly.

Note

eDA103 contains two AsiSI sites, but only AsiSI site in HygR needs to be removed as a part of integration/insertion array. Another AsiSi site (in pUC19 vector) is not taking under a consideration.

2.1 01:30:00 PCR amplification for further Gibson assembly reaction 2h

PrimeSTAR GXL Premix **Catalog #**R051B

PrimeStarHS 2x premix (Takara) High Fidelity

	components	for Gibson 1	for Gibson 2
	water	up to 50 uL	for Gibson 1
	Primestar2x	25	25
	oligos 327/320		1.25/1.25
	oligos 319/326	1.25/1.25	
	eDA103 (74pg/uL)	1.5	1.5

	PROGRAM TAK-2.30	time reaction	cycles
	98C	10sec	33x
	55C	10 sec	
	68C	2min 30 sec	
	4C	hold	

PCR reaction treatment with DpnI for 00:30:00 at 37 °C

2.2 00:30:00 PCR clean-up QIAquick PCR Purification Kit (Qiagen)**QIAquick PCR Purification Kit** 30m

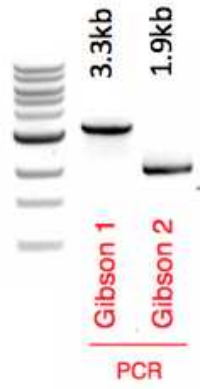
Elution with water 50uL.

DNA Molar concentration of purified PCR products was calculated using bioline conventor **DNA concentration convertor**

Gibson 1 fragment 86 ng/uL

Gibson 2 fragment 46 ng/ul

PCR products verified on 1% agarose gel (4 uL loaded)



2.3 Gibson assembly of 3.3kb fragment 1 and 1.9kb fragment 2 30m

Gibson Assembly Cloning Kit - 10 rxns **New England Biolabs Catalog #**E5510S

00:30:00 37 °C

	A	B
	Gibson1 319/326 (3.3kb) 40nM 3.3 kb	1
	Gibson2 327/320 (2.05 kb) 40nM	1
	Gibson 2x mix	2.5
	water	0.5

Gibson assembly protocol **protocol NEB Gibson Assembly Master Mix**

Immediately after Gibson reaction is E.coli transformation (DH5alpha chemically LiAc competent cells)**E.coli chemical competent cells**

Selection on LB +100ug/mL ampicillin and growth at Overnight 37 °C

2.4 Single colonies verified by colony PCR 1h

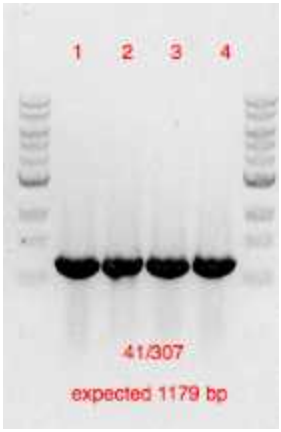
01:00:00

Several colonies obtained on both agar plates. Single colonies transferred on new LB +100ug/mL ampicillin plate, growth overnight and a single colonies submitted for bacterial colony PCR

see protocol **colony PCR**

Oligos 41 and 307 used for primary verification.

Oligo sequences available in the plasmid eDA115 map eda115.dna



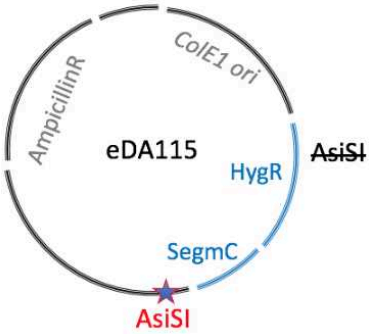
2.5 Positively verified clones re-cultured in LB + 100 ug/m 1 g Carbenoxolone Disodium **biorbyt Catalog #**orb320280 for miniprep (plasmid preparation) Overnight

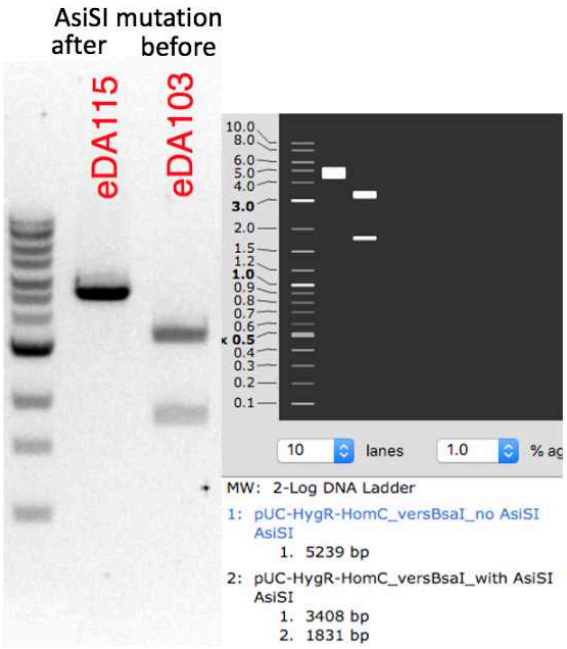
2.6 Plasmid isolation (miniprep) Qiagen **miniprep** 01:00:00 1h

2.7 Restriction digestion verification. Plasmid eDA115 eda115.dna digested with AsiSI (NEB)

AsiSI - 2,500 units **New England Biolabs Catalog #**R0630L

	A	B	C
	AsiSI	1	1
	eDA115 326 ng/uL	3	
	eDA103 103 ng/uL		8
	10 x CutSmart	3	3
	water	up to 30 →	





Tandem paired Hyg-LHRZ part (BsaI-free, AsiSI-free,) ready for cloning with BsaI as a PCR template for a use in the protocol https://www.protocols.io/edit/nanochromosome-arrays-combinatory-assembly-cprkvm4w