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Molecular Cloning- Gibson and LR reactions

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We use this protocol and it's working

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Abstract

The endoplasmic reticulum (ER) has a vast proteomic landscape to preform many diverse functions including protein and lipid synthesis, calcium ion flux, and inter-organelle communication. The ER proteome is remodeled in part through membrane-embedded receptors linking ER to degradative autophagy machinery (selective ER-phagy)1,2. A refined tubular ER network3,4 is formed in neurons within highly polarized dendrites and axons5,6. Autophagy-deficient neurons in vivodisplay axonal ER accumulation within synaptic ER boutons,7 and the ER-phagy receptor FAM134B has been genetically linked with human sensory and autonomic neuropathy8,9. However, mechanisms and receptor selectivity underlying ER remodeling by autophagy in neurons is limited. Here, we combine a genetically tractable induced neuron (iNeuron) system for monitoring extensive ER remodeling during differentiation. With this system, we imaged fixed iNeuron cultures, imaged these via confocal fluorescence microscopy, and quantified ER structures in axons.

Materials

Gateway™ LR Clonase™ II Enzyme mix (cat **11791020)**
TE Buffer Invitrogen (cat **AM9858)**
Thermo Fisher One Shot™ OmniMAX™ 2 T1 Phage-Resistant Cells (Cat. no. C8540-03)
Q5® Hot Start High-Fidelity 2X Master Mix (DNA Polymerase cat#M0494S)
Gibson Assembly using NEBuilder® HiFi DNA Assembly Master Mix (cat# E2621S)
dH2O
S.O.C.E media; Luria-Bertani (LB) broth (cat) or agar
QIAprep Spin Miniprep Kit (cat 27104)
antibiotics

Troubleshooting

Gateway technology cloning

1 LR reaction: Add pDONOR vectors and pDEST vectors to a Gateway™ LR Clonase™ II Enzyme mix supplemented with TE buffer. Gateway™ LR Clonase™ II Enzyme mix (cat **11791020)** https://www.thermofisher.com/order/catalog/product/11791020

• 1–7 µL entry clone (50–150 ng) • 1 µL destination vector (150 ng/µL) • TE buffer pH 8.0, to 8 µL

Incubate reaction overnight at room temperature. Add 1 µL of the Proteinase K solution to each sample and incubate sample sample 37C for 10 minutes.

2 Transformation: Take 1 µL of each LR reaction and add it to 50 µL of Thermo Fisher One Shot™ OmniMAX™ 2 T1 Phage-Resistant Cells (Cat. no. C8540-03). Incubate on ice for 30 minutes. Incubate at 42°C for 30 seconds to heat shock. Add 250 µL of S.O.C. Medium and incubate at 37°C for 1 hour with shaking for recovery. Plate 100 µL of each transformation onto selective LB agar plates.

Gibson technology cloning

3 Design pDonor using insert and backbone fragments and the NEB builder website **https://nebuilder.neb.com/**

4 Perform any necessary PCR reactions using Q5® Hot Start High-Fidelity 2X Master Mix (DNA Polymerase cat#M0494S) and the protocol associated with that PCR enzyme. https://www.neb.com/en-us/products/m0494-q5-hot-start-high-fidelity-2x-master-mix#Product%20Information

5 Linearize backbone as designed in the NEB builder website (either PCR or restriction enzyme digest)

6 Perform Gibson Assembly using NEBuilder® HiFi DNA Assembly Master Mix (cat# E2621S) https://www.neb.com/en-us/products/e2621-nebuilder-hifi-dna-assembly-master-mix#Product%20Information
2-3 Fragment mix strategy:
Assembly: Recommended DNA Molar Ratio Vector:insert = 1:2
Total Amount of Fragments 0.03-0.2 pmol*
NEBuilder HiFi DNA Assembly Master Mix 10 µl
Deionized H2O Total Volume 20 µl****
Incubate samples in a thermocycler at 50°C for 60 minutes

7 Transfrom Gibson reaction:

Take 2 µL of each Gibson reaction and add it to 50 µL of NEB 5-alpha(cat#C2987H). Incubate on ice for 30 minutes. Incubate at 42°C for 30 seconds to heat shock. Add 250 µL of S.O.C. Medium and incubate at 37°C for 1 hour with shaking for recovery. Plate 100 µL of each transformation onto selective LB agar plates.

Prep and full plasmid sequencing of final products

8 At either stage (after Gibson or LR reaction). Take colonies and start overnights (LB+antibiotic E.coli cultures). Miniprep overnight cultures with QIAprep Spin Miniprep Kit (cat 27104). Test digest for good plasmid candidates that look like they have the correct insert. Full plasmid sequence plasmids to confirm proper plasmid construction.