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Designing Overexpression Plasmids for Cellular Studies

In 1 collection

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We use this protocol and it's working

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Abstract

This protocol provides a detailed stepwise method for generating fluorescently tagged constructs using Gibson Assembly, enabling studies of protein localization and function in primary cultured neurons.

Materials

Plasmids and Genes:

- CMV mRuby3-Synapsin1a **addgene** **Catalog** #187896
- DDHD2 **Genscript** **Catalog** #OHu23306
- CPT2 **Genscript** **Catalog** #OHu17939D

Vectors and Promoters:

- plenti hSyn PGK1-HALO **addgene** **Catalog** #220910
- **Restriction Enzymes:** AgeI, SalI, XhoI, PmeI (Acquired from NEB)
- QIAquick Gel Extraction Kit (250) **Qiagen** **Catalog** #28706
- **Gibson Assembly:**
  NEBuilder HiFi DNA Assembly Master Mix - 50 rxns **New England Biolabs** **Catalog** #E2621L
- **PCR Reagents:** High-fidelity DNA polymerase

NEB Stable Competent E. coli:

- NEB Stable Competent E.coli (High Efficiency) - 20×0.05 ml **New England Biolabs** **Catalog** #C3040H
- PCR machine
- Gel electrophoresis apparatus
- UV gel imager
- Heat block or thermal cycler

PCR Amplification of Inserts

1 Design primers for PCR amplification of target genes, ensuring the inclusion of overlapping sequences compatible with Gibson Assembly and restriction sites as needed:

1.1 eGFP-DDHD2: From DDHD2_OHu23306C_pcDNA3.1(+)-N-eGFP, add XhoI and PmeI.

1.2 CPT2: From CPT2_OHu17939D_pcDNA3.1+/C-(k)DYK, add XhoI and PmeI.

2 Amplify target genes using a high-fidelity DNA polymerase to minimize errors.

3 Verify the PCR product sizes via gel electrophoresis and purify the bands using gel extraction and cleanup kit.

Restriction Digestion of Vectors

4 Digest the pLV vector with appropriate restriction enzymes:

4.1 For mRuby-Synapsin: AgeI and SalI.

4.2 For eGFP-DDHD2 and CPT2: XhoI and PmeI.

5 Resolve the digested vector on an agarose gel and purify the linearized vector using Qiagen gel extraction kit.

Gibson Assembly Reaction

1h

6 Prepare the Gibson Assembly reaction using NEBuilder® HiFi DNA Assembly Master Mix:

6.1 Mix linearized pLV vector and purified PCR-amplified inserts at a 1:3 molar ratio.

6.2 Add 10 µL of NEBuilder® HiFi DNA Assembly Master Mix.

6.3 Adjust the total reaction volume to 20 µL with nuclease-free water.

7 Incubate the reaction at 50 °C for 01:00:00. 1h

Transformation of Competent E. coli 1h

8 Transform 5 µL - 10 µL of the Gibson Assembly reaction into competent E. coli.

9 Plate the transformed cells on LB agar plates with appropriate antibiotics.

10 Incubate the plates Overnight at 37 °C. 1h

Verification of Clones

11 Screen colonies by colony PCR or restriction digestion to confirm successful assembly of the constructs.

12 Sequence positive clones to verify the integrity of the constructs.

Plasmid Preparation 1h

13 Grow positive colonies in LB broth with antibiotics Overnight. 1h

14 Extract plasmid DNA using a plasmid purification kit.

Note

- Primer design is critical for successful Gibson Assembly; ensure adequate overlapping sequences (>15 bases) for seamless assembly.
- Use high-fidelity enzymes for PCR to prevent mutations in amplified inserts.
- Handle restriction enzymes and Gibson Assembly reagents on ice to maintain activity.
- Verify plasmid sequences to ensure correct insert orientation and integrity.