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FAST-HDR Vector System User Guide

FAST-HDR Plasmid Extraction.

Please follow the Addgene protocol "*Streaking and Isolating Bacteria on an LB Agar Plate*" (https://www.addgene.org/protocols/streak-plate/) to generate bacterial colonies from the stab bacterial culture with a FAST-HDR plasmid. Culture 4 colonies in LB media, extract the plasmids from an aliquot of the culture, and perform double digestion with a mix of BamHI-KpnI for agarose gel analysis. The expected bands' sizes for each plasmid are included in the following table:

Plasmid	Size	Bands After Double Digestion with BamHI-KpnI
FAST-HDR mClover3 Puromycin	5717 bp	2676 bp, 1679 bp, 681 bp
FAST-HDR mRuby3 Zeocin	5423 bp	2676 bp, 1385 bp, 681 bp
FAST-HDR mTAGBFP2 Blasticidin	5438 bp	2676 bp, 1400 bp, 681 bp
FAST-HDR NanoLuc Puromycin	5447 bp	2676 bp, 1409 bp, 681 bp
FAST-HDR HaloTag Puromycin	5855 bp	2676 bp, 1817 bp, 681 bp

Select a plasmid with the right digestion pattern and save a glycerol stock from the culture of that colony for future use.

Procedure for cloning recombination arms into a FAST-HDR Vector.

Use the following templates for designing the recombination arms by replacing the (X) with the desired sequence of the recombination arm and order the complete sequences as gBlocks from Integrated DNA technologies (IDTDNA).

Left Arm template:
GACGTTGTAAAACGACGGCCAGTGGGTACCxxxxxxxxxxxxxxxxxxxGGTACCCAAGG
CGGTGGAGAATTC

Right Arm template:
CAAGTCCCTGCGGTGTCTTTGCTTGGATCCxxxxxxxxxxxxxxxxxxxGGATCCCGGGC
CCGTCGACTGCAGAGGCCT

1. Digest 500 ng of the selected backbone HDR plasmid with BamHI and KpnI in a final volume of 20 µL (Thermo Fisher FastDigest enzymes or New England Biolabs CutSmart enzymes are recommended for dual digestion in a common buffer). Run the reaction at 37 ˚C for 1 h followed by 85 ˚C for 5 min. This reaction can be freeze at -20 ˚C and can be use multiple times.
2. Dilute the gBlock of each recombination arm in TE buffer at a final concentration of 25 ng/µL.
3. Perform Gibson assembly reaction by mixing 2 µL of the digested plasmid reaction, 0.5 µL of the Left arm, 0.5 µL of Right arm, 2 µL water and 5 µL of NEBuilder Master Mix (New England Biolabs), and incubate at 50 ˚C for 15 minutes.
4. Transform 2 µL of the Gibson assembly reaction into a chemically competent E. coli (5-alpha from New England Biolabs or similar competent cell not resistant to ccdB).
5. Grow overnight at 35-37 ˚C in ampicillin agar plates.
6. The next day, select four colonies, grow overnight in Terrific Broth with ampicillin for plasmid extraction and validation by sequencing with the following primers.
   -for sequencing the Left arm: 5' GCAGATTGTACTGAGAGTGCACCATA 3'
   -for sequencing the Right arm 5' CAGGTTTTGCTTTTTGGCCTTTCCC 3'

Procedure for cloning the sgRNA sequence into the FAST-HDR CRISPR Vector.

Use the following template for designing the sequence containing the desired sgRNA sequence:
(GCGATCCGAGTTCAAATCTCGGTGGAACCTxxxxxxxxxxxxxxxxxxxGTTTTAGAGCT
AGAAATAGCAAGTTAAAAT). You can use any sequence of 20 nucleotides provided by a CRISPR cutting prediction software and is not necessary to start the sequence with a G as with other CRISPR plasmids. Replace the (X) with the desired sequence and order the molecule as an ultramer oligo (4nM) from Integrated DNA technologies (IDTDNA).

1. Digest 500 ng of the FAST-HDR CRISPR plasmid with BpiI in a final volume of 20 µL (Thermo Fisher FastDigest). Run the reaction at 37 ˚C for 1 h followed by 85 ˚C for 5 min. This reaction can be freeze at -20 ˚C and can be use multiple times.

2. Dilute the ultramer oligo in 40 µL of molecular biology grade water to make a stock

solution. Dilute 1 µL of the stock solution into 250 µL of molecular biology grade water to make working solution.

3. Perform Gibson assembly reaction by mixing 2 µL of the digested plasmid reaction, 1.5 µL of the ultramer working solution, 1.5 µL of water and 5 µL of NEBuilder Master Mix (New England Biolabs), and incubate at 50 ˚C for 15 minutes.
4. Transform 2 µL of the Gibson assembly reaction into a chemically competent E. coli (5-alpha from New England Biolabs or similar competent cell not resistant to ccdB).
5. Grow overnight at 35-37 ˚C in ampicillin agar plates.
6. The next day, select three colonies, grow overnight in Terrific Broth with ampicillin for plasmid extraction and validation by sequencing with this Forward primer: 5' GCGTCGATTTTTGTGATGCT 3'

Procedure for exchanging the labeling tag or the resistance gene in the FAST-HDR vector system.

To allow exchanging of the labeling tag or the resistance antibiotic gene in the FAST-HDR system, the sequence cannot contain the following restriction sites:

● BamHI ● KpnI ● EcoRI ● XbaI

Replacing the sequence of the labeling tag. Replace the (X) in the template (GTCGACGGTACCCAAGGCGGTGGAGAATTCxxxxxxxxxxxxxxGAATTCGGATCTGG AGCAACAAACTTCTCA) with the sequence of the desired labeling tag, and avoid including a stop codon or a partial codon at the end of the inserted sequence.

Order the complete sequence as a gBlock DNA fragment from Integrated DNA Technologies (IDTDNA).

1. Digest 500 ng of the selected backbone HDR plasmid with EcoRI in a final volume of 20 µL (Thermo Fisher FastDigest enzymes or New England Biolabs CutSmart enzymes are recommended). Run the reaction at 37 ˚C for 1 h followed by 85 ˚C for 5 min.
2. Dilute the construct of the labeling gene in TE buffer at a final concentration of 25 ng/µL.
3. Perform Gibson assembly reaction by mixing 2 µL of the digested plasmid reaction, 1.5 µL of the labeling gene construct, 1.5 µL of water and 5 µL of NEBuilder Master Mix (New England Biolabs), and incubate at 50 ˚C for 15 minutes.
4. Transform 2 µL of the Gibson assembly reaction into a chemically competent E. coli strain resistant to ccdB (DB3.1 from Abbexa Ltd is recommended).
5. Grow overnight at 35-37 ˚C in ampicillin agar plates.
6. The next day, select three colonies, grow overnight in LB media with ampicillin for plasmid extraction and validation by sequencing with the following Reverse primer: 5' CGGGATTCTCCTCCACGTC 3'

Replacing the sequence of the antibiotic resistance gene. Replace (X) in the following template with the sequence of the desired resistance gene starting with ATG and finishing with a stop codon:
GACGTGGAGGAGAATCCCGGGCCTTCTAGAxxxxxxxxxxxxxxTCTAGATAAATTCGT CAGTAGGGTTGTAAA.

1. Digest 500 ng of the selected backbone HDR plasmid with XbaI in a final volume of 20 µL (Thermo Fisher FastDigest enzymes or New England Biolabs CutSmart enzymes are recommended). Run the reaction at 37 ˚C for 1 h followed by 85 ˚C for 5 min.
2. Dilute the construct of the antibiotic resistance gene in TE buffer to a final concentration of 25 ng/ µL.
3. Perform Gibson assembly reaction by mixing 2 µL of the digested plasmid reaction, 1.5 µL of the antibiotic resistance gene construct, 1.5 µL of water, and 5 µL of NEBuilder Master Mix (New England Biolabs), and incubate at 50 ˚C for 15 min.
4. Transform 2 µL of the Gibson assembly reaction into a chemically competent E. coli strain resistant to ccdB. (DB3.1 from Abbexa Ltd is recommended)
5. Grow overnight at 35-37 ˚C in ampicillin agar plates.
6. The next day, select three colonies, grow overnight in LB media with ampicillin for plasmid extraction and validation by sequencing with the following primers:
   Forward: 5' TTCGGATCTGGAGCAACAAA 3'
   Reverse: 5' GGGAAAGGCCAAAAAGCAAAACCTG 3'