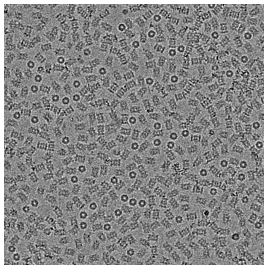
Feb 06, 2024

Cloning, Protein Expression, and Purification of 20S CPs and Assembly Intermediates



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We use this protocol and it's working

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Abstract

This protocol details methods for cloning, expression, and purification of 20S CPs and assembly intermediates for biochemical and structural analysis.

Guidelines

Please familiarise yourself with the laboratory safety rules and guidelines and follow these while performing the experiment. Please wear appropriate PE while performing the experiment.

Materials

X-tremeGENE^{TM} HP DNA Transfection Reagent - ROCHE
cOmplete PROTEASE INHIBITOR COKTAIL - ROCHE
Strep-Tactin® Sepharose® resin - IBA

Safety warnings

Please refer to the Safety Data Sheets (SDS) for health and environmental hazards. Liquid nitrogen (LN2) and other cryogens can cause severe damage to the skin and eyes. Always wear personal protective equipment when handling these cryogens.

Cloning of baculovirus transfer vectors

1w

1 Baculovirus transfer vectors were assembled utilizing a combination of the biGBac and MultiBac systems

Citation

Weissmann F, Petzold G, VanderLinden R, Huis In 't Veld PJ, Brown NG, Lampert F, Westermann S, Stark H, Schulman BA, Peters JM
(2016)
. biGBac enables rapid gene assembly for the expression of large multisubunit protein complexes.

https://doi.org/10.1073/pnas.1604935113 LINK

Citation

Vijayachandran LS, Viola C, Garzoni F, Trowitzsch S, Bieniossek C, Chaillet M, Schaffitzel C, Busso D, Romier C, Poterszman A, Richmond TJ, Berger I
(2011)
. Robots, pipelines, polyproteins: enabling multiprotein expression in prokaryotic and eukaryotic cells.

https://doi.org/10.1016/j.jsb.2011.03.007 LINK

The MultiBac vector pACEBac1 was used as both library vector and acceptor vector for step 1 biGBac assembly reactions with the following primers:

ACEBac-1EC-BBA.rev
ATTTAAATCTTTAGACCATAGAGCGTTCTCGCGAATCGATACTAGTGTTTAAACTCGCTACCTTAGGACC

ACEBac-2EC-BBA.fwd
ATTTAAATAAACCTAATGATGCCTGATGTTTCCTAGGGTATACCCATCTAATTGGAACCAGATAAGTGAAATC

ACEBac-3EC-BBA.fwd
ATTTAAATAAACGGTTCACATAGCTTAGTTTCCTAGGGTATACCCATCTAATTGGAACCAGAT
AAGTGAAATC

ACEBac-4EC-BBA.fwd
ATTTAAATAAACACTGACATTGACTTGGTTTCCTAGGGTATACCCATCTAATTGGAACCAGAT
AAGTGAAATC

ACEBac-5EC-BBA.fwd
ATTTAAATAAATCTATATCTCAATCGGGGTTCCTAGGGTATACCCATCTAATTGGAACCAGAT
AAGTGAAATC

2 Clone all 20S CP subunits and assembly chaperones into pACEBac1 using Gibson assembly

1d

For affinity purification add C-terminal TEV-cleavable twin strep tags on β2 (PSMB7) and β7 (PSMB4)

2.1 Screen for positive clones and sequence verify by Sanger sequencing

1d

3 Preparation of bigBac assembly inserts:

6h

Amplify expression cassette from library vectors (step 2) by PCR with the following primers:

Cas1-ACEBac.fwd
AACGCTCTATGGTCTAAAGATTTAAATCGACCTACTCCGGAATATTAATAGATCATGG

Cas2-ACEBac.fwd
AAACTGGATACTATTGCACGTTTAAATCGACCTACTCCGGAATATTAATAGATCATGG

Cas3-ACEBac.fwd
AAACCTAATGATGCCTGATGTTTAAATCGACCTACTCCGGAATATTAATAGATCATGG

Cas4-ACEBac.fwd
AAACGGTTCACATAGCTTAGTTTAAATCGACCTACTCCGGAATATTAATAGATCATGG

Cas5-ACEBac.fwd
AAACACTGACATTGACTTGGTTTAAATCGACCTACTCCGGAATATTAATAGATCATGG

Cas1-ACEBac.rev
AAACGTGCAATAGTATCCAGTTTATTTAAATGGTTATGATAGTTATTGCTCAGCGGTGG

Cas2-ACEBac.rev
AAACATCAGGCATCATTAGGTTTATTTAAATGGTTATGATAGTTATTGCTCAGCGGTGG

Cas3-ACEBac.rev
AAACTAAGCTATGTGAACCGTTTATTTAAATGGTTATGATAGTTATTGCTCAGCGGTGG

Cas4-ACEBac.rev
AAACCAAGTCAATGTCAGTGTTTATTTAAATGGTTATGATAGTTATTGCTCAGCGGTGG

Cas5-ACEBac.rev
AACCCCGATTGAGATATAGATTTATTTAAATGGTTATGATAGTTATTGCTCAGCGGTGG

3.1 Purify all inserts by agarose gel extraction 2h

4 Preparation of biGBac step 1 assembly acceptor vectors:
linearize the acceptor vector pACEBac1 by PCR with the following primers: 6h

for cloning of 5 expression cassettes:
ACEBac-1EC-BBA.rev and ACEBac-5EC-BBA.fwd

for cloning of 4 expression cassettes
ACEBac-1EC-BBA.rev and ACEBac-4EC-BBA.fwd

for cloning of 3 expression cassettes
ACEBac1-1EC-BBA.rev and ACEBac-3EC-BBA.fwd

4.1 Purify all linearized vectors by agarose gel extraction 2h

5 Clone multi expression cassette Baculo transfer vectors listed below by biGBac step 1 assembly with expression cassette inserts from step 3 and acceptor vectors from step 4 1d

pACEBac1-POMP-PSMG1-PSMG2-PSMG3-PSMG4
pACEBac1-PSMA1-PSMA2-PSMA3-PSMA4,
pACEBac1-PSMA5-PSMA6-PSMA7,
pACEBac1-PSMB1-PSMB2-PSMB3-PSMB4,
pACEBac1-PSMB1-PSMB2-PSMB3-PSMB4-TEV-2xSTII,
pACEBac1-PSMB5-PSMB6-PSMB7,

pACEBac1-PSMB5-PSMB6-PSMB7-TEV-2xSTII.

5.1 Screen for positive clones and sequence verify by Sanger sequencing 1d

6 Assemble finale multi expression cassette baculo transfer vectors by multibac assembly 2d

pACEBac1-PSMA1-PSMA2-PSMA3-PSMA4-PSMA5-PSMA6-PSMA7
pACEBac1-PSMB1-PSMB2-PSMB3-PSMB4-TEV-2xSTII-PSMB5-PSMB6-PSMB7
pACEBac1-PSMB1-PSMB2-PSMB3-PSMB4-PSMB5-PSMB6-PSMB7-TEV-2xSTII

6.1 Digest aceptor vectors listed below with I-CeuI, CIP and purify by agarose gel extraction 4h

pACEBac1-PSMA5-PSMA6-PSMA7
pACEBac1-PSMB5-PSMB6-PSMB7
pACEBac1-PSMB5-PSMB6-PSMB7-TEV-2xSTII

6.2 Digest donor vector listed below with I-CeuI and BstXI, and purify inserts by agarose gel extraction 4h

pACEBac1-PSMA1-PSMA2-PSMA3-PSMA4
pACEBac1-PSMB1-PSMB2-PSMB3-PSMB4-TEV-2xSTII
pACEBac1-PSMB1-PSMB2-PSMB3-PSMB4

6.3 Ligate the following vector/insert pairs listed below and transform in DH5alpha *E. coli* 1d

pACEBac1-PSMA5-PSMA6-PSMA7 and EC1-4 PSMA1-PSMA2-PSMA3-PSMA4
pACEBac1-PSMB5-PSMB6-PSMB7 and EC1-4 PSMB1-PSMB2-PSMB3-PSMB4-TEV-2xSTII
pACEBac1-PSMB5-PSMB6-PSMB7 TEV-2xSTII and EC1-4 PSMB1-PSMB2-PSMB3-PSMB4

6.4 Screen for positive clones and sequence verify by Sanger sequencing 1d

Baculo virus amplification and insect cell expression 1w

7 Culture Sf9 insect cells (Thermo Fisher Scientific) for virus amplification in serum-free Ex-cell 420 medium (Sigma-Aldrich)

Culture Trichoplusnia ni (Thermo Fisher Scientific) in protein free ESF 921 insect cell culture media (Expression Systems LLC)

All steps were carried out according to standard protocols

Citation

Fitzgerald DJ, Berger P, Schaffitzel C, Yamada K, Richmond TJ, Berger I (2006)
. Protein complex expression by using multigene baculoviral vectors.

https://doi.org/ LINK

Citation

Bieniossek C, Richmond TJ, Berger I (2008)
. MultiBac: multigene baculovirus-based eukaryotic protein complex production.

https://doi.org/10.1002/0471140864.ps0520s51 LINK

8 For bacmid preparation transform baculo transfer vector from the section above into EMBACY *E. coli* and plate on LB-agar plates with ampicillin, kanamycin, tetracyclin, gentamycin, IPTG, and XGal — 1d

8.1 Prepare Overnight cultures from white colonies in LB with ampicillin, kanamycin, and gentamycin — 1d

8.2 Prepare bacmids by alkaline lysis and subsequent isopropanol and [M] 70 % (v/v) EtOH precipitation — 2h

8.3 Air dry bacmid pellets, resuspend DNA in milliQ water, and store at 4 °C until usage — 1h

9 P1 virus production — 2d 12h

9.1 For P1 viruis production seed 0.7 - 0.8×10^6 cells/well in 3 mL medium in a six well plate and leave for 00:15:00 min at 27 °C — 15m

9.2 Prepare bacmids by diluting 1 µg bacmid DNA in 20 µL milliQ water, and add 200 µL of medium — 5m

9.3 For each bacmid mix 100 µL medium with 15 µL Extreme Gene HP DNA transfection reagent (Roche) — 5m

9.4 Add 112 µL of Medium Extreme Gene Mix to each bacmid DNA — 5m

9.5 Add 156 µL of the DNA-Extreme Gene Mix to each well in the six-well plate — 5m

9.6 Wrap the plates with Parafilm and incubate for 60:00:00 h at 27 °C — 2d 12h

9.7 Transfer medium containing P1 virus from each well into a 15 mL tube and store at 4 °C until further usage — 10m

10 P2 and P3 virus amplification — 2d

10.1 Seed 0.8 - 1.0 x 10^6 Sf9 cells/ml into an appropriate conical flask and infect cells with 0.5 - 1.0% P1 or P2, respectively — 1h

10.2 Incubate cell suspensions at 27 °C at 80 rpm for 48:00:00 h — 2d

10.3 Harvest P2 and P3 by centrifugation, transfer medium containing virus into a 50 mL tube or 250 mL bottle and store at 4 °C until further usage — 15m

11 Protein Expression in High FiveTM insect cells — 2d 12h

11.1 Seed 2.0 x 10^6 High FiveTM cells/ml into an appropriate conical flask and infect with 0.5 - 1.0 % P3 virus — 30m

11.2 Incubate cell suspensions at 27 °C at 80 rpm for 60:00:00 h — 2d 12h

11.3 Harvest High FiveTM cells by centrifugation, resuspend cell pellets in PBS, transfer to a 50 mL tube, harvest cells by centrifugation, discard supernatant, snap freeze cell pellets in LN2, and store util further usage at -80 °C — 30m

Purification of 20S CPs and 20S CP assembly intermediates — 8h

12 Purification of mature 20S CPs together with their assembly intermediates — 8h

12.1 Thaw cell pellets form section 2 - step 11, resuspend in buffer A 25 mM HEPES pH 7.5 (KOH), [M] 150 millimolar (mM) NaCl, [M] 1 millimolar (mM) DTT, and add 1 tablet cOmplete protease inhibitor mix (Merck) per 10 mL cell pellet resuspended in 40 mL buffer A in a 50 ml tube — 30m

12.2 Lyse cells by sonication on ice — 10m

12.3 Centrifuge lysate at 22k at 4 °C for 01:00:00 h — 1h

12.4 In parallel wash Strep-Tactin® Sepharose® resin (IBA) 3 times with buffer A — 45m

12.5 Incubate supernatants from step 12.3 with resin for 01:00:00 h at 4 °C — 1h

12.6 Wash resin 3 times with buffer A by centrifugation at 2000 x g for 00:15:00 min at 4 °C — 15m

12.7 Load resin on a gravity flow column and elute proteins with [M] 2.5 micromolar (µM) *d*-Desthiobiotin in buffer A — 15m

12.8 Pool fractions of interest, concentrate in a spin protein concentrator with a MWCO of 100 kDa to max [M] 5-8 mg/mL — 1h

12.9 In parallel equilibrate a Superose 6 10/300 GL column (Cytiva) with buffer A — 1h

12.10 Apply concentrated eluate from step 12.8 onto the SEC column at a flow rate of 1 ml/min, record absorption at 280 nm, and fractionate elution a 300 µL — 1h

12.11 Analyze SEC fractions by SDS-PAGE, pool fractions of interest, snap freeze in LN2, and store util further usage at -80 °C for biochemical assays — 2h

For structural analysis use fractions directly after SEC

Citations

Step 7

Fitzgerald DJ, Berger P, Schaffitzel C, Yamada K, Richmond TJ, Berger I. Protein complex expression by using multigene baculoviral vectors.
https://doi.org/

Step 7

Bieniossek C, Richmond TJ, Berger I. MultiBac: multigene baculovirus-based eukaryotic protein complex production.
https://doi.org/10.1002/0471140864.ps0520s51