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Expression and Purification of a Mammalian Protein: Cytosolic Domain of IRE1α from Insect Sf21 Cells

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Abstract: Eukaryotic proteins can be expressed in different heterologous systems. However, mammalian proteins in general have specific post-translational processing requirements that may not be fulfilled by a regular bacterial expression system. In this study, we use an insect cell system to express a mammalian protein of interest. Spodoptera frugiperda (Sf21) cells were used in conjunction with a baculoviral expression system to produce the cytosolic domain (CD) of IRE1, an endoplasmic reticulum (ER) stress sensor protein. Inositol Requiring Enzyme 1 (IRE1) is a dual function kinase and endoribonuclease protein that cleaves X-box binding protein (XBP1) mRNA. We used the pFastBac plasmid to insert the coding sequence into a recombinant bacmid shuttle vector which was then used to infect Sf21 cells. The expressed protein was then purified with an MBPTrap column to obtain >85% pure protein.

Keywords: Sf21 cells; baculoviral expression; IRE1; pFastBac; MBP-tagged protein purification

1. Introduction

Heterologous expression of proteins is defined as the expression of proteins in an organism that does not naturally express the protein. The different systems in use for such purposes can be broadly classified as bacterial, yeast, insect and mammalian systems. Cells in culture can be manipulated to express the protein of interest in transient or stable mode. Some factors that need to be considered while choosing the expression system are the required yield of the protein, post-translational modifications, functionality, and speed of expression.

Proteins of prokaryotic origin are best expressed in bacterial systems. *E. coli* can express and adequately process prokaryotic proteins and is the go-to system for cheap, scalable and high yield expression. If the expression of eukaryotic proteins is required, additional factors need to be taken into consideration. The most important factor would arguably be the level of post-translational modifications (PTM) required for a functional form of the protein. Proteins requiring extensive PTMs will not be processed correctly in bacterial systems and will most likely aggregate in inclusion bodies[1]–[3] . Adding a fusion protein tag to the protein of interest may sometimes help to resolubilize the proteins. However, in the interest of saving time and effort, it is advisable to switch to higher eukaryotic systems such as insect or mammalian cells. Another advantage to eukaryotic systems is the presence of extensive protein folding machinery that is vital for the function of the protein [4].

Table 1 compares the different heterologous protein expression systems with respect to the type of protein, post-translational modifications and ease of large-scale production [5]–[10].

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46 **Table 1: Characteristics of heterologous protein expression in bacterial, yeast, insect**
47 **and mammalian cell systems ranked according to desirability**
48

	Bacterial	Yeast	Insect	Mammalian
Type of protein				
Prokaryotic	Y	Y	Y	Y
Eukaryotic	Y	Y	Y	Y
Secreted	Y	Y	Y	Y
Post-translation modifications				
Phosphorylation	Y	Y	Y	Y
Glycosylation	N	Y	Y	Y
Acetylation	N	Y	Y	Y
Acylation	N	Y	Y	Y
Refolding required?	Y	Y	N	N
Production				
Cell growth	Rapid (30 mins)	Rapid (1.5 hrs)	Moderate (18 hrs)	Slow (24hrs)
Yield	High	High	Moderate	Low
Time to expression	Rapid	Moderate	Moderate	High
Cost	Low	Expensive	Expensive	Very expensive
Ease of scale-up	Easy	Easy	Difficult	Very difficult

49

50
51
52 Bacterial systems have desirable characteristics for large scale production but are not
53 able to process and correctly fold proteins requiring extensive PTMs [11]. Depending on the
54 application of the protein, Chinese hamster ovary (CHO) or Human embryonic kidney (HEK
55 293) cells are typically the system of choice for eukaryotic proteins [12]. This is true,
56 especially for therapeutic proteins or antibodies. However, the yield is very low compared to
57 insect or bacterial cells – typically on the order of 100mg/L of expressed protein [13]. The
58 level of protein expression is higher in insect cells up to 100mg/ L [14], [15].
59 Insect cells and mammalian systems have a wide range of post-translational
60 modifications. An excellent tool to determine all possible post-translational modifications of
61 a protein of interest is dbPTM (http://dbptm.mbc.nctu.edu.tw/). Insect and mammalian
62 systems are both capable of all PTMs with one exception. Insect cells and mammalian cells
63 have similar phosphorylation and O-linked glycosylation patterns. They can authentically
64 process phosphorylation modifications, partly due to the presence of phosphatases. However,
65 in terms of N-glycosylation, proteins in insect cells are high-mannosylated while mammalian
66 cells have a complex glycosylation pattern [4], [12]. **Figure 1** depicts the difference in N-
67 linked glycosylation patterns in insect cells vs. mammalian cells. Regular insect Sf9 and Sf21
68 cells will have a high mannose pattern of glycosylation but mimic Sf9 (available from
69 ThermoFisher) cells are engineered to make complex N-glycans with terminal sialic acid.
70 Varied and complex sugars such as N-acetylneuraminic acid, galactose, fucose, mannose are
71 added in mammalian cells in a branched configuration as opposed to the addition of only
72 mannose residues in insect cells.

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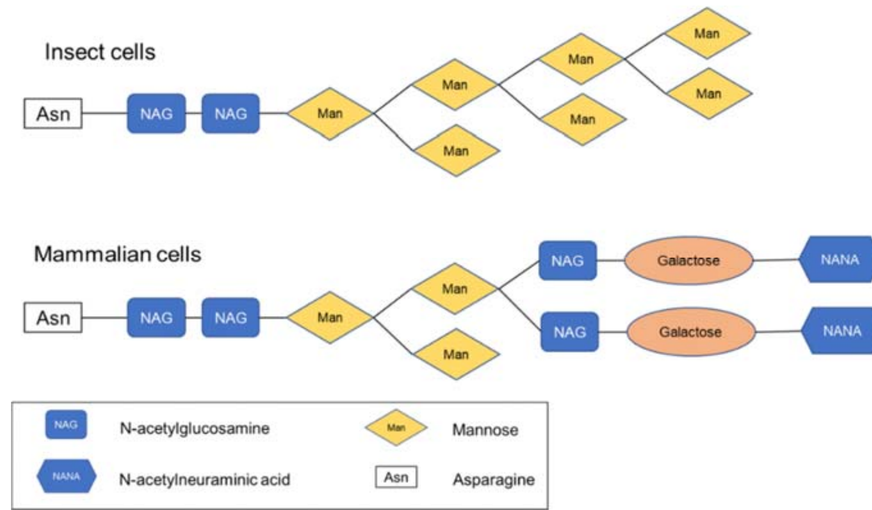

76
77 **Figure 1: N-linked glycosylation patterns for insect cells and mammalian cells**
78

79 In this study, we developed a protocol to express the Inositol Requiring Enzyme-1α
80 (IRE1α) protein, an ER stress sensor. IRE1 is a type I transmembrane protein primarily
81 composed of three domains, the luminal domain, a transmembrane domain and cytosolic
82 domain (CD)[16].
83 IRE1α is a bifunctional protein with kinase and endoribonuclease activity on the CD.
84 We have developed a protocol to produce IRE1α-CD (547-977 aa) in insect Sf21 cells using
85 baculovirus expression. IRE1α has been expressed in *E. coli* cells in our lab (unpublished
86 data) as well as by other groups[17], [18]. However, IRE1α expressed in *E. coli* and yeast
87 systems is in a hyperphosphorylated state[18], [19]. All the purification methods used thus
88 far for purifying this protein have used the 6X His tag. The cell line used was Sf9 [18], [22].
89 However, there are no detailed methods or protocol papers published for this procedure, that
90 is, using the Sf9 cells and 6X His tag method.
91 Post-translational modifications for IRE1 were predicted using algorithms developed by
92 the Center for Biological Sequence Analysis, Technical University of Denmark (http://www.
93 cbs.dtu.dk/ [1]. The predicted N-glycosylation, O-glycosylation, C-mannosylation and
94 glycation sites are scored for the likelihood of modification (**Table 2**). The scores that cross
95 the threshold (which is set by the algorithm at 0.5) are denoted as “positive” hits.
96

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Table 2: Predictions for O-glycosylation, C-mannosylation, N-glycosylation and glycation sites on CD-IRE1.

O-glycosylation			
start	end	score	comment
2	2	0.792	#POSITIVE
3	3	0.796	#POSITIVE
5	5	0.791	#POSITIVE
15	15	0.189	
16	16	0.506	#POSITIVE
24	24	0.111	
38	38	0.015	
61	61	0.043	
73	73	0.040	
85	85	0.006	
102	102	0.018	
121	121	0.110	
126	126	0.102	
127	127	0.213	
128	128	0.181	
135	135	0.052	
151	151	0.107	
164	164	0.034	
178	178	0.107	
180	180	0.741	#POSITIVE
183	183	0.643	#POSITIVE
188	188	0.111	
198	198	0.148	
206	206	0.026	
208	208	0.047	
213	213	0.013	
223	223	0.070	
226	226	0.040	
232	232	0.064	
245	245	0.252	
276	276	0.510	#POSITIVE
288	288	0.454	
300	300	0.102	
300	300	0.102	

C-mannosylation			
start	end	score	comment
191	191	0.18	.
287	287	0.271	.

Glycation			
start	end	score	comment
22	22	-0.943	.
28	28	-0.707	.
53	53	0.801	YES
87	87	-0.875	.
110	110	0.832	YES
144	144	-0.529	.
158	158	0.699	YES
160	160	0.863	YES
170	170	-0.773	.
171	171	0.83	YES
202	202	-0.95	.
231	231	-0.962	.
253	253	-0.799	.
265	265	0.806	YES
273	273	-0.94	.
282	282	-0.878	.
291	291	-0.83	.

N-glycosylation			
start	end	score	comment
204	204	0.5427	+

2. Experimental Design

2.1 Materials

All the materials are listed separated by each sub-section of the procedure.

<u>Cloning of IRE1α-CD into pFastBac plasmid</u>

i. pFastBac His6 MBP N10 TEV LIC cloning vector (4C) (Addgene plasmid #30116)
ii. SspI-HF (New England Biolabs, cat # R3132S)
iii. CutSmart buffer (New England Biolabs cat# B7204S)
iv. QIAquick PCR Purification Kit (Qiagen, cat# 28104)
v. Deoxynucleotide (dNTP) set includes dGTP, dCTP (New England Biolabs, cat # N0446S)
vi. Bovine Serum Albumin (BSA), Molecular Grade (New England Biolabs, cat# B9000S)
vii. T4 DNA Polymerase (New England Biolabs, cat# M0203S)
viii. Q5 High Fidelity 2X Master Mix (New England Biolabs, cat# M0492S)
ix. OneShot Top10 chemically competent *E. coli* DH5α (ThermoFisher, cat# C404003)
x. Luria Broth (Sigma-Aldrich, cat# L3397)
xi. Luria Agar (Sigma-Aldrich, cat# L3272)
xii. LB agar plates containing ampicillin (Sigma-Aldrich, cat # L5667-10EA)

<u>Preparation of recombinant bacmid</u>

xiii. MAX Efficiency *E. coli* DH10Bac competent cells (ThermoFisher cat# 10361012)

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122 xiv. Antibiotics and their stock solutions are listed in **Table 3**. Aliquot and store at -20ºC.
123
124 **Table 3: Antibiotic concentrations and stock solutions**

Component	Dissolve in	Company (Cat. No)	Stock conc.	Special considerations
Kanamycin	Water	Sigma (60615)	50 mg/mL	
Tetracycline	Ethanol	Sigma (87128)	10 mg/mL	Light sensitive
Gentamicin	Water	Sigma (G1914)	7 mg/mL	
X-gal**	Dimethyl formamide	Sigma (XGAL-RO)	20 mg/mL	Light sensitive, DO NOT filter sterilize, Make solution in a glass vial or polypropylene tube
IPTG	Water	Sigma (I6758)	200 mg/mL	Filter sterilize

125 *** There are other options for X-gal such as Bluo-gal (ThermoFisher, cat # 15519028). It is more expensive*
126 *but more sensitive and turns a deeper, more obvious blue that aids in colony identification***
127
128 xv. S.O.C medium (ThermoFisher cat# 15544034)
129 xvi. PureLink HiPure Plasmid Miniprep Kit (Invitrogen, cat# K210002)
130 Transfection of recombinant bacmid into Sf21 cells
131 i. Sf-900-III serum-free media (ThermoFisher, cat# 12658019)
132 ii. Cellfectin II reagent (ThermoFisher, cat# 10362100)
133 iii. Gelcode Blue Stain Reagent (ThermoFisher, cat# 24590)
134 iv. IRE1α (14C10) Rabbit mAb (Cell Signaling Tech, cat# 3294)
135 Protein purification using MBPTrap column
136 v. Xtractor cell lysis buffer (Takara, cat# 635671)
137 vi. cOmplete, Mini, EDTA-free protease inhibitor cocktail (Sigma, cat# 11836170001)
138 vii. MBPTrap HP column (GE, cat# 28-9187-78)
139 viii. D-(+)- Maltose monohydrate (Sigma, cat# 63418-25G)
140 ix. Tris-Cl (Sigma, cat # 10812846001)
141 x. NaCl (Sigma, cat # S3014)
142 xi. Dithiothreitol (DTT) (Sigma, cat# DTT-RO)
143 xii. EDTA (Sigma, cat # 324504)
144 xiii. Phosphate buffered saline (PBS) (Sigma, cat# P7059-1L)
145 xiv. Binding buffer for MBPTrap HP column: 20 mM Tris-HCl, 200 mM NaCl, 1 mM
146 DTT, 1 mM EDTA, pH 7.4
147 xv. Elution Buffer for MBPTrap HP column: Binding buffer + 10mM maltose
148 xvi. Regeneration buffer: 0.5M NaOH
149

150 ## 3. Procedure

151 **Figure 2** depicts a flowchart of the steps involved in the expression and purification of
152 IRE1α-CD in Sf21 cells using baculoviral vectors. The procedure is divided into 5 subunits.
153 The time required for each subunit is indicated in brackets in the left column (**Figure 2**).

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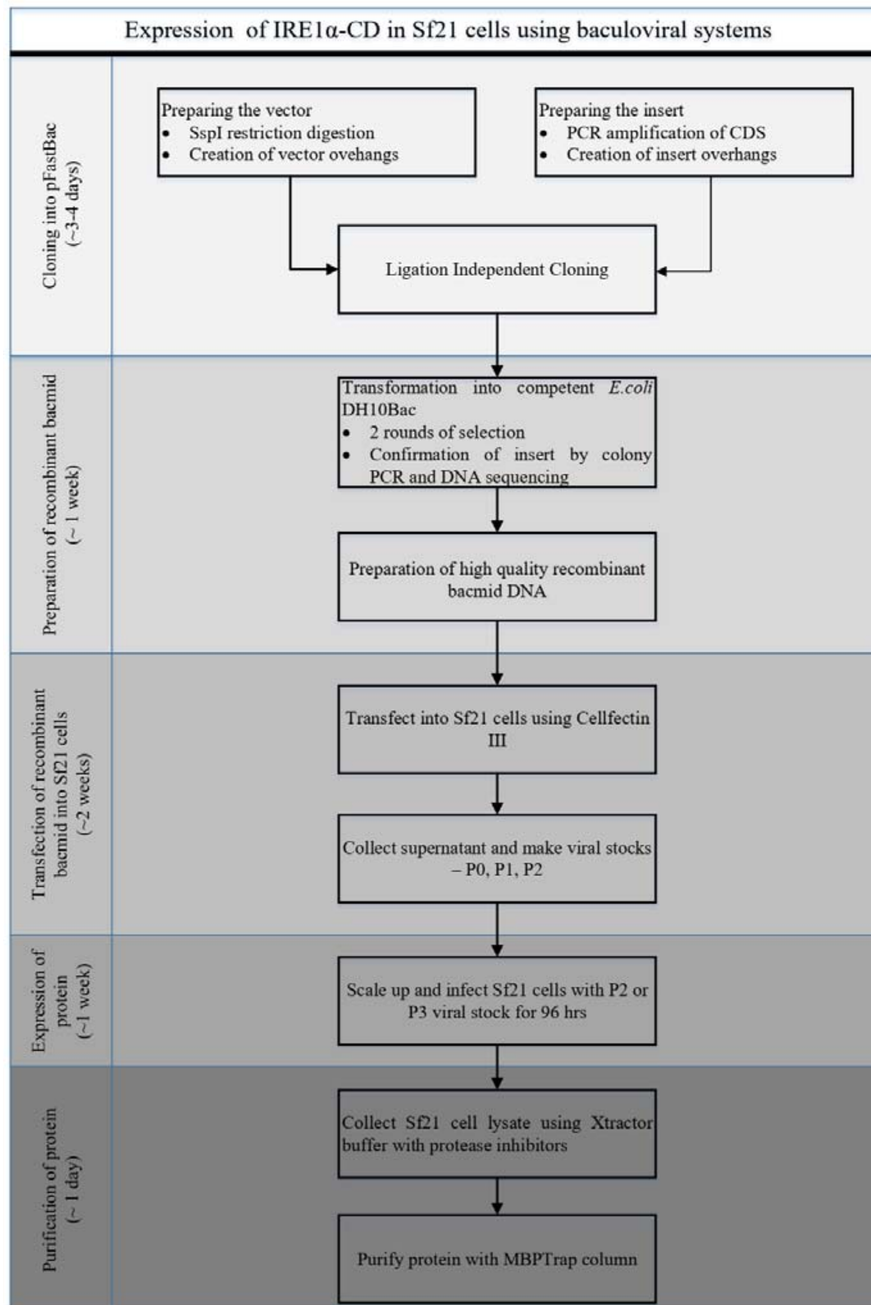

154
155 **Figure 2: Flowchart for all the steps involved in the expression and purification of**
156 **proteins from Sf21 cells**

157 *3.1 Cloning of IRE1α-CD into pFastBac plasmid*

158 *Note:*
159 pFastBac His6 MBP N10 TEV LIC cloning vector (4C) was a gift from Scott Gradia
160 (Addgene plasmid #30116; http://n2t.net/addgene:30116; RRID: Addgene_30116). Another
161 version of this plasmid (5C) is available so that two proteins can be expressed simultaneously.
162 The sequence for IRE1α- CD was obtained from NCBI (Gene id: 2081). Primers were
163 designed to clone the CD fragment from 547 aa-977 aa from a pCDNA-hIRE1 plasmid
164 containing a full-length IRE1 coding sequence.
165

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166 **Table 4: PCR primers for insertion of IRE1-CD (547aa-977aa) into pFastBac plasmid**

CD_IRE1_forward	5′- TACTTCCAATCCAATGCAGGCAGCAGCCCCTCCCTGGAAC-3′
CD_IRE1_reverse	5′- TTATCCACTTCCAATGTTATTAGAGGGCGTCTGGAGTCAC-3′

167
168 **Table 4** shows the primers designed for the insertion of IRE1α-CD into the empty
169 pFastBac plasmid. The underlined tag on each primer is to facilitate ligation independent
170 cloning (LIC) and is specific to the pFastBac backbone. The rest of the primer is specific to
171 the insert coding sequence (CDS). The LIC technique makes use of the endonuclease activity
172 of T4 DNA polymerase to generate sticky overhangs for ligation between the vector plasmid
173 and insert DNA. This technique avoids the use of ligases.
174
175 **Protocol:**
176 *Preparing the vector pFastBac plasmid*
177 i. Linearize the plasmid with SspI-HF (NEB) (**Table 5**). The high-fidelity (HF) versions
178 of restriction enzymes are faster and more efficient. Incubate at 37°C for 15 mins – 2
179 hrs to completely digest all the plasmid DNA. Heat inactivation is performed for 20
180 mins at 65°C.
181 **Table 5: Restriction digestion with SspI-HF**

Component	Amount	Volume (μL)
pFastBac plasmid DNA (250ng/uL)	1 μg	4
10X CutSmart buffer	Final concentration 1X	5
SspI-HF	10 units	1
Water	Make up to 50 μL	40

182 ii. Use a PCR purification kit (QIAquick) to purify the large linearized fragment of
183 plasmid DNA.
184 iii. To create pFastBac vector overhangs, mix the following components in PCR tubes,
185 the order of addition is as listed (T4 DNA polymerase is added last) (**Table 6**).
186 Incubate at 12°C for 30 minutes followed by heat inactivation at 75°C for 20 minutes.
187
188 **Table 6: Mix to create pFastBac vector overhangs**

Reagent	Final conc.	Volume (μL)
10x NEB 2.1	1x	4
Eluted linearized pFastBac DNA	10-50 ng/ μL	20-30
dGTP (100mM)	2.5 mM	1
DTT (100mM)	5 mM	2
BSA (10 ug/ul)	0.25 μg/ μL	0.6
T4 DNA polymerase	0.075 units/ μL	1
Sterile dH2O to 40ul		

189
190 *Preparing the insert IRE1α-CD*
191 iv. Set up a PCR to amplify the IRE1α-CD insert using the primers in **Table 4** as outlined
192 in **Table 7**. Confirm amplification by running the PCR product on a 1.5% agarose
193 gel.

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194 **Table 7: PCR mix for preparing IRE1α-CD insert**

Component	Volume (μL)
Q5 2X Master Mix	25
CD_IRE1_forward primer (10 μM)	2
CD_IRE1_reverse primer (10 μM)	2
pCDNA-hIRE1 (50 ng/ μL)	1
Nuclease free water (make up to 50 μL)	20

195 The PCR program should be run for 98°C 1 min, followed by 35 cycles of 98°C for 30
196 sec, 61°C for 10 sec, 72°C for 90 sec, and a final extension of 72°C for 5 mins.
197 v. Create the insert overhangs in a way similar to the vector overhangs in the presence of
198 dCTP (instead of dGTP).
199 vi. After heat inactivation, mix both vector and insert together (1:3 ratio). The total volume
200 of the combined vector and insert should be between 5-10 uL. Incubate for 5 mins at
201 room temperature and then add 1uL of 25 mM EDTA followed by another incubation
202 for 5 mins.
203 vii. Transform 2 μL of this mixture into competent OneShot Top10 *E. coli* DH5α (100 μL),
204 spread onto LB agar plates containing 100 μg/mL ampicillin and incubated at 37°C for
205 16-24 hrs.
206 viii. Select individual colonies (5-10) and prep the plasmid DNA. Confirm the insertion of
207 IRE1α- CD by DNA sequencing using primers specific to the IRE1α-CD sequence (seq
208 for- 5'- aagcagctccagttcttccaggac-3')
209

210 *3.2 Preparation of recombinant bacmid*

211 *Note:*
212 Once the insert has been cloned into the pFastBac empty plasmid, it needs to be
213 transformed into *E. coli* DH10Bac cells to form the recombinant bacmid. *E. coli* DH10Bac
214 competent cells are sold by ThermoFisher. The genotype is F-mcrA Δ (mrr-hsdRMS-
215 mcrBC) Φ80lacZΔM15 Δ lac X74 recA1endA1 araD139 Δ (ara, leu)7697 galU galK λ - rps
216 L nupG /pMON14272 / pMON7124. DH10Bac cells have a baculovirus shuttle vector and a
217 helper plasmid. This machinery is required for the generation of recombinant bacmid after
218 transformation of pFastBac-IRE1α-CD into the cells. The baculovirus shuttle vector
219 (bMON14272) also encodes kanamycin resistance, and the helper plasmid (pMON7142) has
220 tetracycline resistance. The presence of the Φ80lacZΔM15 Δ lac marker enables the use of
221 blue/white colony screening to determine integration of the insert into the bacmid. pFastBac
222 has the Tn7 element which includes the polyhedrin promoter, the gene of interest and
223 gentamicin resistance.
224 It is also possible to use regular *E. coli* DH10Bac cells and make them chemically
225 competent (see **Supplemental file**).
226

227 **Protocol:**
228 i. Dissolve LB agar powder in water and autoclave at 121°C for 15 mins.
229 ii. Let it cool to 55°C (there should be no lumps of agar).
230 iii. Add all the antibiotics and chemicals in the working concentrations listed in **Table 8**.
231

232 **Table 8: Working concentrations of antibiotics**

Component	Stock conc.	Working conc.	Dilution
Kanamycin	50 mg/mL	50 μg/mL	1:1000
Tetracycline	10 mg/mL	10 μg/mL	1:1000

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Gentamicin	7 mg/mL	7 µg/mL	1:1000
X-gal	20 mg/mL	200 µg/mL	1:100
IPTG	200 mg/mL	40 µg/mL	1:200

iv. Let harden, store at 4°C in the dark.

v. Thaw a vial of competent *E. coli* DH10Bac cells on ice in a 15 mL round bottom sterile polypropylene tube.

Do not use 1.7 mL or 2 mL microcentrifuge tube

vi. Add 100ng pFastBac-IRE1-CD plasmid DNA to the vial.

vii. Incubate on ice for **30** mins.

viii. Heat shock for **45 s** at **42°C.**

ix. Transfer tubes back on ice and chill for 5 mins.

x. Add **900 µL** SOC medium.

xi. Incubate tubes in a shaking incubator, 37°C at 200 rpm for at least **4 hrs**.

This 4 hr long outgrowth step is necessary to allow the bacteria to generate the antibiotic resistance proteins encoded on the plasmid backbone

xii. Make three 10-fold serial dilutions of 800 µL cells. Plate 100uL of each dilution onto LB plates with kanamycin, tetracycline, gentamicin, X-gal and IPTG.

xiii. Incubate plates at 37°C for **48 hrs** to allow enough time for colony formation and color development. Pick white colonies.

Do not pick colonies before 48 hrs because it may be difficult to distinguish between white and blue colonies

Pick colonies that are large and well isolated. Avoid picking colonies that are gray or darker in the center as they may contain a mixture of empty bacmid and recombinant bacmid

xiv. Replate selected colonies for an additional round of selection on fresh LB agar plates overnight at 37°C.

xv. Once the white phenotype is confirmed, inoculate in LB media with kanamycin, gentamicin, and tetracycline and grow overnight.

xvi. Isolate recombinant bacmid DNA using Purelink HiPure Plasmid Kit by Invitrogen with a modified protocol (see **Supplemental file**).

3.3 Transfection of recombinant bacmid into Sf21 cells

Note:

Sf21 cells are suspension cells grown at 28°C, without the need for CO_2 incubators. They are grown in Sf-900 III media. The media can be supplemented, if needed, with serum but bovine serum should not be used (fetal bovine serum (FBS) is preferred). The serum needs to be inactivated to inactivate complement fragments that can inactivate baculoviruses. Transfection reagents can be lipid based (e.g. cellfectin, fugene). Calcium chloride can be used for baculovirus transfection, but it has less efficiency than lipid-based reagents.

Protocol:

Transfection of bacmid into Sf21 cells

i. A day before transfection, passage Sf21 cells so that they are at a density of 3 x 10^5 cells/mL.

Actively dividing cells have a higher transfection efficiency and produce more protein

ii. Plate cells at a concentration of 8 x10^5 cells/well of a 24-well plate.

iii. Allow the cells to attach for 1 hr and replace media with fresh media (without FBS).

iv. Use 2 µg of bacmid DNA per well. Dilute in 75 µL of Sf 900-III media. Incubate for 15 mins.

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280 v. Use 8 µL of cellfectin II in 75 µL media. Incubate for 15 mins.
281 *Make sure the cellfectin II is mixed thoroughly before use, but do not vortex the tube*
282 vi. Mix together the two tubes and incubate for 15-30 mins.
283 vii. Add mixture dropwise to the wells of the 24-well plate.
284 viii. 24 hrs later, replace transfection media with fresh SF-900 III media containing 10%
285 FBS.
286 *Serum proteins in FBS act as substrates for proteases*
287 ix. Check for signs of infection (SIF) after 24 hrs up to 96 hrs.
288 *SIF may not be obvious in P0 infection so continue infection for 5 days and collect the
289 supernatant and re-infect for 5 more days to amplify the viral stock*
290 x. After changing media, collect supernatant after every 24 hrs for 24, 48, 72 and 96 hrs.
291 This supernatant is the P0 viral stock. Store at 4ºC in the dark.
292 xi. Use the P0 viral stock to infect newly plated Sf21 cells to generate P1 stock. Add 150
293 µL of P0 stock dropwise on top of the cells (plated in a 6-well plate), gently swirl a few
294 times and incubate the plate at 28ºC.
295 xii. Look for SIF in 24 hours post infection time. After 5 days, collect only P1 viruses with
296 SIF and store viral stocks in the dark at 4ºC.
297 xiii. In a similar fashion, collect P2, P3 and P4 stocks for increased baculoviral titer with an
298 increasing number of cells infected (6-well plate → 150 cm dish→ T25 flask). Store all
299 at 4ºC protected from light.
300
301 *Scale-up of heterologous protein expression*
302 i. Seed Sf21 cells in a T75 (vented) flask as described in the transfection procedure. Make
303 sure they are actively dividing cells in the log phase of growth.
304 ii. Add 150 µL of the collected P1 stock.
305 iii. Incubate at 27ºC for 5 days or until 30-40 % of the cells have lysed.
306 iv. Collect cells as well as supernatant. **This will be the P2 viral stock.**
307 v. Use P2 viral stock to infect spinner flasks with Sf21 cells.
308 vi. If no signs of infection are observed, infect Sf21 cells with the P2 stock of baculoviruses
309 and collect the P3 viral stock (see **Figure 5** for SIF). We concentrated the baculovirus
310 viral supernatants with successive passages (P1,P2,P3) and used the passage that gave
311 us visible signs of infection at 24 hrs with expression of protein. The viral titer
312 (expressed as plaque-forming units/mL or pfu/mL) can also be determined with a plaque
313 assay. Briefly, cells in a tissue culture dish are infected and overlayed with agarose. After
314 the cells are grown for about 10 days, the plaques can be counted to determine the
315 pfu/mL concentration.
316

317 *3.4 Purification of MBP fusion protein*

318 *Note:*
319 For this protocol, use Sf21 cells infected with P3 stock of baculovirus for 96 hrs. The cell
320 pellet is collected and lysed with Xtractor buffer, and an MBPTrap column is used to extract
321 MBP-tagged IRE1α-CD. The MBP tag can be cleaved from the protein by incubating with 1
322 unit of AcTEV protease (ThermoFisher, cat# 12575015) for 1 hr at 30ºC for each 3µg protein.
323

324 **Protocol:**
325 i. Prepare Xtractor buffer with protease inhibitor of choice. In this purification protocol, we
326 used cOmplete EDTA free protease inhibitor cocktail.
327 ii. Pellet Sf21 cells at 100 x g for 15 mins, remove supernatant media and wash pellet once
328 with phosphate buffered saline (PBS). Add 5 mL Xtractor buffer to 1 g cell pellet.

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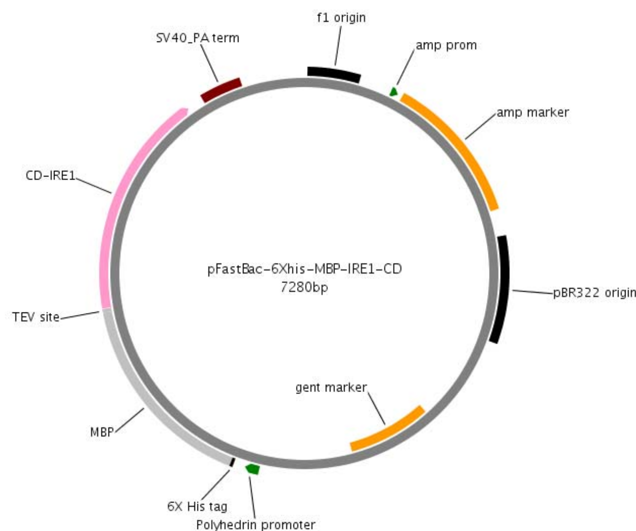
329 iii. Mix vigorously by vortexing and rock at room temperature for 15-30 mins. Make sure
330 that cell lysis is complete by observing under the microscope.
331 iv. Centrifuge at 16,000 g for 20-30 mins to pellet the cell debris.
332 v. Take the clarified supernatant and filter through a 0.22 μm filter immediately before
333 loading onto the MBPTrap HP column.
334 vi. Prior to loading the clarified lysate, equilibrate the MBPTrap HP column with 7 column
335 volumes (CV) of binding buffer at a flow rate of 1 mL/min
336 vii. Load clarified lysate onto the column. The binding capacity of the 1 mL MBPTrap HP
337 column is protein dependent but can bind approximately to 5 mg-7 mg of MBP-tagged
338 protein. The flow rate should be decreased to 0.5 mL/min.
339 viii. Wash with 10 CV of binding buffer at a flow rate of 1 mL/min. If real-time A280 readings
340 are possible, wash until no discernible absorbance at 280 nm is observed.
341 ix. Add 5 CV of elution buffer at a reduced flow rate of 0.5 mL/min, and assay elute fractions
342 to determine fractions with the highest concentration of the protein of interest with a
343 Bradford or BCA assay.
344 x. Regenerate the column with 3 CV distilled water followed by 3 CV of 0.5 M NaOH.
345 Wash away the NaOH with 5 CV distilled water. The column is now ready to be used
346 again.
347

348 **4. Expected Results**

349 The pFastBac- IRE1α-CD plasmid features are shown in **Figure 3** [20]**.** IRE1α-CD is
350 fused to the C-terminus of an MBP protein under the polyhedrin promoter. A 6X His tag is
351 also present at the N-terminal of the MBP protein. The MBP protein and IRE1α-CD coding
352 sequences are separated by a Tobacco Etch Virus (TEV) protease site. The plasmid has an
353 ampicillin (amp) and gentamicin (gent) selection marker for selection during the preparation
354 of recombinant bacmid. The pFastBac backbone has a Tn7att transposition element that
355 guides the insertion of the coding sequence into the bacmid. After transformation into *E. coli*
356 DH10Bac, colonies were grown for 48 hrs. Colonies show up within 24 hrs but take an
357 additional day to develop a blue color. Large white colonies were selected and re-streaked
358 onto a Tet-Kan-Gent-IPTG-X-gal plate.



359
360 **Figure 3: Plasmid map for pFastBac-IRE1α-CD**

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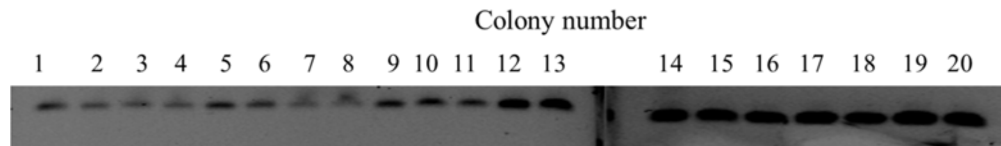
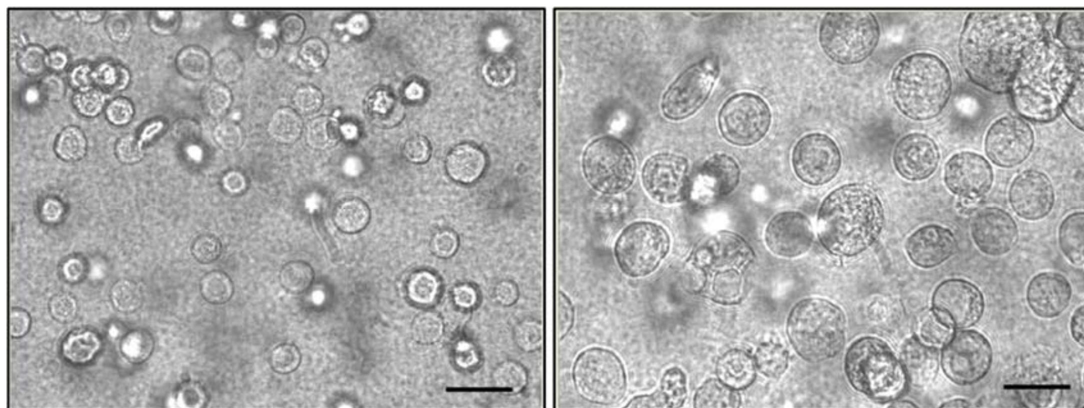
361
362 After this additional round of selection, a colony PCR was performed to make sure that
363 IRE1α-CD was incorporated into the recombinant bacmid (**Figure 4**). Primers specific to an
364 internal region in IRE1α-CD were used. As expected, a band was seen in all of the white
365 colonies picked, indicating that the gene of interest was inserted in the bacmid. Bacmids were
366 prepped and sent for DNA sequencing. Once the IRE1α-CD sequence was confirmed,
367 glycerol stocks were made for future use (see **Supplemental file**).
368



369
370 **Figure 4: Colony PCR of white *E. coli* transformant colonies**
371
372 The recombinant bacmid was extracted from *E. coli* DH10Bac cells and precipitated with
373 100% isopropanol to obtain pure bacmid samples. Sf21 cells were transfected and monitored
374 for signs of infection for 5 days and multiple passages of viral titer. **Figure 5** shows the Sf21
375 cells changing on infection with P3 baculoviruses. The cells become larger in size, and the
376 nuclei appear to occupy more of the cell. The Sf21 cells show more granularity as the
377 infection progresses.
378



379
380 **Figure 5: Signs of infection in untransfected Sf21 cells (left) and P3 treated Sf21 cells**
381 **(right) for 24 hrs. Scale bar = 45µm**
382
383 We checked the expression of IRE1α-CD after transfection by collecting cell lysate after
384 P0 and P1 infections. **Figure 6** shows a GelCode Blue-stained SDS-PAGE (8%) of cell
385 lysates after transfection and infection with baculoviruses to express IRE1α-CD. As evident
386 in the figure, the IRE1α-CD protein is produced along with other contaminating insect cell
387 proteins, also stained by GelCode Blue and therefore needs to be purified for downstream
388 assays. The proportion of IRE1α-CD protein expressed in P1 infected cells was more than
389 P0 infected cells, reflecting the higher concentration of baculoviral titer. The baculovirus
390 stock was amplified to P3 to obtain high protein expressing infected Sf21 cells.
391 The P3 viral supernatant was collected, filtered with a 0.22 µm filter and stored at 4°C
392 in the dark for subsequent infection. Sf21 cells were passaged the day before infection so that
393 they were in log phase at a density of 3x10^{5} cells/mL. 150 µL of P3 supernatant was added
394 for each mL of Sf21 cells. The time required for the expression of a protein depends on each
395 protein of interest expressed.

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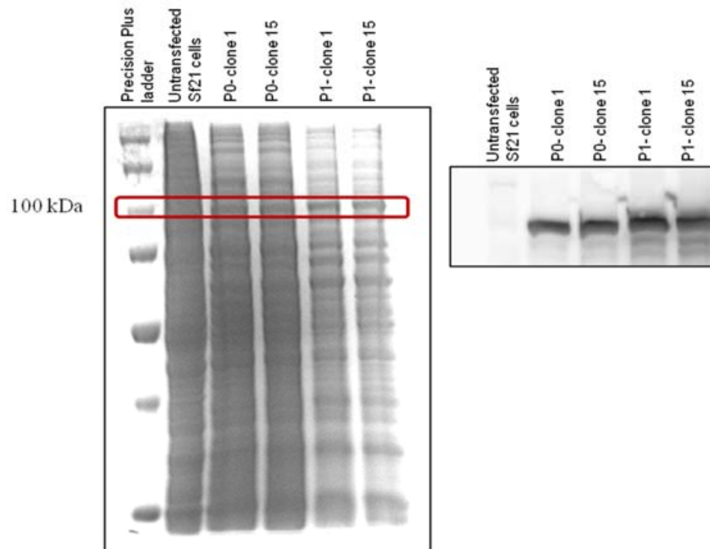
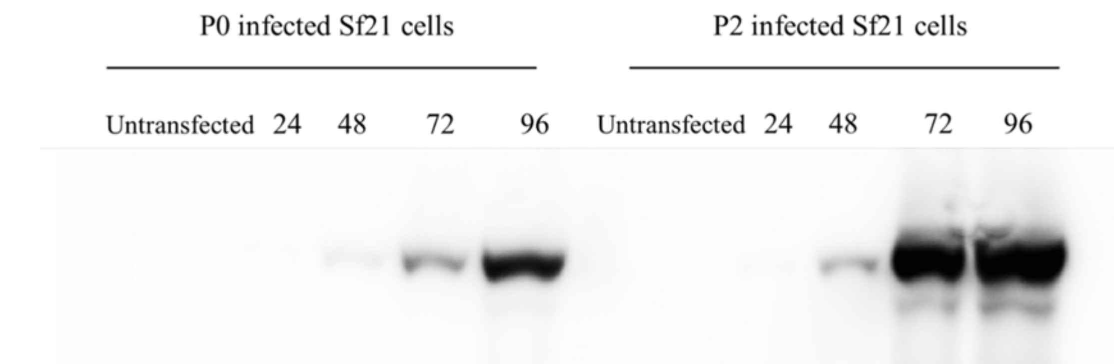


396
397 **Figure 6: Expression of MBP- IRE1α-CD protein prior to purification. (Left) SDS-**
398 **PAGE of total protein lysate for untransfected Sf21 cells, P0 and P1 infected clones 1**
399 **and 15. The 100 kDa band is marked and the putative 6X His- MBP- IRE1α-CD**
400 **protein band is prominent in P1 infections. (Right) Western blot using an anti-IRE1**
401 **antibody**
402
403 **Figure 7** shows the western blot for the different time points throughout protein
404 expression to determine the optimum time point for harvesting the cells. For IRE1α-CD, in
405 P0 infected cells, maximum expression was observed at 96 hrs. Upon increasing infection
406 titer by using P2 stocks, the overall level of protein expression increased. The optimal time
407 point for harvesting was still after 96hrs of infection.
408



409
410 **Figure 7: Expression of IRE1-CD protein. Western blot showing the time course of**
411 **protein expression in P0 (left) and P2 (right) infected Sf21 cells using anti-IRE1α**
412 **antibody**
413
414 Once the viral titer and time for expression were optimized, the cell lysates were
415 collected for purification of the protein. We used the MBP tag on the IRE1α-CD to aid in the
416 purification. Filtered and clarified lysate was run through the MBPTrap column to obtain
417 IRE1α-CD protein. The protein was of >85% purity based on densitometric analysis of the
418 bands seen in an SDS-PAGE gel. Once IRE1α-CD is purified, it can be used for binding
419 assays to determine binding partners for the protein [21]. **Figure 8** below shows the various

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fractions from the purification process outlined in the manuscript. The addition of an MBP-tag makes for a very specific purification. The yield of the expressed protein obtained after purification differs by the plasmids used, by baculoviral stocks and by the type of protein being expressed. The final volume of Sf21cells infected for scale-up can be adjusted based on the yield of protein required.

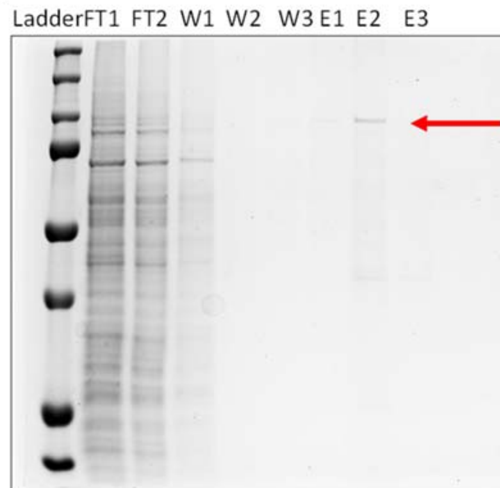

Figure 8: A GelCode Blue stained SDS-PAGE showing the various fractions from the purification procedure of IRE1-CD. FT- Flowthrough, W- Wash fractions, E- Elute fractions. The ladder is the Precision Plus blue ladder (Bio-Rad, cat# 1610373). The E2 fraction shows a pure protein band at ~100kDa as expected

Sf21 insect cells combined with the pFastBac baculoviral system make a very robust, optimizable system for expression of eukaryotic proteins. MBP-IRE1α-CD was successfully expressed and purified from Sf21 cells. This protocol can be easily modified to express any mammalian protein in Sf21 insect cells by modifying the PCR primers shown in **Table 4** for the desired CDS. Long term expression of the protein can be achieved by making frozen stocks of baculovirus infected Sf21 cells. The MBP tag can be cleaved off using AcTEV protease if it interferes with downstream assays and the protein can be cleaned up using a Ni-NTA column. An AKTA-FPLC automated system can be used to aid in purification of higher volumes of cell lysate. If a secretion tag is used and the protein of interest is secreted in the media, a 5mL MBPTrap column can be used with the AKTA FPLC system to automate the purification process.

5. Summary

Further characterization of the expressed IRE1α protein is required to establish its structure, phosphorylation status and *in vitro* XBP1 splicing activity conclusively. The phosphorylation status of the protein can be identified by various methods. Feldman et al., used mass spectrometry techniques to identify the phosphorylated sites on IRE1α [22]. This technique in conjunction with a Phos-tag gel would lend more information on the ratio of phosphorylated to unphosphorylated species in the purified protein[23], [24]. Using the Phos-tag ligand developed by Fujifilm Wako-Chem, an SDS-PAGE gel can be used to separate phosphorylated proteins from unphosphorylated forms. The Phos-tag ligand binds to the phosphate group and slows down migration of the phosphorylated band. This leads to a

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452 separation of the phosphorylated and unphosphorylated species into separate bands. To
453 confirm that the band pattern is because of distinct phosphorylation, purified protein samples
454 can be treated with λ- phosphatase enzyme with activity towards phosphorylated serine,
455 threonine and tyrosine residues. On treatment and subsequent application to a Phos-tag SDS-
456 PAGE gel, the slower migrating band should disappear. *In vitro* XBP1 splicing can be
457 assayed with a hairpin-RNA cleavage assay or by a fluorescence-based assay with a FRET-
458 paired oligonucleotide [18].

459 If the protein structure needs to be determined, circular dichroism or small-angle X-
460 ray scattering techniques can be used. Circular dichroism is a very useful tool to determine
461 secondary structure as well as native folding of expressed or fusion proteins. Specifically,
462 secondary structure can be determined using far-UV spectra and protein folding
463 characteristics can be determined by monitoring spectra at different temperatures or in the
464 presence of different denaturing agents [25].

465

466

467 **Author Contributions:** Conceptualization, A.O. and C.C.; Methodology, A.O.; Validation, A.O., G.J. and
468 C.C; Writing—original draft preparation, A.O.; Writing—review and editing, A.O., G.J, C.C.; Funding and
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476 to publish the results.

477 .

478

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549 # Supplemental file
550

551 ## I. Preparation of competent *E coli* DH10Bac cells

552 **Materials needed:**
553 i. *E. coli* DH10Bac (streak from glycerol stock onto a Luria agar plate containing
554 tetracycline/kanamycin/gentamycin, get isolated colonies and inoculate single
555 colony) in LB broth containing tet/kan/gent grown at 37°C at 200 rpm.
556 ii. Ice cold centrifuge tubes, P10, P200, P1000 tips, microcentrifuge tubes and cryovials
557 iii. Refrigerated centrifuge
558 iv. Dimethyl sulfoxide (DMSO)
559 v. FSB buffer: (Sterile filter through a 0.22 µm filter and store at 4°C)

Components	Company	Cat. no	Final concentration
Potassium acetate pH 7.5	Sigma-Aldrich	P1190	10 mM
$MnCl_2$	Sigma-Aldrich	M1787	45 mM
$CaCl_2$	Sigma-Aldrich	C5670	10 mM
KCl	Sigma-Aldrich	P9541	100 mM
$[Co(NH_3)_6]Cl_3$	Sigma-Aldrich	481521	3 mM
Glycerol	Sigma-Aldrich	G5516	10 %

560
561 **Protocol:**
562 i. Grow *E.coli* DH10Bac cells overnight until the OD is between 0.3-0.6. The LB
563 media should contain kanamycin and tetracycline at the working concentrations
564 listed in Table 8 of the main text.
565 ** It is very important not to overgrow the culture**
566 ii. Cool the cells in wet ice for 10-20 minutes.
567 **From now on the cells should remain always ice cold**
568 iii. Pellet the cells by centrifugation, 10 min at 2500 rpm, 4°C.
569 iv. Resuspend the cells gently in 24 ml of ice-cold FSB.
570 v. Incubate on wet ice for 10-15 minutes.
571 vi. Pellet then cells again as above.
572 vii. Resuspend the cells in 8 ml of cold FSB.
573 viii. Add 280 uL DMSO.
574 ix. Incubate for 5 minutes on wet ice.
575 x. Add 280 uL DMSO.
576 xi. Incubate for further 5 minutes on wet ice.
577 xii. Aliquot the cells in 200-400 uL batches to sterile single-use cryovials.
578 xiii. Store in -80°C.
579 **Do not store in liquid nitrogen**
580
581

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582 **II. Preparation of glycerol stocks for *E coli* DH10Bac cells with recombinant bacmid**

583 **Materials needed:**

584 i. Glycerol (Sigma-Aldrich, cat # G5516)
585 ii. Cryovials (Sigma-Aldrich, cat# 5000-0020)

586 **Protocol:**

587 i. Prepare a 60% glycerol solution in water. Filter sterilize using a 0.22 µm filter or
588 autoclave the solution at 121°C for 15 mins.
589 ii. Grow *E. coli* DH10Bac cells with recombinant bacmid in LB media for 14-16 hrs.
590 The LB media should contain kanamycin, gentamicin and tetracycline at the working
591 concentrations listed in **Table 8** of the main text.
592 iii. Add 750 µL of cells and 250 µL of the glycerol solution to a cryovial.
593 iv. Invert a few times to mix properly.
594 v. Store at -80°C.

595
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597

598 **III. Preparation of recombinant bacmid from *E. coli* DH10Bac cells**

599 **Materials needed:**

600 i. PureLink HiPure Plasmid Miniprep kit (buffers R3, L7, N3) (ThermoFisher, cat#
601 K210002)
602 ii. 100% isopropanol

603 **Protocol:**

604 i. For recombinant bacmids, use 15–25 mL of an overnight *E. coli* DH10Bac with
605 recombinant bacmid grown in Luria Broth.
606 ii. Pellet the cells at 5000 × g for 7 minutes to harvest the cells. Remove all medium and
607 wash with PBS if necessary.
608 iii. Add 0.4 mL Resuspension Buffer (R3) with RNase A to the cell pellet in the tube and
609 resuspend the cells. Gently shake the tube until the cell suspension is homogeneous.
610 iv. Add 0.4 mL Lysis Buffer (L7). Place the cap on the tube and ensure it is secure. Mix
611 gently by inverting the capped tube until the lysate mixture is thoroughly
612 homogenous. **Do not vortex.**
613 v. Incubate the lysate at room temperature for 5 minutes. **Do not exceed 5 minutes**.
614 vi. Add 0.4 mL Precipitation Buffer (N3) and mix immediately by inverting the tube
615 until the mixture is thoroughly homogeneous. **Do not vortex.** Centrifuge the lysate at
616 >12000g for 10 mins at RT.

617 **Don't load onto the column provided with the kit. It decreases yield of bacmid and
618 does not increase the quality of bacmid obtained**

619 vii. Take the supernatant gently without disturbing the pellet Add 0.63mL ice cold
620 isopropanol to the supernatant. Mix well and incubate at RT for 15 mins.

621 **Alternatively, keep overnight at -20°C for precipitation**

622 viii. Centrifuge tube at >12000g for 30 mins
623 ix. Air dry the pellet for 10 mins, then resuspend the purified bacmid DNA in 60 uL
624 water.
625 x. Solubilize bacmid DNA for 1 hr at 65°C or 4°C overnight.

626 **4°C overnight is much better than 65°C**

627 xi. Measure concentration of bacmid with Nanodrop.

628

629