BRP Vectors and Competent Cells

Mo Bi Tec
MOLECULAR BIOTECHNOLOGY

Contents

1. Background 3

2. Advantages 4

3. Description of Plasmids 5

3.1. pJL3 5

3.2. pSW1 5

4. Control of Vector Function 6

5. Protocols 7

5.1. Promoter selection 7

5.2. *E. coli* host 7

5.3. Growth conditions 7

5.4. Competent cells and transformations 8

6. Literature 9

7. Example: Excretion of Human Growth Hormone 10

7.1. Summary 10

7.2. Plasmids, bacterial strains and media 10

7.3. Co-transformation, cell growth 10

7.4. SDS-PAGE and immunoblotting 10

7.5. Large scale culture and purification of the excreted protein 11

8. Order Information, Shipping, and Storage 11

9. Contact and Support 12

1. Background

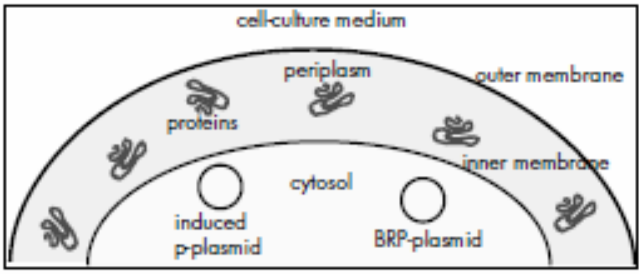
The BRP vectors express the bacteriocin release protein (BRP), resulting in a permeabilization of the outer and inner membranes of *E. coli.* As a consequence, some cytoplasmic and periplasmic proteins are released into the culture medium. This has considerable advantages for protein production and purification.

Being a Gram-negative bacterium, *E. coli`s* cell envelope consists of an inner and outer membrane. The excretion of proteins into the cell-external culture medium is generally avoided by the two membranes. The BR-Protein opens the *E. coli* envelope in a controlled way and enables protein excretion into the culture medium.

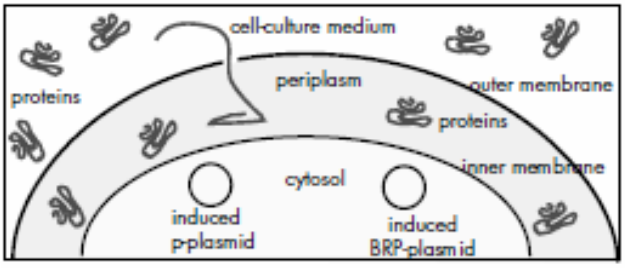
The gene for the bacteriocin release protein BRP is 84 bp long. The resulting small protein of 28 amino acids activates phospholipase A of the outer membrane resulting in the formation of permeable zones in the *E. coli* cell envelope. Since proteins can pass through these zones, some cytoplasmic and periplasmic proteins are released into the culture medium. The rate of permeability is controlled, and proteins produced in the cell in large amounts are released specifically.

Periplasmic Proteins

1A Co-transformed cell containing the BRP- and a recombinant plasmid

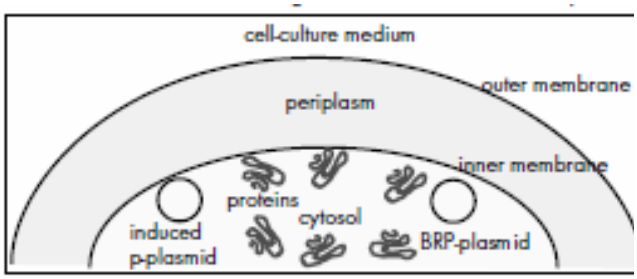


1B Induced cell containing the BRP- and a recombinant plasmid



Cytosolic Proteins

2A Co-transformed cell containing the BRP- and a recombinant plasmid



2B Induced cell containing the BRP- and a recombinant plasmid

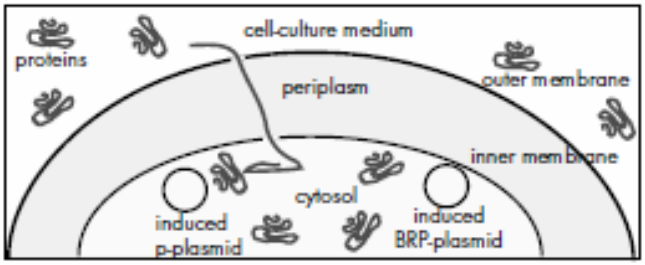


Figure1: Schematic presentation: Function of the BRP vector. 1A-1B: Release of periplasmic proteins. 2A-2B: Release of cytosolic proteins into the culture medium.
p-plasmid: plasmid containing the DNA sequence coding for the protein of interest.

The BRP gene is cloned under the control of the inducible lpp-lac promoter/operator system. Thus, the expression of the BRP gene and, consequently, the release of proteins into the culture medium is regulated by varying the IPTG (isopropyl-1-thio-ß-D-galactopyranoside) concentration in the medium for the vector pJL3 or, alternatively, varying the mitomycin C concentration for the vector pSW1. For selection the BRP vectors carry antibiotic markers (pJL3: chloramphenicol, pSW1: tetracycline).

BRP vectors are co-transformed with the vector containing the recombinant gene of interest. Therefore, pSW1 and pJL3 do not contain a multiple cloning site. Both BRP plasmids carry the p15A1 origin of replication and are, as a result, compatible with, e.g., commonly used ColE1 vector systems.

Now MoBiTec offers also pre-made competent cells already transformed with a BRP vector. This has the advantage that the cells are supplied ready for transformation with the vector carrying the gene of interest. The time consuming protocols in chapter 5.1. to 5.4.1. can therefore be omitted. For order information on these competent cells, please refer to chapter 8.

2. Advantages of BRP Vectors and Cells

The secretion of proteins into the culture medium is of advantage in systems where proteins have to be isolated from Gram-negative cells. This is especially the case when heterologous recombinant proteins are produced in *E. coli*.

The advantages include:

1. Cloned proteins may no longer accumulate in the cytoplasm of *E. coli*. Thus, problems associated with the lethality of recombinant proteins, their preferential degradation, and inclusion body formation are avoided.
2. The medium provides larger space for accumulation of the protein of interest.
3. The culture medium has fewer proteases which could degrade the protein.
4. Protein purification is simplified. Only a selection of proteins is exported through the envelope. Thus, export into the medium is the first step in the purification of the protein of interest. Also further purification steps are simplified.
5. Export into the culture medium facilitates large-scale biotechnological (continuous) production since the bacterial cells remain intact.

3. Description of Plasmids

3.1. Plasmid pJL3

The plasmid pJL3 encodes chloramphenicol resistance and contains the Bacteriocin Release Protein under control of the lpp-lac tandem promoter/operator (Ref. 1, 2). Depending on the host strain moderate induction with IPTG (10-40 µM) will result in releasing the periplasmic and certain cytoplasmic proteins. Full induction might result in quasi-lysis of host cells. This lysis can be hampered by adding 10-20 mM Mg^{2+} ions to the culture medium (preferably normal broth, for instance, Lab-Lemco broth (Oxoid) supplemented with 0.5% NaCl and 0.5% Na lactate).

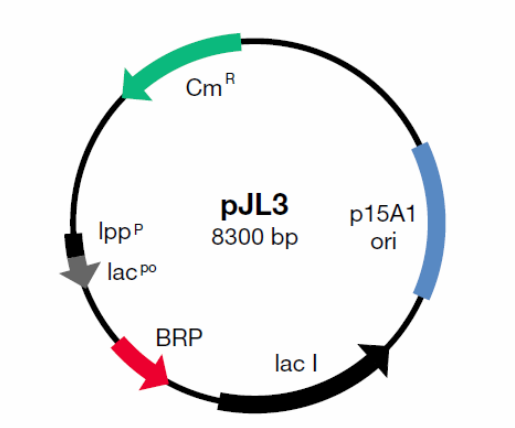


Figure 2: The Bacteriocin Release Protein (BRP) expression plasmid pJL3. The plasmid has a size of 8,300 bp. It contains the hybrid *E. coli* lipoprotein promoter lppp and the lac promoter operator lacpo system to control the expression of BRP. The expression of the BRP gene is regulated by the lac repressor encoded by the *lacI* gene. The vector carries the chloramphenicol resistance marker Cm^R and the p15A1 origin of replication.

3.2. Plasmid pSW1

The plasmid pSW1 encodes tetracycline resistance and contains the BRP gene under control of the mitomycin C inducible pCloDF13 promoter (Ref. 3). Induction with 10-25 ng of mitomycin C per ml of culture will give moderate expression of the BRP and more or less specific release of periplasmic and certain cytoplasmic proteins. Full induction (0.5 µg per ml) might result in quasi-lysis (this is dependent on the host strain). The presence of 10-20 mM Mg^{2+} inhibits the lysis of the host cells.

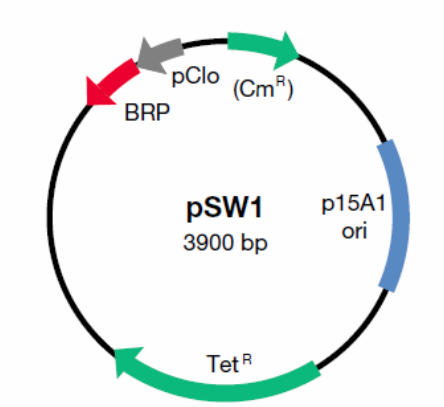


Figure 3: Bacteriocin Release Protein (BRP) expression plasmid pSW1. The plasmid has a size of 3,900 bp. It contains the pCloDF13 promoter (pClo). The vector carries the tetracycline resistance (*Tet*^{R}), a part of the chloramphenicol resistance (*Cm*^{R}), and the p15A1 origin of replication.

4. Control of Vector Function

Growth of transformed cells on media containing the appropriate antibiotic is not sufficient alone as evidence that the plasmid is active and BRP is expressed and functions. First, the *E. coli* host cells should be checked for absent of the BRP function. Then, BRP vectors should be transformed into the host cells and these cells are then checked for inducible BRP function. To check if the cells express BRP, a quasi-lysis test is performed. The basis of this test is the decline in culture turbidity and cell viability when BRP is expressed in the absence of divalent metal ions.

Quasi-lysis test:
E. coli culture samples (1 ml) are taken from the fermenter and used to inoculate two 250-ml flasks of broth (without additional Mg^{2+} or other divalent metal ions) containing the appropriate antibiotic. Incubate at 37 °C until an OD_{660} of about 0.1 is reached. Fully induce one of the flasks. At one hour intervals for the next five hours, the OD_{660} is read, and samples are taken for plating out (on solid broth agar plus antibiotic minus inducer). Typical results are shown in fig 4.

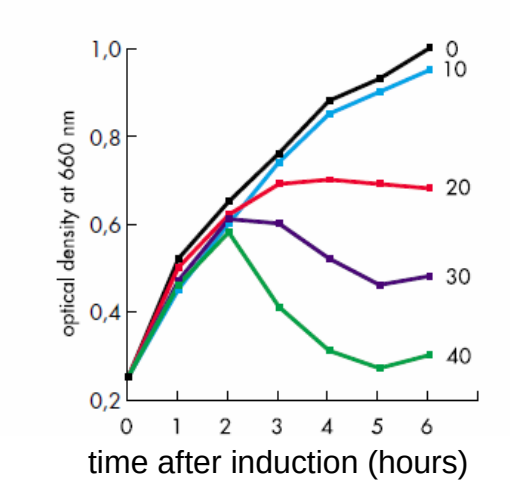


Figure 4: Effect of induction with IPTG on the growth of *E. coli* harboring a BRP vector. IPTG was added at t = 0. The various concentrations of IPTG used (µM) are indicated in the figure. See reference 1.

5. Protocols

Note:

order numbers BRPSW1 and BRPJL3:
if you purchased lyophilized vector DNA start with chapter 5.1.

order numbers COMSW1 and COMJL3:
if you purchased competent *E. coli* cells pre-transformed with a BRP vector, start with chapter 5.4.2.

5.1. Promoter selection

We suggest using separate promoters for the BRP and the protein to be produced/ or secreted. This will give you the opportunity to induce a full production of the protein of interest and to use a moderate induction of the BRP (which prevents early lysis of producer cells).

5.2. *E. coli* host

In general, for the BRP vectors nearly any normal laboratory strain can be used, as long as it is not phospholipase A- and, for pSW1, also not *recA*$^-$. Selected *E. coli* strain must, of course, be suitable for the second plasmid containing your cloned gene, which is co-transformed with the BRP vector. The *E. coli* background suggested for co-transformation in Ref. 4 (Van Tiel-Menkveld *et al*., J.Bacteriol. 140, 415 (1979)) is thr leu thi lacY tonA supE. We also had good results with *E. coli* K12 RV308 (su-, DlacX74, galISII::OP308, rpsL) as described in Ref. 6 (Schoner *et al.*, BioTechnology 3, 151-154 (1985)). We provide *E. coli* K12 N3406 (thr leu thi lacY tonA supE) as competent cells which are already transformed with a BRP vector.

5.3. Growth Conditions

5.3.1. Antibiotics

Double transformants containing either pJL3 or pSW1 and another plasmid encoding a (foreign) protein to be expressed and released should be cultured in the presence of two antibiotics to keep a selective pressure upon both of two plasmids. For pJL3 we used 34 µg/ml chloramphenicol, and for pSW1 15-20 µg/ml tetracycline.

5.3.2. Induction

Plasmid pJL3: the lac system is inducible with isopropyl-ß-D-thiogalactopyranoside (IPTG). Amounts lower than 7.5 µM end concentration hardly have any effect. 15-20 µM is effective, and larger amounts, up to 40 µM, result in cell lysis (pJL3).

Plasmid pSW1: the mytomycin C-inducible pCloDF13 promoter becomes active between 10 and 25 ng/ml end concentration. Full induction occurs at 0.5 µg/ml.

5.3.3. Broth

Normal broth, e.g., Lab-Lemco broth (Oxoid) supplemented with 0.5% NaCl and 0.5% Na lactate containing no fermentable sugars is strongly advised; do not use rich media in protein export runs. We suggest TY broth.

5.3.4. Temperature

Generally, *E. coli* strains should be grown at 37 °C. If the optimal growth conditions for the host organism are lower, the strain can also be incubated below 37 °C. Higher temperatures over longer incubation time can lead to the loss of plasmids. Short induction periods at 42 °C (heat shock promoters) are tolerable.

Note: MoBiTec offers a convenient 2 Liter Bench-Top Fermenter.

5.4. Competent cells and transformation

Note: MoBiTec also offers competent cells, which are already transformed with a BRP vector.

Two different strains, packaged in color-coded tubes, are available:

Red: *E. coli* K12 N3406 transformed with pSW1 (order #COMSW1)

Yellow: *E. coli* K12 N3406 transformed with pJL3 (order #COMJL3)

Genotype *E.coli* K12 N3406: thr leu thi lacY tonA supE

In each kit 5x200 µl competent cells are supplied. The cells are shipped on dry ice and require storage at -80 °C. The transformation efficiencies are indicated on the data sheet for each lot.

5.4.1. Preparing competent cells yourself (Ref. 7; Hanahan J. Mol. Biol. 166, 557-580 (1983))

Pick a single colony from a freshly streaked SOB agar plate and disperse in 1 ml SOB medium by vortexing. Inoculate the cells into an Erlenmeyer flask containing SOB medium (culture volume to flask volume of 1:10 to 1:30). Incubate at 37 °C with moderate agitation until the cell density is 4 to 7x10^7 viable cells/ml.

Collect the culture in 50 ml centrifuge tubes and chill on ice for 10 to 15 min. Pellet the cells by centrifugation at 750 to 1000 g for 12 to 15 min at 4 °C. Carefully drain the pelleted cells (remove recalcitrant drops). Resuspend the cells in 33% of the original culture volume of TFB by vortexing moderately and incubate for 10 to 15 min. Pellet the cells and drain as above.

Resuspend in TFB to 8% of the original volume. Add DMSO & DTT solution (1 M DTT, 90% DMSO (v/v) and 10 mM potassium acetate) to 3.5% (v/v) and swirl the tube immediately.

Incubate on ice for 10 min. Add a second equal aliquot of DMSO & DTT solution as above to give a 7% final concentration. Incubate the tubes on ice for 10 to 20 min. Pipet 200 µl aliquots into chilled tubes.

5.4.2. Transformation

Note: If the pre-made competent cells, which are already transformed with a BRP vector, are purchased, only the vector of interest has to be transformed into the competent *E. coli* cells.

If the BRP vectors are purchased as lyophilized DNA, the protocol below has to be performed for both, the BRP vector and the vector of interest. This transformation can be performed in one or two steps.

Transformation protocol for competent cells:

1. Thaw competent cells on ice.
2. Gently mix the cells by hand. Aliquot 100 µl of the cells into a pre-chilled 15 ml tube.
3. Prepare a fresh 1:10 dilution of β-mercaptoethanol (stock 14.2 M) with distilled water (dH_20).
4. Add 1.7 µl of diluted β-mercaptoethanol to the 100 µl competent cells, yielding a final concentration of 25 mM.
4. Gently swirl the contents of the tubes. Incubate the cells on ice for 10 minutes, swirling gently every 2 minutes.
5. Add 0.1-50 ng of DNA to the cells and swirl gently. As a control, use an aliquot without adding DNA.
6. Incubate the cells on ice for 30 minutes.
7. Heat pulse the tubes in a 42 °C water bath for 45 seconds. The duration of the heat pulse is critical for obtaining high efficiencies.
8. Incubate the tubes on ice for 2 minutes.
9. Add 0.9 ml of pre-heated (42 °C) SOC medium and incubate the tubes at 37 °C for 1 hour with shaking at 225-250 rpm.
10. When transforming the DNA, use a sterile spreader rod to plate ≤ 200 µl of the transformation mixture on the appropriate antibiotic plates. For the control aliquot, use LB agar plates.

Notes:
A) If desired, the cells may be concentrated by centrifugation at 1,000 rpm for 10 minutes. The pellet should then be resuspended in 200 µl of SOC medium before plating.

B) If the transformant is ampicillin resistant, the transformation mixture may also be plated on LB-ampicillin-methicillin agar plates if satellite colonies are observed.

6. Literature

1. J. Luirink, F. K. de Graaf and B. Oudega; Mol. Gen. Genet. 206, 126-132 (1987).
2. H. M. Hsiung, A.Cantrell, J. Luirink, B. Oudega, A. J. Veros, & G. W. Becker Bio/Technology 7, 267-271 (1989).
3. E. J. Verschoor, J. Luirink, S. de Waard, F. K. de Graaf & B. Oudega Antonie van Leeuwenhoek 55, 325-340 (1989).
4. G. J. van Tiel-Menkveld, A. Rezee & F. K. de Graaf; J.Bacteriol. 140, 415-423 (1979).
5. M. Dagent and S. D. Ehrlich, Gene 6, 23-28 (1979).
6. R. G. Schoner, L. F. Ellis, and B. E. Schoner, Bio/Technology 3, 151-154 (1985).
7. D. Hanahan, J. Mol. Biol. 166, 557-580 (1983).
8. Peng Yu and Ka-Yiu San; Biotechnol. Prog. 8, 25-29 (1992).
9. van der Wal, F.J. *et al*., Biotech.Letters 17(8), 815-820 (1995).
10. van der Wal, F.J. *et al*., Appl. Microbiol. Biotechn. 44, 459-465 (1995).
11. van der Wal, F.J. *et al*., FEMS Microbiol. Rev. 17, 381-399 (1995).

7. Example: Excretion of Human Growth Hormone

7.1. Summary

The gene encoding human growth hormone (hGH) was cloned adjacent to a sequence for the signal peptide of the *E. coli* outer membrane protein OmpA in the expression vector pIN-III-OmpA. The resulting hybrid protein was translocated across the cytoplasmic membrane, leaving a correctly processed and folded hGH in the bacterial periplasm (Hsiung *et al.*, Biotechnol. 4, 991 (1986)). By introducing a second, compatible, plasmid containing the pCloDF13--derived BRP gene under lac promotor control (pJL3), mature hGH could be directed into the culture medium, from which it could be easily purified (Hsiung *et al.*, Biotechnol. 7, 267 (1989)).

7.2. Plasmids, bacterial strains, and media

Plasmid pOmpA-hGH2 codes for the hGH precursor OmpA-hGH and uses a lpplac promotor-operator system regulated by the lac repressor. Plasmid pLT29-OmpA-hGH contains a heat-inducible runaway replicon. For plasmid pJL3 see above.

E. coli RV308 was the host strain for all of these recombinant plasmids. Cells harboring both pJL3 and pOmpA-hGH were grown in TY broth (Difco) with ampicillin (100 µg/ml) and chloramphenicol (34 µg/ml.). Cells harboring both pJL3 and pLT29-OmpA-hGH were grown in TY broth with kanamycin (50 µg/ml) and chloramphenicol (34 µg/ml).

7.3. Co-transformation, cell growth

The BRP expression vector pJL3 was co-transformed into *E. coli* together with the hGH expression vector pOmpA-hGH2. Double transformants were selected on TY plates containing both chloramphenicol and ampicillin. Isolates were analyzed for hGH release following induction of both BRP and hGH. Induction was facilitated by growth of a 1:50 dilution of overnight culture in 2 ml TY broth at 30 °C for 1.5 h followed by addition of IPTG (20 µM) and $MgSO_4$ (10 µM). The culture was then grown at 37 °C for 3 h.

When cells were grown in a rich (TY) medium without IPTG, the cells grew well, but almost no hGH was released into the medium. The optimal IPTG concentration for the BRP induction and hGH release was found to be around 20 µM, which resulted in a yield of 4.5 µg/ml A_{550} cells. With lower IPTG concentrations (5 µM), the cells grew to a higher density, but the amount of hGH released was lower. Higher IPTG concentrations (40 µM) resulted in both a retardation of cell growth as well as lower hGH yields. When double transformed *E. coli* cells were grown in minimal (M9 plus casamino acids) medium, the synthesis and excretion of hGH was severely reduced.

7.4. SDS-PAGE and Immunoblotting

Following the 3 h induction phase, 1 ml aliquots of cell culture were withdrawn and pelleted in 1.5 ml microfuge tubes. Cell lysates were prepared by resuspension of cell pellets in 200 µl of SDS sample buffer (2% SDS, 30% glycerol, 1 M 2-mercaptoethanol, 6 M urea in 0.125 M Tris/HCl, pH 6.8). Culture medium was concentrated 10-fold in a Centricon 10 microconcentrator, whereupon an SDS sample buffer (20% glycerol, 10% 2-mercaptoethanol, 6% SDS in 0.125 M Tris/HCl, pH 6.8) was added up to a final volume of 200 µl. Samples were boiled for 5 min and 10 µl aliquots run on a 15% SDS

polyacrylamide gel at 40 mA for 3 h (see Figure 4A). Duplicated gels were blotted onto nitrocellulose and hGH subsequently detected using rabbit anti-hGH serum (see Figure 4B).

7.5. Large scale culture and purification of the excreted protein (hGH)

Double transformed bacterial cells were grown in 1.8 l of TY medium to a cell density A_{550} of 0.4, then induced with 20 µM IPTG for 3 h. A MoBiTec 2 Liter fermenter is useful here. The culture medium was collected and concentrated to 115 ml using an Amicon stirred cell with a YM 10 membrane. The released hGH was purified by a one-step procedure from concentrated culture medium.

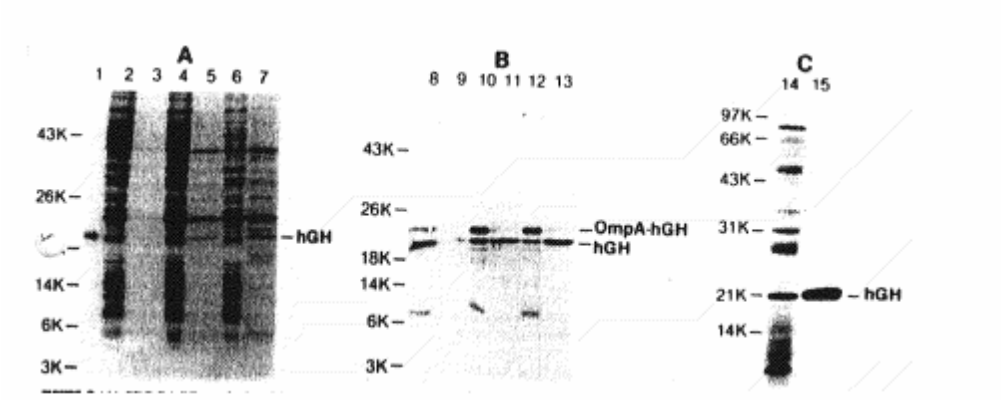


Figure 5: (A) SDS-PAGE analysis of hGH excretion by *E. coli* RV308 cells harboring the two plasmids pJL3 and pOmpA-hGH2. Samples were prepared as described above. **Lane 1:** hGH standard; **lane 2:** total cell lysate from cells grown without IPTG; **lane 3:** culture medium concentrate from cells grown without IPTG; **lane 4:** total cell lysate from cells grown in the presence of 5 µM IPTG; **lane 5:** culture medium concentrate from cells grown in the presence of 5 µM IPTG; **lane 6:** total cell lysate from cells grown in the presence of 20 µM IPTG; **lane 7:** culture medium concentrate from cells grown in the presence of 20 µM IPTG. (B) Release of hGH into the culture medium by immunoblotting analysis. The samples loaded on lanes 8 to 13 are the same as those loaded in lanes 2 to 7. (C) Non-reducing SDS-PAGE analysis. Lane 14: culture medium concentrate (16 µg total protein) from large scale prep; **lane 15:** purified hGH.

8. Order Information, Shipping, and Storage

Order#	Product	Quantity
BRPJL3	BRP Plasmid, pJL3, lyophilized DNA	5 µg
BRPSW1	BRP Plasmid, pSW1, lyophilized DNA	5 µg
shipped at RT; store at 4 °C		
COMSW1	E.coli K12 transformed with pSW1	5x200 µl
COMJL3	E.coli K12 transformed with pJL3	5x200 µl
shipped on dry ice; store at -80 °C		

9. Contact and Support

MoBiTec GmbH ◆ Lotzestrasse 22a ◆ D-37083 Goettingen ◆ Germany

Customer Service – General inquiries & orders
phone: +49 (0)551 707 22 0
fax: +49 (0)551 707 22 22
e-mail: order@mobitec.com

Technical Service – Product information
phone: +49 (0)551 707 22 70
fax: +49 (0)551 707 22 77
e-mail: info@mobitec.com

MoBiTec in your area: Find your local distributor at www.mobitec.com

Version: 08/2015