



EnBase™ Flo

Shake Flask Set

5 x 100 ml

User Manual EBLM100 – Rev. 1.6

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EnBase™ technology is patented to University of Oulu
(PCT/FI2007/050648, pending). Licensed by BioSilta Oy.

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EnBase™ Flo Shake Flask Set Contents

EnBase™ Flo Shake Flask Set Components and Storage Conditions

ITEM	CONTENTS	STORAGE
1. Oxygen permeable membrane covers	10 pcs. for cultivation, aseptically packed	RT
2. EnZ I'm (3000 U/L):	0.4 ml, sterile	+4 °C, 3 months
3. EnBase™ Flo Medium	5 x 100 ml of balanced medium, sterile	RT, 5 weeks
4a. Medium supplement: Thiamine	0.5 ml, sterile	RT, 3 months
4b. Medium supplement: Magnesium solution	1 ml, sterile	RT, 3 months
5. EnBase™ Medium for dilution	20 ml of medium 3., sterile	RT, 5 weeks
6. Medium booster	50 ml of complex medium additives, sterile	RT, 5 weeks

Storage of the set components: See the best before-date and storage conditions from the packages. RT: Room temperature, 18-25 °C

2. EnZ I'm is stable at room temperature but should be **stored at + 4 °C on arrival** to avoid contamination.

3. EnBase™ Medium: After addition of Thiamine (**4.**), use fresh.

Disposal of spare media: The medium components are not harmful in small concentrations and can be discarded according to local rules without precautions.

EnBase™ Product Family

EnBase™ technology is currently available in shake flasks, 50 ml tubes, 96-well plates, 24-, 48-, and 96 deep-well plates. Please see the ordering information in this manual.

If you need a specific cultivation format for your research, ask for a custom product and continue to benefit from the EnBase™ technology.

The Theory Behind the EnBase™ Technology

EnBase™ is an enhanced novel microbial cultivation system providing high cell density cultivation coupled with high recombinant protein yields in a range of cultivation vessels from 96 microwell plate to bioreactor.

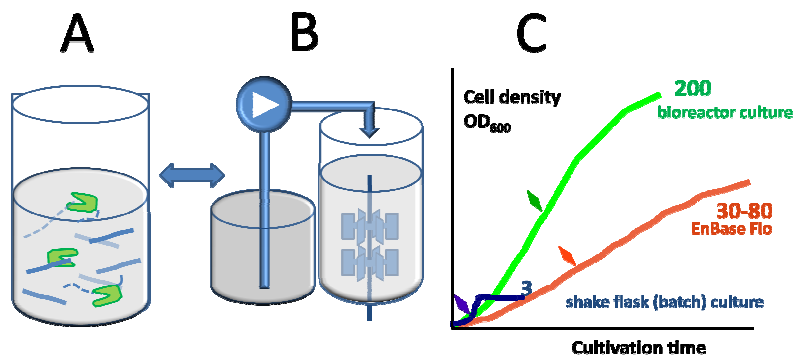
EnBase™ exploits the principle of fed-batch cultivation, traditionally used within bioreactors to provide controlled growth at micro-scale by enzymatic substrate release.

In a typical bioreactor process the main substrate feed (mostly glucose) is added by a pump, in EnBase™ glucose is delivered by enzymatic

degradation of a polymeric substrate (obtained either from a gel or lately as a soluble polymeric substrate in EnBase™ Flo products). By utilising controlled growth within EnBase™, accumulation of growth limiting compounds from overflow metabolism, and oxygen starvation can be significantly reduced or eliminated.

Typical cell densities within growth

processes	OD ₆₀₀
✓ Fed-Batch Bioreactor	50 - 200
✓ EnBase™	25 - 50
✓ Batch Shake Flask	1 - 10



Schematic view of the EnBase™ substrate delivery system of Liquid EnBase™ (A) compared to the setup in fed-batch bioreactor (B). The graph (C) shows the typical growth profiles and final cell densities of standard shake flask cultivation (blue line), fed-batch process in a bioreactor (green line) and EnBase™ cultivation (red line).

Use of EnBase™

EnBase™ turns a shake-flask, microwell plate or deepwell plate into an efficient mini bioreactor system. EnBase™ can be used for process optimisation, scale-up, for miniature production of plasmid DNA, recombinant proteins, and for the screening of genomic libraries. EnBase™ can be used with different type of growth media, typically mineral salt medium (MSM) for process optimisation and boosted EnBase™ medium for growth to high cell densities.

The EnBase™ medium is based on an optimised mineral salt medium (MSM) composition, where small amounts of complex medium additives (yeast extract, peptones) have been added to ensure a fast adaptation of cells to the medium. Balanced growth and favourable pH after overnight cultivation make bacteria ready for efficient recombinant protein production. Many auxotrophic (amino acid deficient) *E. coli* strains can be cultivated in EnBase™.

EnBase™ products are ready-to-use, out-of-box solutions with the ready-optimised conditions. However, where desired the user can customise the amount of EnZ I'm and further influence how many cells are available after overnight cultivation for your recombinant protein production process.

The recombinant protein production process. With EnBase™ you can induce recombinant protein production at high cell densities without bringing down the protein productivity per cell. Medium conditions and the physiological state of the cells are kept optimal, induction cell densities of OD₆₀₀ 5 to 15 work with EnBase™ and simplify the workflow. Favourable cell growth conditions enable long induction times. BioSilta recommends up to 24 h induction for recombinant protein production. Longer induction time may have a beneficial effect on the production of certain proteins and may increase the proportion of soluble (bioactive) recombinant protein. The standard recombinant protein production method is a two-day process with a minimal hands-on time. You prepare an overnight culture with EnBase™ medium, which is ready for the induction/boosting step (inducer i.e. IPTG, medium boosters and EnZ I'm are then added). The induction can last until next morning, when the cells are ready for harvesting. If slow cell growth or acidic conditions are beneficial for specific protein production, the medium booster solution can be omitted for maximal protein production.

EnBase™ cultivations reviewed:

- ✓ Slow glucose release controlled by EnZ I'm addition ensures defined cell growth in a fed-batch manner.
- ✓ Oxygen limitation and overflow-metabolism are avoided, thereby optimal conditions for recombinant protein production are provided.
- ✓ Addition of medium boosters and an extra dose of EnZ I'm during the recombinant gene expression provide the required biosynthetic activity to the bacteria.
- ✓ Balanced use of carbon source (glucose) and peptones (medium boosters) for optimal cell maintenance, growth and protein synthesis.
- ✓ Two-phase cultivation for an efficient protein production:
 1. The production of a high amount of cells by overnight cultivation (more than 10-fold cell mass when compared to that normally used for induction)
 2. Addition of medium boosters together with the inducer provides enough energy and constituents for efficient recombinant protein synthesis.

Detailed scientific info at: www.biosilta.com

About EnBase™ Flo Shake Flask Cultivation

Beware!

- ❖ *Growth is linear*
 - ✓ *It takes longer time to reach high cell densities than in ordinary batch cultivation, fast is not always the best in this case.*

- ❖ *Inoculation and shaking speed*
 - ✓ *Use inoculants being in exponential growth phase*

 - ✓ *250 rpm in shakers with circular orbit of 25 - 50 mm are recommended.*

 - ✓ *Shaking speed depends on the shaker and cultivation shake flask used. Maximise the agitation but avoid wetting the closure.*

- ❖ *Shake flask*
 - ✓ *Use 1L.shake-flask: 100 ml cultivation volume is 10 % of the flask volume which provides good oxygen transfer to the medium.*

 - ✓ *Cover the flask with a membrane-cover that enables good and safe oxygen transfer. Note however that wetting of any kind of membrane cap results in poor gas exchange and potential failure of the cultivation. In the case of membrane wetting, replace the membrane.*

Take care that in the beginning of your cultivation the concentration of any carbon sources or complex compounds is minimised. Remove the medium of your pre-culture by centrifugation.

With all inoculum options it is important to have initial $OD_{600} > 0.1$.

Note:

Items needed but not provided:

- **1 liter Shake-Flasks**

- **Inducer**, e.g. IPTG
1.1 ml of 100 mM IPTG solution (0.131 g IPTG + 5.5 ml distilled H₂O, sterilised by membrane-filtration)

- EnBase is not limited to the lacI-based expression systems;
Induction can be adapted to your existing method

❖ Induction

- ✓ Recommended induction OD_{600} for EnBase™ cultivation is 5-10. For efficient recombinant protein production, we recommend the addition of an extra EnZ I'm and nutrient dose at the time of induction for maximum performance (provided in the shake flask set).
- ✓ EnBase™ Flo provides good conditions for a long induction, therefore expression times from 6 to 24 hours are recommended. To enable correct protein folding and high proportion of soluble protein, a temperature shift (from 30 to 25 °C or lower) can be used after the induction is started.

❖ Harvest

- ✓ Recommended expression time is 6 to 24 h.
- ✓ Induction and harvest times need to be optimised for each specific expression system and customer culture conditions.

Note:

- **The control system** of EnBase™ is based on the idea of fed-batch cultivation, in which the growth is controlled by glucose limitation. Therefore it is essential to remove residual glucose, from the inoculum cultures prior to inoculation.
- **In all bacterial cultivations** it is needed to use fresh cells that are in the exponential growth phase to prevent long lag phase or inhibited growth by glucose accumulation.

EnBase™ Flo Cultivation Procedure

The culture is started with a volume of 100 ml. After induction the total volume will be about 110 ml due to addition of medium booster (10 ml) and supplements.

1. Prepare the inoculum:
 - A. Collect the cells from LB-agar plate cultivated overnight at 37 °C by rinsing with 3-4 ml EnBase™ Medium (bottle 3).
 - or*
 - B. Inoculate straight from a glycerol stock (minimum OD₆₀₀ of glycerol stock OD₆₀₀ > 10).

Don't use overnight-preculture in which the cells are already in stationary phase!

2. Add aseptically to 100 ml of EnBase™ Flo Medium (vial 3.)
 - antibiotics if needed
 - 0.1 ml thiamine (vial 4a.)
 - 0.2 ml magnesium solution (vial 4b)
 - 20 µl of EnZ I'm (vial 2.) until final concentration of 0.6 U/L .

3. Pour this into the **1L** cultivation shake flask and inoculate. Recommended initial OD₆₀₀ for EnBase™ cultivation is 0.1-0.2.

It is important to use only 10 % of the flask volume for maximal oxygen transfer!

4. Seal the flask with a membrane cover (1.)
5. Cultivate bacteria (30 °C, 250 rpm) for 16 to 20 h. During that time the recommended induction cell density OD₆₀₀ of 5 to 10 is reached.

Start cultivation immediately to avoid glucose accumulation. Glucose release is started after step 2.

INFO

- **Recommended EnZ I'm concentration is based on cultivation at 30 °C and pH 7. Under other conditions it is beneficial to optimise the EnZ I'm dosing.**
- **Medium supplements:**
Some compounds are introduced to the medium to avoid precipitation and unfavourable chemical reactions

6. Prepare the Booster/Inducer mixture: (can be done in advance)

- ❖ Take 10 ml of Medium Booster (vial 6.) and add 55 μ l of EnZ I'm (vial 2.) to provide extra 1.5 U/L final concentration in 110 ml cultivation volume¹
- ❖ Add inducer (e.g. 1.1 ml of 100 mM IPTG solution) to the Medium Booster (vial 6.)

After (overnight) cultivation (step 5.), add 10 ml medium booster/inducer mixture to the cultivation flask (Booster: 10 % of the cultivation volume).

7. Continue cultivation at the temperature, which is known to be optimal for production and correct folding of your protein. Harvest after 6 to 24 h expression.

¹ EnZ I'm concentration recommendations: Optimal EnZ I'm concentration is dependent on the cultivation conditions, i.e. oxygen transfer capacity, and also of the bacterial strain and medium. Therefore it may be beneficial to optimise the concentration in the specific conditions to reach best yield.

Hard Facts

TECHNOLOGY

Glucose limited fed-batch. Glucose is released enzymatically from the polymer supplied in the cultivation medium.

SHAKE FLASK AND CULTURE VOLUME

Recommended cultivation volume is 10 % of the shake flask volume to reach maximum aeration of the cultivation. Therefore use of 1 liter shake flask, baffled or non-baffled, is recommended for this procedure.

ORGANISMS/STRAINS

Applicable strains: W3110, RB791, RV308, BL21 and its DE3 derivatives, Rosetta. Attention: Redox mutants like Origami™ or Rosetta-gami™ strains reach lower cell densities due to higher cell maintenance requirements. Contact customer service (info@biosilta.com) if you want to use amino acid auxotroph strains like DH5α or DH10B for EnBase™ cultivations!

MEDIUM

A balanced growth medium is used in the first phase of cell cultivation. For protein production, this medium is supplemented with a booster solution, EnZ I'm and inducer.

STERILITY

All medium components are sterilised by autoclaving or by sterile filtration. They are produced by using aseptic techniques.

CULTIVATION CONDITIONS

Shaking speed is dependent on the shaker type and flask geometry. For standard 1L shake flasks shaking speed of 250 rpm is effective. The cultivation temperature of 30 °C can be used for the overnight culture but after the induction the conditions need to be optimised for each expression system.

REFERENCES

EnBase™ technology: Panula-Perälä, J., Šiurkus, J., Vasala, A., Wilmanowski, R., Casteleijn, M., Neubauer, P. (2008). Enzyme controlled glucose auto-delivery for high cell density cultivations in microplates and shake flasks. *Microbial Cell Factories* 7, 31.

Troubleshooting

Problem	Cause	Remedy
Long lag-phase	Non-viable cells in the inoculation or low initial cell density	Use fresh cells collected from overnight plate or use pre-culture. Start the EnBase™ cultivation with initial OD ₆₀₀ between 0.1 and 0.2.
Slow start of growth	Glucose accumulation in the beginning of cultivation may cause overflow metabolism and synthesis of growth-inhibiting metabolites.	1. Reduce EnZ I'm concentration 2. Check that the inoculum does not contain glucose.
Poor growth	Medium acidification due to bad oxygen transfer: wetting of closures might be one reason	Prevent medium spilling to the membrane cap by decreasing the shaker speed
Poor growth	Too old cells were used as inoculum	Ensure that your pre-culture has not reached stationary phase or the glycerol stock contains high proportion of living cells.

Please consult BioSilta (info@biosilta.com) if you are not getting beneficial results in your application. We are here to support you.

Ordering Information

Try EnBase™

EnBase™ Optimisation Set

EnBase™ Optimisation set	EnBase™ 24 deep-well plate for optimisation, 3 different EnBase™ media, EnZ l'm, booster solution, user manual.
EBOS01	1 EnBase™ optimisation set
EBOS02	2 EnBase™ optimisation sets

Gel EnBase™ Products

Shake Flask and Starter Culture

EnBase™ Mini Shake Flask	4 (8) pieces of EnBase™ mini shake flasks, 100 ml (200 ml) EnBase™ medium, EnZ l'm, booster solution, user manual.
EBMF04	EnBase™ Mini Shake Flask set, 4 flasks (4 x 20 ml culture)
EBMF08	EnBase™ Mini Shake Flask set, 8 flasks (8 x 20 ml culture)

EnBase™ Starter Tube	12 (24) pieces of EnBase™ Tubes, 100 ml (200 ml) EnBase™ medium, EnZ l'm, booster solution, user manual.
EBST12	EnBase™ Starter-Tube set, 12 tubes (12 x 5 ml culture)
EBST24	EnBase™ Starter-Tube set, 24 tubes (24 x 5 ml culture)

EnBase™ Plated Products

EnBase™ 96 Well Plate	6 (12) pieces of EnBase™ 96 micro-well plates, 100 ml (200 ml) of EnBase™ medium, EnZ l'm, booster solution, user manual.
EB96S06	EnBase™ 6 plate cultivation set (culture volume 150 µl / well)
EB96S12	EnBase™ 12 plate cultivation set (culture volume 150 µl / well)

EnBase™ 48 Deep-Well Plate	4 pieces of EnBase™ 48 deep-well plates, membrane covers, 4 x 80 ml of EnBase™ medium, EnZ l'm, booster solution, user manual.
EB48D04	EnBase™ 48DWP, 4 plate set (culture volume 1.5 ml / well)

EnBase™ 24 Deep-Well Plate	4 pieces of EnBase™ 24 deep-well plates, membrane covers, 4 x 80 ml of EnBase™ medium, EnZ l'm, booster solution, user manual.
EB24D04	EnBase™ 24DWP, 4 plate set (culture volume 3 ml / well)

Liquid EnBase™ Products

Shake Flask Sets

EnBase™ Flo Shake Flask 5 x 100 ml	5 x 100 ml EnBase™ Flo medium, membrane covers for shake flasks, EnZ I'm, booster solution, user manual.
EBLM100	EnBase™ Flo set for five 1 liter flasks (5 x 100 ml culture)

EnBase™ Flo Shake Flask 2 x 500 ml	2 x 500 ml EnBase™ Flo medium, membrane covers for shake flasks, EnZ I'm, booster, user manual.
EBLM500	EnBase™ Flo medium (2 x 500 ml in 5L flask or 5 x 200 ml in 2L flask)

Plate-Sets

EnBase™ Flo 96 Well Plate	6 (12) pieces of 96 micro-well plates, 2 x 100 ml (4 x 100 ml) of EnBase™ Flo medium, EnZ I'm 3000 units/L, booster solution, user manual.
EBF9606	EnBase™ Flo, 6 plate set (culture volume 300 µl / well)
EBF9612	EnBase™ Flo, 12 plate set (culture volume 300 µl / well)

EnBase™ Flo 96 Deep-Well Plate	4 pieces of 96 deep-well plates, membrane covers, 4 x 80 ml of EnBase™ Flo medium, EnZ I'm, booster solution, user manual.
EBF96D04	EnBase™ Flo 96DWP, 4 plate set (culture volume 700 µl / well)

EnBase™ Flo 48 Deep-Well Plate	4 pieces of 48 deep-well plates, membrane covers, 4 x 80 ml of EnBase™ Flo medium, EnZ I'm, booster solution, user manual.
EBF48D04	EnBase™ Flo 48DWP, 4 plate set (culture volume 1.5 ml / well)

EnBase™ Flo 24 Deep-Well Plate	4 pieces of EnBase™ 24 deep-well plates, membrane covers, 4 x 80 ml of EnBase™ medium, EnZ I'm 3000 units/L, booster solution, user manual.
EBF24D04	EnBase™ Flo 24DWP, 4 plate set (culture volume 3 ml / well)

R1.2

If you need a specific cultivation format for your research, ask for a custom product and continue to benefit from the EnBase™ technology.

Further information, orders and inquiries:

W: www.biosilta.com

E: sales@biosilta.com

T: +358-44-557 1862

Quick Reference Guide

Always study the instructions of this manual carefully before starting cultivation. This *Quick reference guide* can be used as a memory aid when the procedure is familiar.

- ❖ Prepare pre-culture if necessary

1st day:

- ❖ Prepare culture medium
 - ✗ Add thiamine, magnesium solution, antibiotics
- ❖ Add EnZ I'm
- ❖ Inoculate
- ❖ Start cultivation, close the shake flask with membrane

2nd day:

- ❖ Add Inducer/ booster/ EnZ I'm mixture
(or use cultivation as a pre-culture)

3rd day:

- ❖ Harvest



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