EnBase™ OptiSet

User Manual EBOS01 – Rev. 4.4

For research use only. Not for human or diagnostic use.

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Preface

Dear researcher,

EnBase™ OptiSet is a handy tool which helps you to select an optimal cultivation medium for the production of your recombinant protein.

It includes a total of 8 different conditions for testing. One of the test media is the medium which you routinely use for recombinant protein production. The other medium/cultivation options utilize the patented EnBase technology of BioSilta. EnBase™ enables controlled growth of bacteria by enzymatic glucose feeding.

This optimisation procedure is very simple and requires low hands-on time. Investing 2 hours with this OptiSet product will help you to

a) find a cultivation method which is optimal for the type of protein which you are expressing
b) find a method which yields a substantial (ten-fold) increase in bacterial cell number and product yield
c) find a method which can be easily scaled up to larger volumes
d) find a method which allows you to reduce the volume of your cultures

Additionally, the use of EnBase™ technology allows you to use higher induction cell densities and longer induction times than those which are typically used in shake flask cultivations. By using recommended cultivation procedures, you will obtain increased flexibility, and the growth of bacteria will follow your schedule and expectations. Controlled slow growth of bacteria will bring you a new level of control. In finding an optimal way of cultivating bacteria and producing recombinant proteins, you can save several days of work by having a reliable high yield cultivation method in your hands.

Please read the following pages carefully including the overview of the product and the detailed protocol on how to optimize your cultivation conditions.

EnBase™ OptiSet

Optimisation set components and storage conditions

ITEM	CONTENTS	STORAGE
1. EnBase™ 24 deep-well Plate	1 plate	RT, 5 weeks
1a. Oxygen permeable membrane covers	4 pcs. for cultivation	RT
2. EnZ I'm (3000 U/L):	200 µL, sterile	**+4 °C, 3 months**
3. EnBase™ Medium for dilution	20 ml, sterile	RT, 5 weeks
4a. Medium supplement: Thiamine	100 µl, sterile	RT, 3 months
4b. Medium supplement: Magnesium	200 µl, sterile	RT, 3 months
5. Medium Booster	900 µl, sterile	RT, 5 weeks
6. Medium Booster	3.18 ml, sterile	RT, 5 weeks

Description of other vials

ITEM	CONTENTS	STORAGE
7. EnBase™ Mineral Salt Medium (MSM 2)	20 ml, sterile	RT, 5 weeks — Colour Code Green
8. EnBase™ Balanced Medium (MSM 10)	20 ml, sterile	RT, 5 weeks — Colour Code Red
9. EnBase™ Flo Medium	20 ml, sterile	RT, 5 weeks — Colour Code Yellow
10. Empty vial for standard medium	*Sterile, use for your own standard medium*	Colour Code Blank
Vials for harvesting the cells	24 pcs. of 2 ml coloured tubes, non-sterile.	RT

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.

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

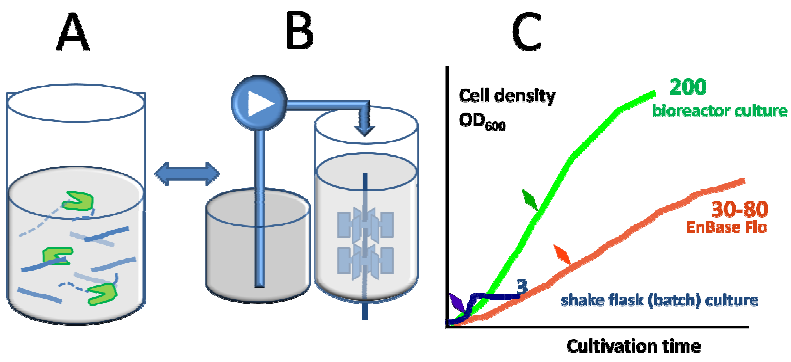
Disposal of spare media: The medium components are not harmful in small concentrations and can be discarded according to local rules without precautions.

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.

Typical cell densities within growth processes	**OD_{600}**
✓ **Fed-Batch Bioreactor**	**50 - 200**
✓ **EnBase™**	**25 - 50**
✓ **Batch Shake Flask**	**1 - 10**

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.



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 OD_{600} 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™ OptiSet Cultivation

Beware!

- *The greatest success with this EnBase™ set is reached if*
  - ✓ *Cultivation conditions are optimal*
  - ✓ *Inoculants being in exponential growth phase are used*

- *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.*

- *Plate cover*
  - ✓ *Remove the plastic covers from the plate and cover the plate for the cultivation with the oxygen permeable membrane cover which offers sufficient oxygen transfer to the plate.*

- *Time of induction and harvest*
  - ✓ *Recommended induction and harvest time for EnBase™ OptiSet cultivations are designed for a convenient working schedule and not for most optimal conditions when performed separately. Different cultivation modes are here combined in one time frame. Induction and harvest time for the own standard cultivation (C4-6, D4-6) can be adapted to optimal conditions for the target protein.*

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:

- ***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.*

Cultivation Facilities

Before starting, take care that your cultivation conditions are suitable for deepwell plate cultivation.

Shaker: Amplitude: 50 mm, shaking speed: 200 rpm. In case of other shaker type the agitation needs to be optimised so, that spilling is avoided and agitation maximised.

Humidity and Temperature: There should be high relative humidity (70 – 80 %) in the incubation chamber.

- ✓ Humidification of the chamber is recommended to avoid evaporation during the cultivation.
- ✓ It is recommended to keep the cultivation chamber at the cultivation temperature to avoid condensation of liquid to the membrane cover.
- ✓ The time schedule of the OptiSet cultivation is described for a temperature of 30 °C, but also lower temperatures are possible. In that case the cell growth is slower.

Items needed but not provided:

- ✓ Humidified shaker-incubator
- ✓ Pipettes for accurate dispensing of volumes: 10 µL – 3 ml
- ✓ IPTG or other inducer

Hint:

- *Humidity can be obtained by adding open containers with water or wet tissue to the incubators if a humidified chamber is not available.*
- ***Medium supplements:*** *Some compounds are introduced to the medium after sterilisation to avoid precipitation and unfavourable chemical reactions.*

The OptiSet plate and procedure overview

If the OptiSet product and cultivation procedure are already familiar to you, feel free to use these simplified instructions to make the cultivations. They help you to plan your cultivation work schedule. For details, read the following pages.

Table 1. The simplified plate layout. Eight different cultivation systems will be tested: each system in final setup contains three parallel samples

	1	2	3	4	5	6
A	EnBase gel with MSM medium			EnBase Flo		
B	EnBase gel MSM medium Boosted with complex additives			EnBase Flo Boosted with complex additives		
C	EnBase gel with Balanced medium			Your standard medium		
D	EnBase gel with Balanced medium Boosted with complex additives			Your standard medium Boosted with complex additives		

Notes:

Check-List for EnBase cultivation activities

Steps	Activities	Medium Bottles			
		7. MSM2	**8.** MSM10	**9.** Flo	**10.** Standard
Day 1 (preparations)					
1	Prepare the bacterial inoculum	❑			
2	Add the required medium components to the medium bottles; follow the order shown in Table 2	❑	❑	❑	❑
	Add bacteria to the medium	❑	❑	❑	❑
	Add Enz'Im to the medium	❑	❑	❑	
3	Apply medium from bottles to plate wells (6 wells from each bottle) and start cultivation	❑	❑	❑	❑
Day 1 after 2 h cultivation (boosting and induction)					
4	Add medium booster (vial 5.) to 3 wells of standard cultivation				❑
	Add IPTG to all standard medium wells				❑
Day 1 after 3-4 h induction (harvest)					
5	Harvest the cells after 3-4 h induction				❑
Day 2 (boosting and induction)					
6	Prepare booster/enzyme/IPTG mixture (vial 6.)	❑		❑	
	Apply EnZ I'm(vial 2.) to "upper rows"	❑	❑	❑	
	Apply booster/enzyme to "lower rows"	❑	❑	❑	
	Add inducer (IPTG) to all wells	❑	❑	❑	
Day 3 (or late Day 2 for shorter induction)					
7	Harvest the cells	❑	❑	❑	
8	Analyze the results	❑	❑	❑	❑

Cultivation Protocol

Day 1: Medium preparations

1. **Prepare the inoculum** in the morning:
   - **A. Prepare the inoculum**:
     - A. Collect the cells from LB-agar plate cultivated overnight at 37 °C by rinsing with 3-4 ml EnBase™ Medium (vial 3.).
     - *or*
     - B. Inoculate straight from a glycerol stock (minimum OD_{600} of glycerol stock OD_{600} > 10).

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

   Measure optical density of cell suspension. Calculate needed volume for the different media to obtain initial cultivation OD_{600} = 0.15 according to the equation

   $$\frac{0.15 \cdot 20.0 \text{ ml}}{\text{Measured } OD_{600}} = \text{x ml}$$

   x ml is the volume of cell suspension, which will be added into medium bottles.

2. **Add the supplements to the medium bottles** according to the following table (Table 2.). It is important to keep the order and add the enzyme last. Mix bottles carefully after addition.

Table 2. Preparation of cultivation media

Order	Addition of:	Bottle MSM2	Bottle MSM10	Bottle Flo	Bottle Standard
1.	Own standard medium				❑ 20 ml
2.	Needed antibiotics	❑_____ ml	❑_____ ml	❑_____ ml	❑_____ ml
3.	Thiamine, vial 4a.	❑ 20 µl	❑ 20 µl	❑ 20 µl	
3.	Magnesium, vial 4b.	❑40 µl	❑40 µl	❑40 µl	
4.	Cells	❑_____ ml (x ml)	❑_____ ml (x ml)	❑_____ ml (x ml)	❑_____ ml (x ml)
5.	EnZ´Im, vial 2.	❑40 µl	❑20 µl	❑10 µl	

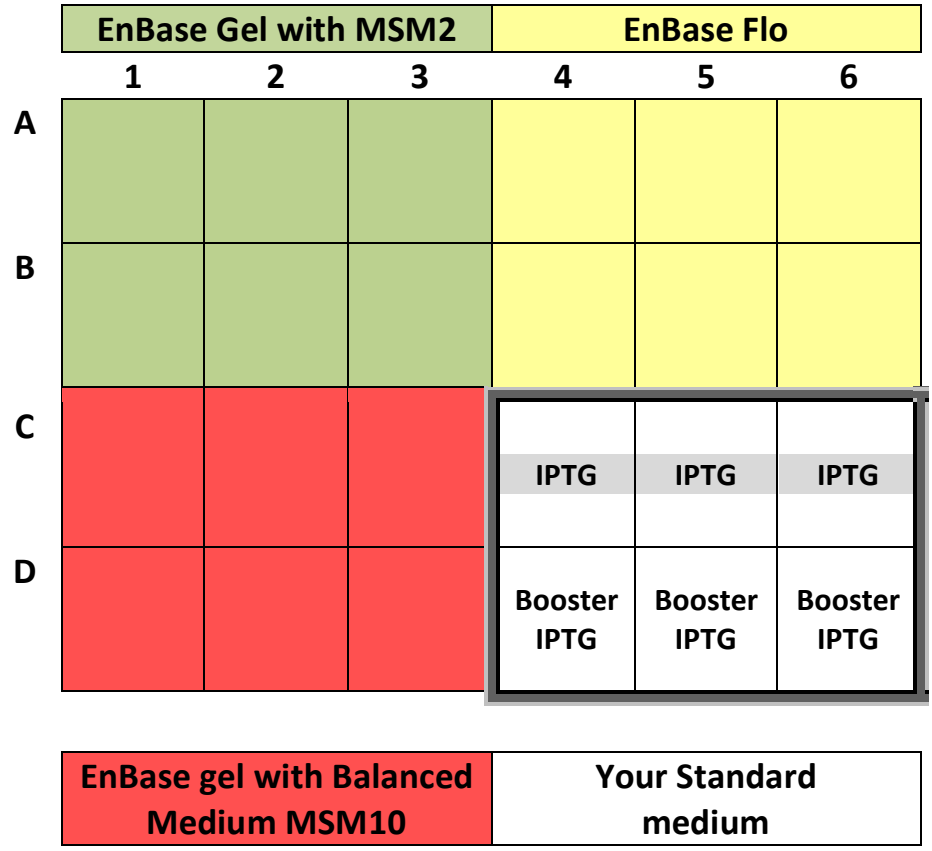
3. **Remove the plastic storage-seal** from the 24-DWP and add 3 ml of the medium-cell mixture per well as marked with colours on the plate layout 1*:*

   Bottle 7. MSM 2 (wells A1, A2, A3, B1, B2, B3: Green)
   Bottle 8. Balanced Medium (MSM10) (wells C1, C2, C3, D1, D2, D3: Red)
   Bottle 9. EnBase Flo medium (wells A4, A5, A6, B4, B5, B6: Yellow) and
   Bottle 10. Your standard medium (wells C4, C5, C6, D4, D5, D6: Blank*)*.

   Cover the plate with the separately provided oxygen permeable membrane and start the cultivation at 30 °C, 200 rpm on shaker with amplitude of 50 mm. (The plastic cover is only for protection of the plate during transport.)

4. After 2 h cultivation add inducer, e.g. IPTG to the wells containing your **standard medium** (wells C4, C5, C6, D4, D5 and D6) and **add 300 µl medium boosters** (vial 5. Medium Booster, 900 µl) to the wells D4, D5 and D6 (see plate layout 1). Continue cultivation.

   Plate layout 1: *at induction of culture with standard medium*



5. 3-4 h after the induction, take aliquot for OD_{600} measurement of cultivation in your standard medium C4, C5, C6 and D4, D5, D6 and harvest the cells into delivered 2 ml colourless reaction tubes.

Day 2: Induction

6. After overnight cultivation (16-20 h) make induction/boosting for the EnBase™ cultivations (see plate layout 2).
   -Make EnZ´Im + Booster mixture: Add 35 µl En Zl'm (vial 2) to the vial 6. (medium Booster 3.18 ml) and mix carefully
   -Pipette 300 µl of the mixed solution to wells shown in **plate layout 2** (wells B1 to B6, D1 to D3).
   -**Add 3 µl Enzyme** (vial EnZ´Im) to the wells A1 to A6, C1 to C3)
   -**Add the required amount of inducer (IPTG) to all cultivations**

Plate layout 2. At induction of cultures with EnBase media

	1	2	3	4	5	6
A	3 µl EnZ´Im	3 µl EnZ´Im	3 µl EnZ´Im	3 µl EnZ´Im	3 µl EnZ´Im	3 µl EnZ´Im
B	300 µl EnZ´Im +Booster	300 µl EnZ´Im +Booster	300 µl EnZ´Im +Booster	300 µl EnZ´Im +Booster	300 µl EnZ´Im +Booster	300 µl EnZ´Im +Booster
C	3 µl EnZ´Im	3 µl EnZ´Im	3 µl EnZ´Im			
D	300 µl EnZ´Im +Booster	300 µl EnZ´Im +Booster	300 µl EnZ´Im +Booster			

Day 3 (or late Day 2): Harvest

7. 24 h after induction (step 6) take aliquots for OD_{600} measurement of the EnBase™ cultivations and harvest cells in delivered 2 ml reaction tubes according to the colour code.

8. Analyze the recombinant protein production according to your desired method (SDS-PAGE, target protein quantification after specific purification steps, or enzyme activity tests). When making preparations for these analysis please realize that EnBase cultivations may yield over 10-fold higher biomass and protein concentrations when compared to your standard cultivation methods.

Notes:

Troubleshooting

Problem	Cause	Remedy
Water droplets form on the membrane lid during the cultivation	Too high agitation splashes the liquid to the lid	Lower the agitation speed
Loss of volume during the cultivation	Evaporation is not prevented but is equal when oxygen permeable membrane is used EnBase™ gel is sucking the liquid phase into itself	Humidify the chamber to decrease evaporation Check the best before date of the plates: after 5 weeks storage the gel functions start to weaken
Slow 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.
Gel brakes down or detaches	Wrong storage temperature Products expired	Gels shall not be frozen or stored at low temperature Check the exp. date

Please consult BioSilta if you are not getting beneficial results in your application.

We are here to support you.

Hard Facts

TECHNOLOGY
Glucose limited fed-batch. Glucose is released enzymatically from the polymer matrix that is attached on the bottom of the wells and from a liquid polymer in EnBase™ Flo.

MAXIMAL CULTURE VOLUME PER WELL
Cultivation volume for the OptiSet is 3.0 mL. Original well volume is 10 mL, of which the EnBase™ gel takes 2 mL.

MAXIMUM NUMBER OF CULTIVATIONS ON ONE PLATE
24

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
Humidity: 70-80 % of relative air humidity in the cultivation chamber to minimize evaporation. Shaking: A: 25 mm or 50 mm, 200 rpm.

REFERENCES
EnBase™: 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.

Ordering Information

Try EnBase™

EnBase™ Optimisation Set

EnBase™ Optimisation set	EnBase™ 24 deep-well plate for optimisation, 3 different EnBase™ media, EnZ I'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 I'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 I'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 I'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 I'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 I'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 in2L 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:

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