EUROMEDEX

BACTH System Kit
Bacterial Adenylate Cyclase Two-Hybrid System Kit

Cat N°: EUK001

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

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Notice to Purchaser

The BACTH system kit is covered by the U.S. Patent No. 6,333,154.
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Materials Supplied

Materials	Amount	Reference
BACTH strains[a]		
DHM1, LB/DMSO stock (reporter strain for BACTH assay)	1 ml	EUB001
BTH101, LB/DMSO stock (reporter strain for BACTH assay)	1 ml	EUB002
Store at – 80 °C		
BACTH vectors[b]		
pKNT25, supercoiled, 10 µg (0,5 µg/µl in TE buffer)	20 µl	EUP-25N
pKT25, supercoiled, 10 µg (0,5 µg/µl in TE buffer)	20 µl	EUP-25C
pUT18, supercoiled, 10 µg (0,5 µg/µl in TE buffer)	20 µl	EUP-18N
pUT18C, supercoiled, 10 µg (0,5 µg/µl in TE buffer)	20 µl	EUP-18C
Store at – 20 °C		
Control plasmids		
pKT25-zip, supercoiled, 1 µg (0,05 µg/µl in TE buffer)	20 µl	EUP-25Z
pUT18C-zip, supercoiled, 1 µg (0,05 µg/µl in TE buffer)	20 µl	EUP-18Z
Store at – 20 °C		

a : Bacterial stocks were grown in an Animal Product Free medium (APF LB Miller ; Euromedex ref. : AE-0173)
b : DNA sequences and plasmid maps of the vectors are provided in Annex III

Storage conditions:

All plasmids : -20°C
Strains : -80°C

Material Not supplied

MacConkey agar base medium

M63 minimal medium

X-gal (5-Bromo-4-chloro-3-indolyl- β-D-galactopyranoside)

IPTG (isopropyl-β-D-thiogalactopyranoside)

ONPG (o-nitrophenol-β-galactoside)

Ampicillin

Kanamycin

Maltose

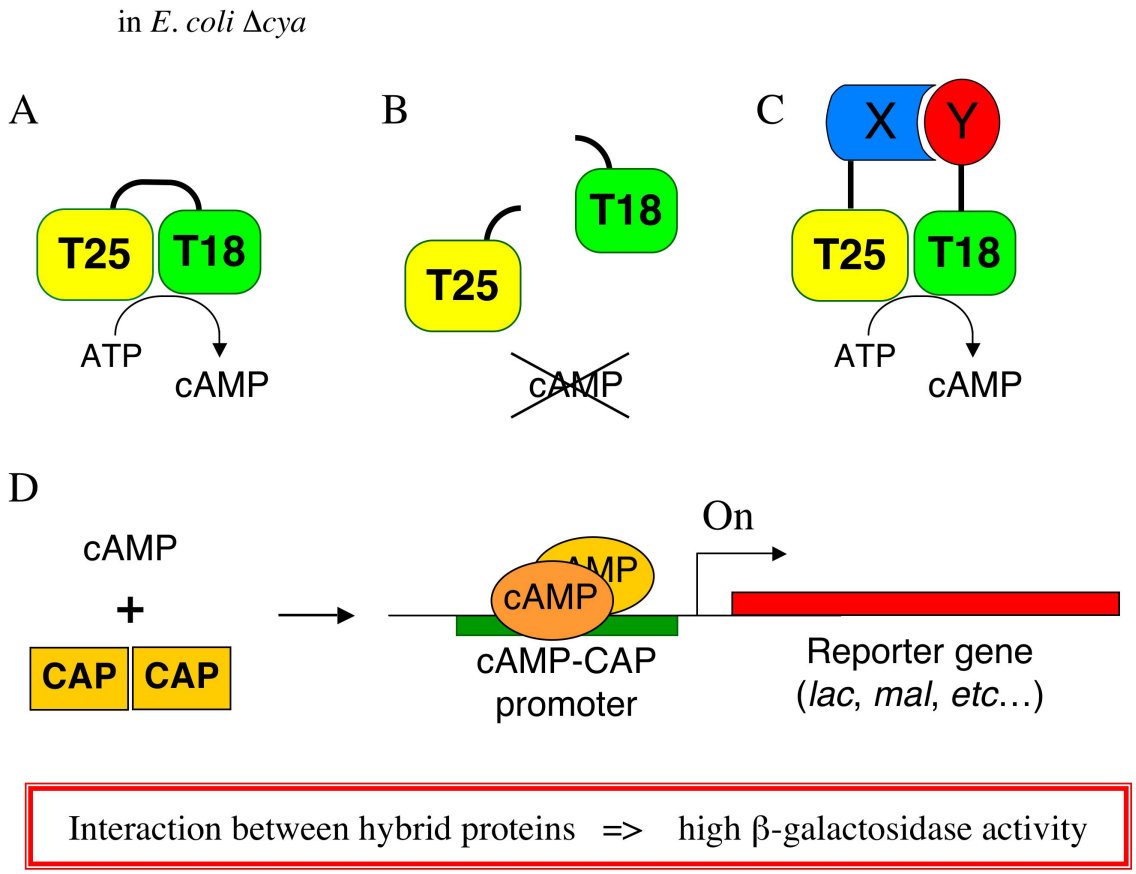
Standard *E. coli* K-12 lacI^q strains

Principle of BACTH system

Euromedex bacterial two-hybrid (BACTH, for "Bacterial Adenylate Cyclase-based Two-Hybrid") system is a simple and fast approach to detect and characterize protein-protein interactions *in vivo*. In contrast to yeast two-hybrid system, which requires specialized expertise, BACTH offers all the advantages of working with *Escherichia coli*: readily accessible microbiology and molecular biology techniques (plasmid preparations, efficiency of transformation, PCR…).

The BACTH system has been developed by the group of Dr. D. Ladant at the Pasteur Institute and is based on the interaction-mediated reconstitution of the adenylate cyclase activity in *E. coli* (1). It exploits the fact that the catalytic domain of adenylate cyclase (CyaA) from *Bordetella pertussis* (2) consists of two complementary fragments, T25 and T18 (Figure 1A), that are not active when physically separated (Figure 1B). When these two fragments are fused to interacting polypeptides, X and Y, heterodimerization of these hybrid proteins results in functional complementation between T25 and T18 fragments and, therefore, cAMP synthesis (Figure 1C). Cyclic AMP produced by the reconstituted chimeric enzyme binds to the catabolite activator protein, CAP. The cAMP/CAP complex is a pleiotropic regulator of gene transcription in *E. coli*. It turns on the expression of several resident genes, including genes of the *lac* and *mal* operons involved in lactose and maltose catabolism (Figure 1D). Therefore, bacteria become able to utilize lactose or maltose as the unique carbon source and can be easily distinguished on indicator or selective media.

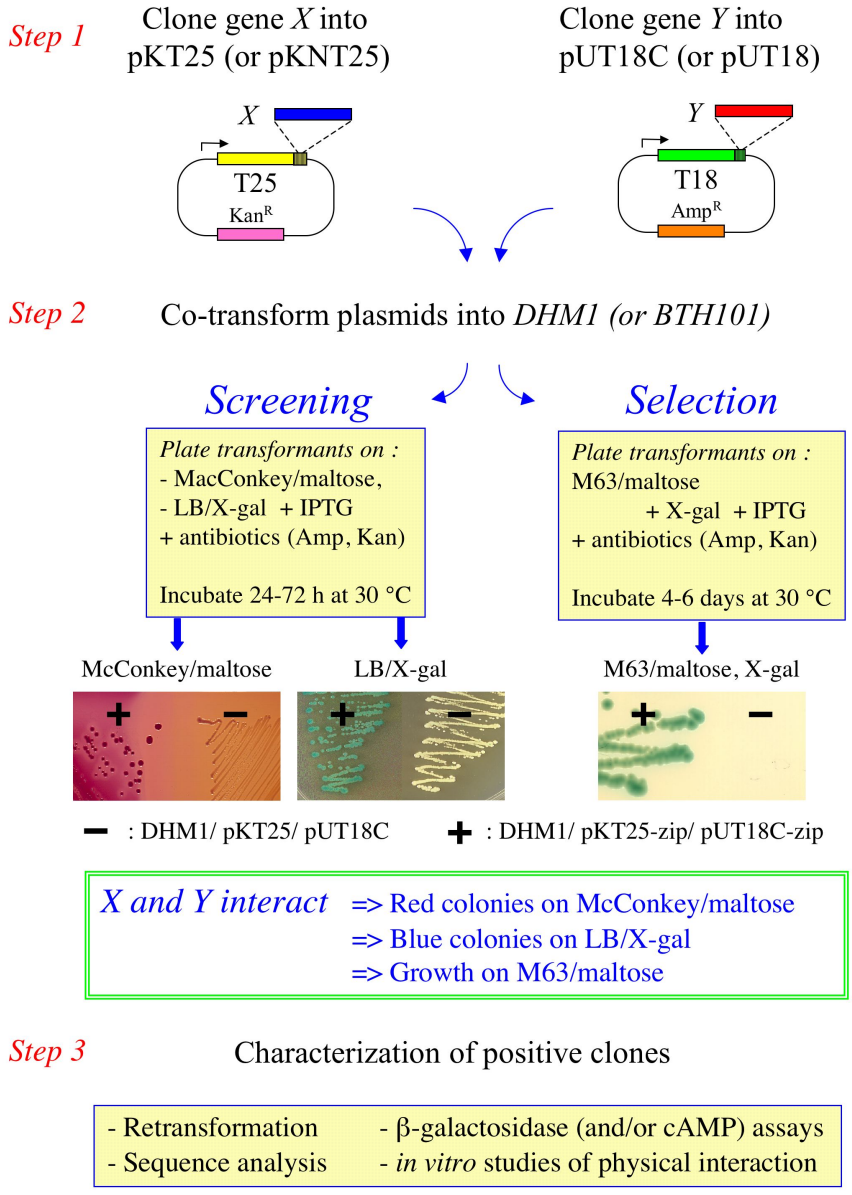
Figure 1 : Principle of the bacterial two-hybrid system.



Outlined of the procedure

Detection of *in vivo* interactions between two proteins of interest with the BACTH system requires the co-expression of these proteins as fusions with the T25 and T18 fragments in bacteria that are lacking endogenous adenylate cyclase activity (*E. coli cya*). This is achieved by using two compatible vectors, one expressing the T25 fusion (pKT25 or pKNT25) the other one expressing the T18 fusion (pUT18 or pUT18C). The bacteria are co-transformed with the two recombinant plasmids and plated on either indicator or selective media to reveal the resulting Cya^+ phenotype (Figure 2). The efficiency of complementation between the two hybrid proteins can be further quantified by measuring cAMP levels (a direct measure of the reconstituted adenylate cyclase enzymatic activity) or by assaying the β-galactosidase enzymatic activities in bacterial extracts, an easy and robust assay that is correlated with cAMP levels produced in the cells, as the expression of β-galactosidase is positively regulated by cAMP/CAP. The hybrid proteins expressed in *E. coli*, can also be characterized by using diverse biochemical approaches, such as immunodetection, immunoprecipitation, copurification, etc....

Figure 2: General methodology to analyze protein-protein interactions with BACTH system



Advantages of the BACTH system

The BACTH system presents several characteristics that makes it a worthy alternative and/or complementary tool to the traditional yeast two-hybrid system:

1 - As the BACTH assay is carried out in *E. coli* , the screening and the characterization of protein-protein interactions are greatly facilitated. It only requires standard molecular biology techniques. *E coli* grows faster than yeast and is easily transformed with high efficiency. In addition, the same plasmid constructs used in library screening to identify a putative binding partner to a given "bait", can be employed to express the chimeric proteins in order to characterize their interaction by *in vitro* binding assays.

2 - The BACTH system relies on a signaling cascade which utilizes the diffusable regulatory molecule, cAMP. As a consequence, the physical association of the two interacting chimeric proteins can be spatially separated from the transcription activation readout. Hence, it is possible to analyze protein-protein interactions that occur either in the cytosol, at the inner membrane level or on the DNA.

Indeed, In the past years the BACTH system has been successfully employed to characterize a wide variety of proteins of different origins (bacterial, eukaryotic, or viral), different sizes (from few tens to several hundreds of amino acids), different cellular locations (cytoplasmic, membrane associated, periplasmic or exported proteins), and different biological functions (enzymes, transporters, chaperones, transcriptional regulators, signaling, etc.) Numerous references of studies that employed BACTH system can be found on the web site of *Proc. Natl. Acad. Sci. U.S.A*. (http://www.pnas.org/cgi/content/full/95/10/5752).

Materials provided

A - Reporter strains

The BACTH kit provides two non-reverting adenylate cyclase deficient *(cya) E. coli* reporter strains, BTH101 and DHM1, (see genotypes below) that are used as host organisms for detection of protein-protein interactions (8, 9). Other *E. coli cya* strains (see *E. coli* strain collection (http://cgsc.biology.yale.edu) might also be tested.

The different genetic backgrounds of these two strains provide different complementation efficiencies and different reporter gene stringencies. BTH101 displays an exquisite BACTH efficiency and fast growth but some unstability of plasmids can appear due to the Rec^+ character of the strain. DHM1 is a *recA* strain with a lower complementation efficiency and slower growth. The frequencies of spontaneous Lac^+ and Mal^+ revertants (because of cAMP/CAP independent promoter mutations) are respectively, 10^{-8} and 10^{-9} in BTH101 and 10^{-7} and 10^{-9} in DHM1.

Strain genotype:

DHM1: F^-, *cya-854*, *recA1, endA1, gyrA96 (*Nal^r*)*, *thi1, hsdR17*, *spoT1*, *rfbD1, glnV44(AS).*

BTH101: F^-, *cya-99*, *araD139, galE15, galK16, rpsL1 (*Str^r*)*, *hsdR2, mcrA1, mcrB1.*

Competent cells preparation

Efficient (>10^8 cfu/μg) electro-competent cells can be prepared according to standard protocols (6). Competent BTH101 and DHM1 cells can be also prepared by $CaCl_2$ technique (6).

Important notice:

Before use, the BTH101 and DHM1 strains from the LB-DMSO stock should be restreaked on either MacConkey/maltose or LB/X-Gal/IPTG plates and grown overnight at 37 °C. White colonies (*i.e. cya*) should be picked up to start the overnight liquid preculture. Any red (on MacConkey/maltose) or blue colonies (on LB/X-Gal/IPTG) that may appear should be avoided (they likely correspond to Lac^+ or Mal^+ revertants or contaminants).
If too many contaminants are present upon re-streaking of the stock, it might be useful to add a selective antibiotic to the MacConkey/maltose or the LB/X-Gal/IPTG plates: DHM1 is resistant to nalidix acid (30 μg/ml) whereas BTH101 is resistant to streptomycin (100 μg/ml).

B - Plasmids

The BACTH technology requires co-expression of two hybrid proteins within the same recipient *cya* bacteria. The BACTH system kit provides two different sets of compatible vectors (4, 8, 9) that allow genetic fusions of proteins of interest at either the N or the C-termini of the T18 fragment (pUT18 and pUT18C) or of the T25 fragment (pKT25 and pKNT25). The nucleotide sequence of these vectors can be downloaded from the Euromedex web site (http://www.euromedex.com).

1. pKT25 encodes the T25 fragment (corresponding to the first 224 amino acids of CyaA) that is expressed under the transcriptional control of a lac promoter. The pKT25 vector is a derivative of the low copy-number plasmid pSU40, expressing a kanamycin resistance selectable marker. A multicloning site sequence (MCS) is inserted at the 3' end of T25 to allow construction of in-frame fusions at the C-terminal end of the T25 polypeptide.

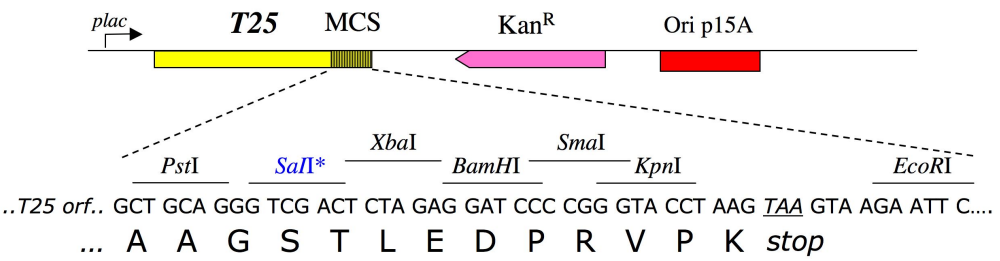
2. pKNT25 encodes the T25 fragment that is fused in frame downstream from a MCS. This allows to create in-frame fusions at the N-terminal end of T25. The vector is a derivative of the low copy-number plasmid pSU40, expressing a kanamycin resistance selectable marker.

3. pUT18 is a derivative of the high copy number vector pUC19, expressing an ampicillin resistance selectable marker, and encodes the T18 fragment (amino acids 225 to 399 of CyaA) that is expressed under the transcriptional control of a lac promoter. The T18 open reading frame lies downstream of a MCS with 9 unique restriction sites. This plasmid is designed to express chimeric proteins in which a heterologous polypeptide is fused to the N-terminal end of T18.

4. pUT18C differs from pUT18 in that the MCS is located at the 3’ end of the T18 open reading frame. This plasmid is designed to create chimeric proteins in which a heterologous polypeptide is fused to the C-terminal end of T18.

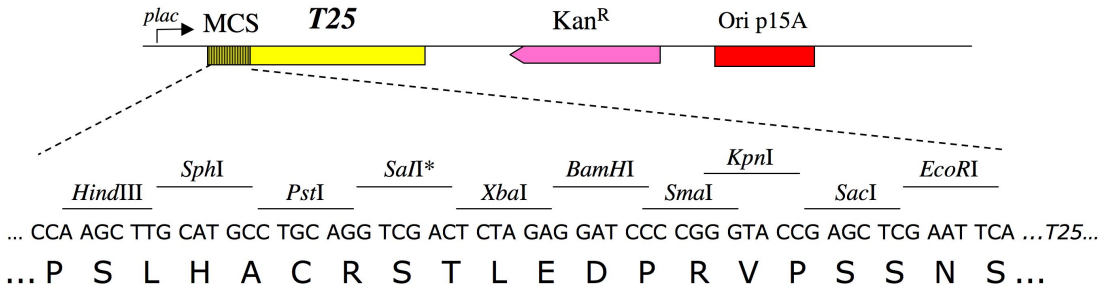
Figure 3 : BACTH plasmid maps

pKT25 (3442 bp)

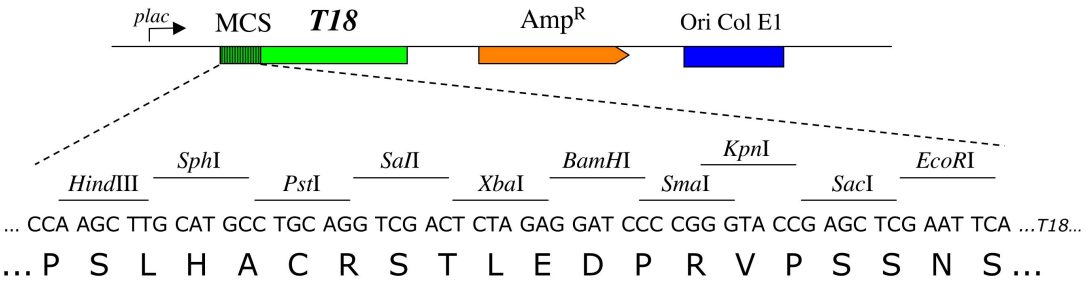


pKNT25 (3469 bp)

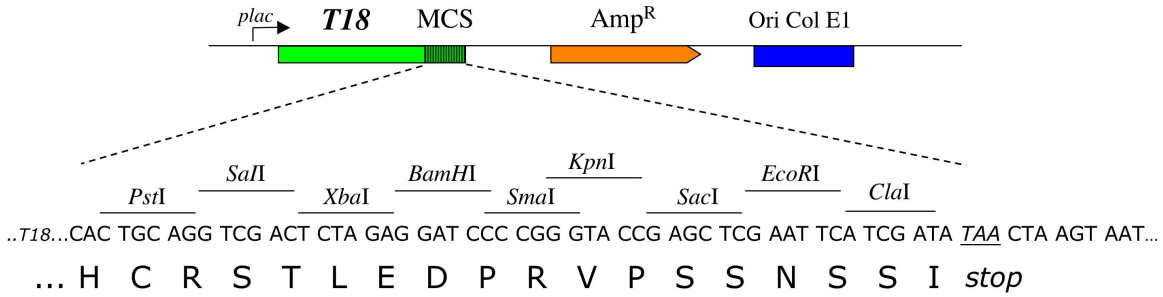
** Caution : a second SalI site is present in T25 ORF*



pUT18 (3023 bp)



pUT18C (3017 bp)



Control plasmids

1. pKT25-zip is a derivative of pKT25 in which the leucine zipper of GCN4 (1) is genetically fused in frame to the T25 fragment (inserted within the KpnI site of pKT25).

2. pUT18C-zip is a derivative of pUT18C in which the leucine zipper of GCN4 is genetically fused in frame to the T18 fragment (inserted between the KpnI and the EcoRI site of pUT18C).

The plasmids pKT25-zip and pUT18C-zip serve as positive controls for complementation. They expressed the T25-zip and T18-zip fusion proteins that can associate as a result of dimeriztion the leucine zipper motifs appended to the T25 and T18 fragments. When pKT25-zip and pUT18C-zip are co-transformed into DHM1 or BTH101, they restore a characteristic Cya^+ phenotype.

Plasmid preparation

Standard *E. coli* K-12 *lac*I^q strains (such as XL1-Blue) are recommended for plasmid preparation.

pUT18 and pUT18C plasmids are derived from pUC19. Standard plasmid preparation protocols for high-copy number plasmids should be followed (6).

pKT25 and pKNT25 plasmids are derived from pSU40, a low-copy number plasmid. Plasmid preparations should be performed accordingly (6).

Materials not provided

Media

In the BACTH system, interaction between the two proteins of interest leads to cAMP synthesis and thus confers a Cya^+ phenotype to the recipient *cya* bacteria. This can be easily scored either on indicator plates (*i.e.* LB-X-Gal or MacConkey media supplemented with maltose or lactose) or on selective plates (synthetic media supplemented with lactose or maltose as unique carbon sources). The time of growth in these two types of media varies significantly.

MacConkey medium

E. coli cya bacteria are unable to ferment lactose or maltose (3): they form white (or pale pink) colonies on MacConkey indicator media containing lactose or maltose, while cya^+ bacteria form red colonies on the same media (fermentation of the added sugars results in the acidification of the medium which is revealed by a color change of the dye phenol red) (5). **Maltose is the most appropriate sugar to work on MacConkey agar base medium**. Note that all MacConkey agar base media are not of equal quality (MacConkey from Difco Laboratories - cat # 216830 – is recommended). 40 g of MacConkey agar are dissolved in 1 liter of distilled water and autoclaved. A stock solution of **glucose-free** maltose (20% in water) is sterilized by filtration. Maltose (1% final concentration) as well as antibiotics (ampicillin at 100 μg/ml and kanamycin at 50 μg/ml) are added to the autoclaved MacConkey medium just before pouring plates (5). IPTG (isopropyl-β-D-thiogalactopyranoside, 0.5 mM) can be included in the medium to induce full expression of the hybrid proteins

LB-X-Gal medium

In *E. coli*, expression of the *lac*Z gene encoding β-galactosidase is positively controlled by cAMP/CAP (3). Hence, bacteria expressing interacting hybrid proteins will form blue colonies on rich LB medium (5) in the presence of the chromogenic substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 40 μg/ml), while cells expressing non-interacting proteins will remain white (pale blue). IPTG (0.5 mM) can be included in the medium to increase β-galactosidase expression. The concentrations of antibiotics indicated above are used in LB-X-Gal medium.

By using these rich indicator media functional complementation can be detected within 24 - 72 hrs at 30°C. Overcrowding of indicator plates should be avoided (maximum 300-500 colonies per plate) otherwise the detection of positive clones might be difficult (5). It should be noted that after prolonged incubation (4-5 days), negative colonies (*i.e. cya*$^-$) will show a weak red (on MacConkey-maltose) or blue spot (on LB-X-Gal) in the center, but will remain colorless at the periphery. **It may be worth testing also the complementation at 37°C**, although in most cases, complementation is **much less efficient at this temperature than at 30°C**.

Synthetic medium

Since Cya$^+$ cells are Mal$^+$, they are able to grow on a minimal medium supplemented with maltose as a unique carbon sources (3, 5).

M63 medium supplemented with maltose is prepared as follows: add 2g $(NH_4)_2SO_4$, 13.6g KH_2PO_4, 0.5mg $FeSO_4 \cdot 7H_2O$ and 15 g agar per liter of water (adjust pH to 7.0 with KOH). After autoclaving, add 1ml of $MgSO_4 \cdot 7H_2O$ 1M, 10 ml of 20% maltose, 2ml of 0.05% vitamin B1 (thiamin), and appropriate antibiotics at lower concentration than for rich media (50 μg/ml ampicillin; 25 μg/ml kanamycin). Growth of Mal$^+$ colonies can be detected after 4 to 8 days of incubation at 30°C. X-Gal and IPTG can be also included in this medium.

Analysis of protein-protein interaction with BACTH system

General methodology

The general methodology to analyze protein-protein interactions with BACTH is the following (see figure 2):

Step 1: the genes encoding the two proteins of interest (X and Y in Fig.2; they are usually amplified by PCR using appropriate primers) are sub-cloned into pKT25 (or pKNT25) and pUT18 (or pUT18C) vectors in frame with the T25 and T18 fragment open reading frames by using standard molecular biology techniques (6). Vectors and recombinant plasmids are commonly propagated at 30°C in standard *E. coli* K12 *recA* strains (such as XL1-Blue). To avoid any problems during construction of the plasmids it is wise to use as host an *E. coli* strain that overproduce the LacI repressor to prevent expression of the hybrid proteins. Plasmid DNA is routinely purified with one of many commercial kits used for mini-preparation of DNA, according to manufacturer's instructions

Step 2: the two recombinant plasmids encoding the T25-X (or X-T25) and T18-Y (or Y-T18) hybrid proteins are transformed into competent BTH101 or DHM1 reporter cells. A high level of competency of *E. coli* cells allows cotransformation of two plasmids. Transformants are plated either on indicator or selective plates and incubated at 30°C or 37°C. **It is noteworthy that in most cases, complementation is much more efficient at 30°C than at 37°C.**

The screening procedure (*i.e.* on indicator plates) is appropriate if only a small number of transformants (less than 500 colonies per plate) is tested (1, 7, 8, 9). If X and Y interact, all colonies should give the same phenotype on the indicator plates: blue on LB-X-Gal or red on MacConkey-maltose. Complementation can be detected in 1 - 4 days. If no interaction occurs, all colonies should be colorless.

The selection procedure is particularly suited for screening libraries (10, 11). For this, DHM1 or BTH101 cells are first transformed with a pKT25 (or pKNT25) derived bait plasmid encoding the T25-bait protein fusion (or with pUT18 or pUT18C derived bait plasmid encoding the T18-bait fusion). New competent cells are then prepared from these transformants and co-transformed with the plasmid libraries constructed in pUT18C or in pKT25 (see ref. 10 or 11). Co-transformants are plated on M63 minimal medium plus maltose (or lactose).

Importantly, after transformation, the cells should be washed at least three times with M63 medium in order to remove all traces of the rich medium used in the transformation procedure. Up to 10^6 cells can be plated on a single dish of M63/maltose (plus appropriate antibiotics). Growth of Mal^+ colonies will be detected after 4-8 days of incubation at 30°C. The X-gal substrate can be added to the medium to facilitate the early visualization of growing colonies (that should also be Lac^+).

Test of interactions

Twenty μl of fresh or thawed electrocompetent BTH101 or DHM1 reporter cells should be mixed with 5 ng of each plasmid. Serial dilutions of the transformation mixture should be plated in order to obtain about 100-200 colonies per plate. It is important that the number of colonies do not exceed 500, if you use MacConkey or X-gal media. As a positive control, competent reporter cells should be co-transformed with the control plasmids pKT25-zip and pUT18C-zip.

Analytical procedures

Quantification of the functional complementation mediated by interaction between two proteins, X and Y, can be obtained by measuring cAMP levels and β-galactosidase activities in liquid cultures (1, 7, 8, 9).

Cyclic AMP can be measured in boiled bacterial cultures by radioimmunoassays or ELISA assays (1). Commercial kits for cAMP determination (ELISA or radioimmunoassays) are available from many companies.

β-galactosidase measurements are performed on permeabilized cells (either exponential or overnight cultures) using o-nitrophenol-β-galactoside (ONPG) as a substrate (see Annex II for a detailed protocol). β-galactosidase activity is usually expressed in units (one unit of corresponds to 1 nmol of ONPG hydrolyzed per min at 28°C) per mg of bacterial dry weight. Under routine conditions, when no interaction occurs, the DHM1 strain expresses about 150 units of β-galactosidase/mg of bacterial dry weight (BTH101 about 100). When hybrid proteins associate, β-galactosidase activities range between 700 to 7000 units/mg, depending on the efficiency of functional complementation (T25-zip/T18-zip complementation should give about 6000-8000 units/mg).

When a large number of β-galactosidase assays has to be carried out, it may be practical to carry out the assays in 96-well microtiter plates as described in Annex II (see also references 11 & 12).

Additional information and further description of experimental procedures can be found in references 13 & 14.

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Annex I : Materials and media

Materials

IPTG, isopropyl β-D-thiogalactopyranoside
= inducer of *lac* promoter. Stock solution of 100 mM in water, sterilized by filtration.
X-gal, (5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside
= chromogenic substrate of β-galactosidase. Stock solution of 20 mg/ml in dimethyl formamide
ONPG, 2-Nitrophenyl β-D-galactopyranoside (substrate of β-galactosidase)

Antibiotics

Bacterial media are supplemented with antibiotics from stock solutions in the following concentrations;

Antibiotics	Stock concentration	Working Concentration
Ampicillin	100 mg/ml in H_20	100 µg/ml
Kanamycin	50 mg/ml in H_20	50 µg/ml
Streptomycin	100 mg/ml in H_20	100 µg/ml
Nalidixic acid	30 mg/ml in ethanol	30 µg/ml

Media

Luria-Bertani (LB) broth

To prepare 1 L LB broth, mix 10g of NaCl, 10g of tryptone, and 10g of yeast extract, adjust pH to 7.0 with NaOH , add deionized H_2O to a final volume of 1 liter and autoclave.
To prepare LB plates, add 15 g of agar per liter of LB broth and autoclave. Allow the medium to cool down to less than 45°C, then add the antibiotics and pour the plates.

LB/X-gal medium

To prepare LB/X-gal plates, the LB/agar medium (above) is autoclaved, allowed to cool down to less than 45°C and supplemented , just before pouring plates, with 40 µg/ml of the X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside) chromogenic substrate and appropriate antibiotics. IPTG (isopropyl-β-D-thiogalactopyranoside, final concentration of 0.5 mM) is usually also added to the medium in order to induce full expression of the hybrid proteins as well as that of the β-galactosidase reporter enzyme.

MacConkey/maltose medium.

To prepare MacConkey/maltose plates, 40 g of MacConkey agar are dissolved in 1 liter of distilled water and autoclaved. A stock solution of **glucose-free** maltose (20% in water) is sterilized by filtration. Maltose (1% final concentration) as well as antibiotics (ampicillin at 100 µg/ml and kanamycin at 50 µg/ml) are added to the autoclaved MacConkey medium just before pouring plates (5). IPTG (isopropyl-β-D-thiogalactopyranoside, final concentration of 0.5 mM) is usually added to the medium in order to induce full expression of the hybrid proteins.
Importante notice: all MacConkey agar base media are not of equal quality. MacConkey from Difco Laboratories - cat # 216830 – is strongly recommended.

M63/maltose minimal medium

To prepare 5x concentrated M63 minimal medium, mix 10 g $(NH_4)_2SO_4$**,** 68 g KH_2PO_4, 2.5 mg $FeSO_4 \cdot 7H_2O$, 5 mg vitamin B1, add deionized H_2O to a final volume of 1 L, adjust pH to 7.0 with KOH , and autoclave.

To prepare M63/maltose plates, autoclave 15 g of agar in 800 ml H_20. Then add 200 ml sterile 5x M63 medium, 0.2-0.4 % maltose, and the appropriate antibiotics at half the usual concentrations (*i.e.* ampicillin 50 µg/ml, kanamycin 25 µg/ml) just before pouring plates.

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Annex II : Assay of β-galactosidase activity

This assay provide a quantitative determination of the efficiency of the functional complementation between pairs of hybrid proteins. Detailed description of the procedure can be found in Miller (5) and Karimova *et al.* (7, 8, 9).

Materials, Solutions, and Reagents:

PM2 assay medium

70 mM $Na_2HPO_4.12H_20$,

30 mM NaH_2PO_4 H_20 ,

1 mM $MgSO_4$

0.2 mM $MnSO_4$, pH 7.0.

Add 100 mM β-mercaptoethanol just before use.

Substrate solution:

ONPG, o-nitrophenol-β-galactoside,

Solution of 4 mg/ml in PM2 medium without β-mercaptoethanol (store at – 20 °C)

Stop solution :

1 M Na_2CO_3

Other reagents

Toluene

SDS 0.1%

Equipment needed

Cotton gauze

Shaking incubator, preset to 30 °C

Water bath preset to 28 °C

Spectrophotometer

Methodology:

1. Bacterial cells to be assayed for β-galactosidase activity are grown in 3-5 ml of LB broth in the presence of 0.5mM IPTG and appropriate antibiotics at 30°C and vigorous agitation for 4 - 6 hours (*i. e.* exponential phase of growth) or overnight (stationary phase).
2. The liquid cultures are diluted 1 to 5 with M63 medium (i.e. 0.5 ml of bacterial culture are added to 2 ml M63 medium) and the optical density (OD) at 600 □m of each cultures is recorded.
3. Bacterial cells are then permeabilized by adding 1 drop (approximately 30 μl) of toluene and 1 drop (30-35 μl) of a 0.1% SDS solution per 2.5 ml of the diluted cell suspensions in a 50 ml glass tube (or Falcon tube).
4. The tubes are vortexed for 10 seconds, lightly plugged with cotton (to allow the toluene to evaporate), and vigorously agitated in a shaker at 37°C for 30-40 minutes.
5. For the enzymatic reaction, aliquots of 0.1 to 0.5 ml of the permeabilized cells are added to 1 ml of PM2 assay buffer (in 5 ml glass tubes)
6. For the enzymatic reaction, 0.1 ml of the permeabilized cells are added to 0.9 ml of PM2 buffer (in a 5 ml glass tube) and the tubes are placed in water bath at 28 °C for 5 min.
   Note: the volume of aliquot of the permeabilized cells can be increased up to 0.5 ml (the volume of PM2 buffer is decreased accordingly to maintain a final volume of 1 ml) if the β-galactosidase enzymatic activity in the sample is low.
   A control tube containing 1 ml of PM2 assay buffer is also prepared to serve as a blank.
7. The enzymatic reaction is started by adding 0.25 ml the ONPG (o-nitrophenol-β-galactoside) substrate solution (0.4% ONPG in PM2 buffer without β-mercaptoethanol) pre-equilibrated at 30°C.
8. After sufficient yellow colour has developed (see below), the reaction is stopped by the addition of 0.5 ml of the 1M Na_2CO_3 stop solution.
9. The optical density at 420 nm (OD_{420}) is then recorded for each tube.
   Note: the reaction is linear up to an absorbance at 420 nm of 1.6. The reading at 420 nm is a combination of absorbance by the o-nitrophenol, that results from hydrolysis of ONPG, and light scattering of the cell debris. This latter can be neglected if small volumes of cell suspensions are used and the 420 nm reading is above 0.3. At lower absorbance the light scattering can be corrected for by obtaining the absorbance at 600 nm from the same reaction mixture and using a correction factor: OD_{420} - 1.5 x OD_{600} which then compensates for light scattering.
10. The enzymatic activities, A (in units /ml), are calculated for each samples according to the following formula:

    A = 200 x (OD_{420} – OD_{420} in control tube) / min of incubation) x dilution factor
    (i.e. if 0.1ml of permeabilized cells was added to 0.9 ml of PM2 buffer in step 6, then dilution factor = 10, so that activity is expressed in units/ml)
    One unit of β-galactosidase activity corresponds to 1 nmol of ONPG hydrolyzed per min at 28°C. The factor 200 in the above formula is the inverse of the absorption coefficient of o-nitrophenol, that is 0.005 per nmol/ml at pH 11.0 (i.e. after addition of Na_2CO_3).
    Results are generally given as units/mg dry weight bacteria. This is determined from the OD at 600 nm of the bacterial culture, considering that 1ml of culture at OD_{600} = 1 corresponds to 300 μg dry weight bacteria.

Other methods for β-galactosidase assay can be found in Miller (5) or Sambrook et al. (6).

Assay of β-galactosidase activity in 96-well format assay.

Additional protocols for β-galactosidase assays in 96-well microtiter plates can be found in references 11-14.

For each set of transformation (i.e. expressing a given couple of T25 and T18 hybrid proteins), the β-Gal assay is performed on eight overnight cultures that are grown at 30□C in 300 μL of LB broth in the presence of 0.5 mM IPTG and appropriate antibiotics in a 96-well microtiter plate (2.2 ml 96-well storage plate, Thermo Fisher Scientific). Before measurement the cultures are diluted 1:5 into M63 medium in the same microplate and 175 μL of the diluted culture from each well are transferred to flat bottom microtiter plate to record the OD_{595}nm absorbance data.

To permeabilize the cells, 200 μL of bacterial suspensions are transferred into a new microtiter plate (1.2 ml polypropylene 96-well storage block, Thermo Fisher Scientific) and 7 μL of 0.05% SDS (sodium dodecyl sulfate) and 10 μL of chloroform are added. The solutions are vigorously intermixed and then incubated under a fume hood at room temperature for 30-40 min (to evaporate chloroform).

For the enzymatic reaction, aliquots (20 μL) of the permeabilized cells are added (in a new microtiter plate) to 105 μL of the PM2 reaction buffer containing 100 mM β-mercaptoethanol, and 0.1% o-nitrophenol-β-galactoside (ONPG). The plate is incubated at room temperature for 20-30 min or until sufficient yellow colour has developed.

The reaction is then stopped by adding 50 μL of 1M Na_2CO_3 and the OD_{405}nm absorbance data are recorded with a microplate reader and analyzed with an appropriate software (or with Microsoft Excel or other spreadsheet programs).

For each well, the enzymatic activity, A (in relative units) is calculated according to:
$A=1000x(OD_{405}-OD_{405}$ in control well) / ($OD_{595}-OD_{595}$in control well) / t(min) of incubation.

Annex III : BACTH vector sequences & maps

pKNT25

3469 bp

T25 fragment (upper cases)

MCS sequence (underlined)

cagctggcacgacaggtttcccgactggaaagcgggcagtgagcgcaacgcaattaatgtgagttagctcactcattagg
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pKT25

3445 bp

T25 fragment (upper cases)

MCS sequence (underlined)

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ccgct

pKT25-zip

3559 bp

T25 fragment (upper cases)

Leucine zipper (underlined)

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pUT18

3023 bp

T18 fragment (upper cases)

MCS sequence (underlined)

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pUT18C

3017 bp

T18 fragment (upper cases)

MCS sequence (underlined)

cagctggcacgacaggtttcccgactggaaagcgggcagtgagcgcaacgcaattaatgtgagttagctcactcattagg
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CTCGCCGGATGTACTGGAAACGGTGCCGGCGTCACCCGGATTGCGGCGGCCGTCGCTGGGCGCAGTGGAACGCCA<u>**CTGCA**</u>
<u>**GGTCGACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTCATCGAT**</u>**ATAA**ctaagtaatatggtgcactctcagtacaat
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pUT18C-zip

3119 bp

T18 fragment (upper cases)

Leucine zipper (underlined)

cagctggcacgacaggtttcccgactggaaagcgggcagtgagcgcaacgcaattaatgtgagttagctcactcattagg
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