

# Construction and expression of GFP conjugated MIM-I-BAR

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**Abstract:** To achieve a visible inverse Bin-amphiphysin-Rvs (I-BAR) domain recombinant of missing in metastasis (MIM) protein, the green fluorescent protein (GFP) encoding gene was cloned at the terminal of MIM-I-BAR as a probe. The DNA was successfully constructed on a 6xHis-tagged prokaryotic expression plasmid. The non-GFP labeled MIM-I-BAR encoding plasmid was also constructed as a control. Being successfully transformed into BL21 (DE3) cells, the GFP-conjugated MIM-I-BAR (MIM-I-BAR-GFP) exhibits strong visible fluorescence, and the expression product can be easily detected by visual inspection, a fluorescence microscope, Western blot or ultraviolet and visible spectrophotometer. Moreover, examination of expression efficiency under various culture conditions revealed that the MIM-I-BAR-GFP gene has a high protein yield at 10 °C, but not at the culture temperature of 37 °C. This property is much different from that of the non-fluorescent MIM-I-BAR gene. This optimal expression condition is also proved to be feasible for protein production in midi-scale. The fluorescent recombinant MIM-I-BAR-GFP protein can serve as a useful tool in scientific research, biomedical application and pharmaceutical development.

**Key words:** missing in metastasis; inverse Bin-amphiphysin-Rvs; green fluorescent protein; plasmid; expression; purification

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Plasma membrane plays a protective and functional role and it is vital for living eukaryocytes. Various cellular activities require membrane deformation, including polarization, endocytosis, filopodia/lamellipodia formation, mobility, etc., many of which are driven by intracellular membrane binding proteins<sup>[1-2]</sup>. Among these proteins, the Bin-amphiphysin-Rvs (BAR) family is an important group<sup>[3-4]</sup>. These kinds of proteins have the N-terminal BAR domain, forming a rigid anti-parallel di-

mer, binding to and deforming the membranes into curvatures<sup>[5-6]</sup>. The inverse BAR(I-BAR) proteins are a subfamily of BAR, forming a convex face and favor membrane protrusions rather than invaginations<sup>[7]</sup>. In the mammalian I-BAR family, the missing in metastasis (MIM) gene has attracted particular concerns due to its low expression in a subset of relatively higher metastatic potent bladder cancer cells<sup>[8]</sup>. The study of MIM protein can bring better understanding to certain human diseases<sup>[9-15]</sup> and promote therapeutic research progress<sup>[16-17]</sup>. The I-BAR domain is critical for the MIM function. It consists of 250 amino acids and binds to PI(4,5)P<sub>2</sub>-enriched membranes, shapes membrane deformations by approaching the interior of tubules, interacts with small GTPase Rac, and cross-links actin filaments<sup>[18-20]</sup>. Recombinant MIM-I-BAR has been proved to be useful in many fields<sup>[20-21]</sup>.

To the best of our knowledge, there still remains a lack of proper methods to accurately identify MIM-I-BAR from other substances. A commercial antibody that specifically binds to MIM-I-BAR is so far unavailable. Even with a specific antibody, it is tedious to track, monitor or quantify the protein from bio-samples for the relatively inconvenient immunological assays. Chemical labeling of MIM-I-BAR is also challenging since it is technically difficult to achieve and considerably expensive for purification. Therefore, directly labeling the protein which can make the preparation and detection more convenient is an urgent requirement. Considering that the green fluorescent protein is a useful probe for protein modification, in this paper we construct such plasmids that can produce the MIM-I-BAR-GFP protein in the prokaryotic expression system by carrying genes encoding both MIM-I-BAR and GFP in a continuous sequence. We also find that the MIM-I-BAR-GFP gene under high expression conditions is quite different from MIM-I-BAR without GFP. Finally, under the optimized system, we prepare a remarkable amount of proteins and immobilize them on beads for storage as well as for further applications.

## 1 Experimental

### 1.1 Chemicals and antibodies

All chemicals unless otherwise indicated were purchased from Sigma Aldrich. The Ni-NTA resin was from Genscript. T4 DNA Ligase and restrict enzymes were

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from NEB. BL21 (DE3) Competent *E. coli* and DNA agarose were from Invitrogen. The DNA Gel Extraction Kit was from AXYGENT. The DH5 $\alpha$  Competent *E. coli*, SDS-PAGE Gel Preparation Kit and Plasmid Midi Preparation Kit were from Beyotime. Coomassie Brilliant Blue R-250 was from KeyGEN. Tryptone and yeast extract were from Oxoid. Pfu DNA polymerase was from Thermo Scientific. The 6xHis monoclonal antibody (clone 4A12E4) was from Lifetechnologies.

### 1.2 Plasmids

The plasmid MIM-I-BAR-GFP used as a gene template was prepared as described previously<sup>[22]</sup>. Plasmids encoding His tagged MIM-I-BAR or MIM-I-BAR-GFP proteins were prepared by ligation of PCR-generated DNA fragments into vectors pET-14b (Novagen). DNA cloning was performed in the DH5 $\alpha$  cells. The primers used in PCR are listed in Tab. 1.

Tab.1 Designed primers for PCR

Gene name		Designed primers
His-MIM-I-BAR	Sense	5'-CAGTTACTCGAGGAGGCTGTGATCGAGAAGG-3'
	Anti-sense	5'-GTCGGATCCTTAGTCCAAAATCACCTGTTAC-3'
His-MIM-I-BAR-GFP	Sense	5'-CAGTTACATATGGAGGCTGTGATCGAGAAGG-3'
	Anti-sense	5'-GTCGGACTCGAGTTACTTGTACAGCTCGTCC-3'

### 1.3 Recombinant proteins

Briefly, BL21 (DE3) competent *E. coli* cells were transformed by the plasmids, and the transformed cells were selected based on their resistance to ampicillin. A single colony of the transformed cells was added to 3 mL of Lennox broth (LB, 1% tryptone, 0.5% yeast extract and 1% NaCl, pH 7.4) medium containing 50  $\mu$ g/mL ampicillin and incubated at 37  $^{\circ}$ C for 16 h at a shaking speed of 200 revolutions per minute. The culture was further inoculated in 100 mL of pre-warmed LB medium with 50  $\mu$ g/mL ampicillin and shaking with a speed of 200 revolutions per minute for another 1 to 2 h at 37  $^{\circ}$ C. When the optical density at the wavelength of 600 nm reached 0.3 or 0.4, the culture was added to with 0.5 mmol/L of isopropyl-beta-D-thiogalactopyranoside (IPTG). After an additional 3 h of incubation, the cells were harvested in phosphate buffered saline (PBS), sonicated and centrifuged. The supernatant was incubated with Ni-NTA resin for 2 h at 4  $^{\circ}$ C followed by three doses of wash with Buffer A (Sinopharm) and stored in Buffer A containing 0.02% NaN<sub>3</sub>. If bead-free proteins were needed, His-tagged proteins were eluted with 250 mmol/L imidazole in Buffer A, and dialyzed using a Centricon YM-10 centrifugal filter (Millipore).

### 1.4 Immunoblotting

After incubation, cells were harvested by centrifuging, re-suspended in PBS, then sonicated and kept on ice be-

fore gel electrophoresis. A portion (50  $\mu$ L) of each sample was mixed with 10  $\mu$ L 6x protein loading buffer (Beyotime), and 10  $\mu$ L of each mixture was fractionated by SDS/PAGE, transferred to a nitrocellulose membrane and subjected to Western blotting with anti-6xHis followed by horseradishperoxidase-conjugated secondary antibody in 100 mmol/L TBST (Tris/HCl, pH 7.5, 150 mmol/L NaCl, and 0.05% (volume fraction) Tween20) containing 5% (mass fraction) non-fat milk powder. The antibody-reactive substances on the membrane were detected by the hypersensitive ECL detection kit (KeyGEN).

### 1.5 Analysis of photoabsorption

Cells in liquid culture medium were collected by centrifuging at 5 000 *g*. After washing and being re-suspended in PBS, the cells were treated by sonication for lysis and then were kept in light resistant container at 4  $^{\circ}$ C. Photoabsorption of the cellular proteins were analyzed by spectrophotometric measurement at 300 to 700 nm using the UV-3600 spectrophotometer (Shimadzu).

## 2 Results and Discussion

The plasmid encoding His-tagged MIM-I-BAR-GFP was constructed as well as the control non-GFP plasmid pHis-MIM-I-BAR. Although GFP can act as a probe at both ends of the target protein, here we used GFP as a C-terminal tag to minimize the effects of MIM-I-BAR dimer on the GFP C-terminal catalytic fluorescent group. With the plasmid MIM-I-BAR in pEGFP plasmid as the reaction template, MIM-I-BAR-GFP and MIM-I-BAR genes containing restricted clone sites were prepared by PCR using designed primers. As shown in Fig. 1 (a), PCR products were subsequently purified by gel extraction, and then were double digested by restriction enzymes to obtain the insert genes. The enzymes were NdeI/XhoI for MIM-I-BAR-GFP and XhoI/BamHI for MIM-I-BAR. The pET-14b vector was cut and purified in the meantime. Ligation was performed using T4 DNA ligase. The molar ratio of the insert gene and the vector gene is 3:1. and the genes were then transformed into DH5 $\alpha$  cells for

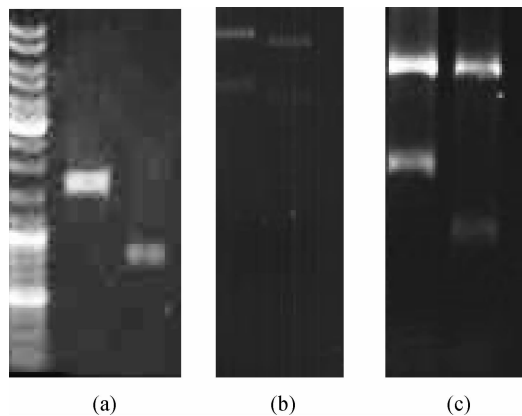
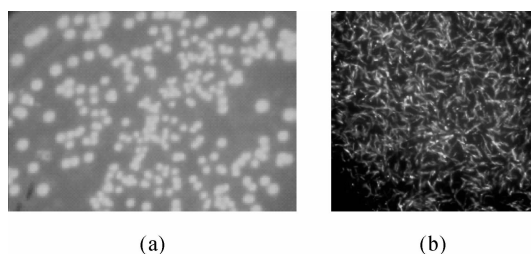


Fig.1 The DNA examination in the molecular cloning procedures. (a) PCR; (b) Clones; (c) Verification

selection under ampicillin. As shown in Fig. 1(b), positive clones were collected, preliminarily checked by double digestion (see Fig. 1(c)), and further confirmed by deoxyribonucleic acid sequencing. The samples from left lane to right lane represent the standard markers, MIM-I-BAR-GFP and MIM-I-BAR, respectively. The molecular size of the DNA bands are confirmed by comparing them with the standard markers, about 1.6 kilo base pairs (kbp) for MIM-I-BAR-GFP and about 0.8 kbp for MIM-I-BAR.

The vector plasmid pET-14b contains 6xHis-tag at the N-terminal of the cloned gene, which can be easily expressed in the BL21(DE3) prokaryocyte system and be purified with the aid of Ni ion beads. After sequence validation and midi-preparation, the two type of plasmids were successfully transformed into BL21(DE3) *E. coli* cells, which survived the selection of ampicillin on tissue culture plates. Interestingly, after being stored at a low temperature, clone cells carrying pHis-MIM-I-BAR-GFP plasmid displayed significant background GFP-linked protein expression as shown in Fig. 2(a). When suspended in the culture medium, the cells with green fluorescence can be easily observed under a fluorescent microscope (see Fig. 2(b)). It not only suggests that the protein is capable of being expressed and correctly folded to obtain intrinsic functions, but also implies that the MIM-I-BAR protein tagged with GFP may not necessarily express under the same condition that is suitable for cell growth, which is around 37 °C.

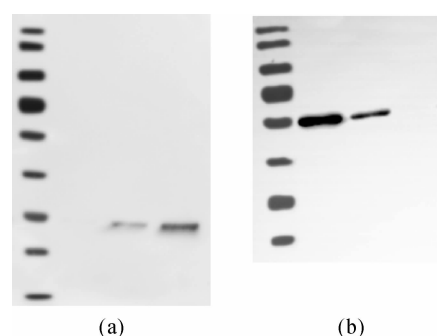


**Fig. 2** The strong green fluorescence of the transformed cells. (a) Clones on the culture plate; (b) Cells under fluorescent microscope

In order to obtain a relatively high protein yield, different temperature points including 10, 20 and 37 °C were examined for protein expression when culturing the transformed *E. coli* with IPTG. The cell samples were collected and subjected to Western blot analysis. Since the pET-14b vector marks each encoded protein a His-tag, the expression products can be detected and quantified using an antibody against His-tag by immunoblotting assay such as Western blot.

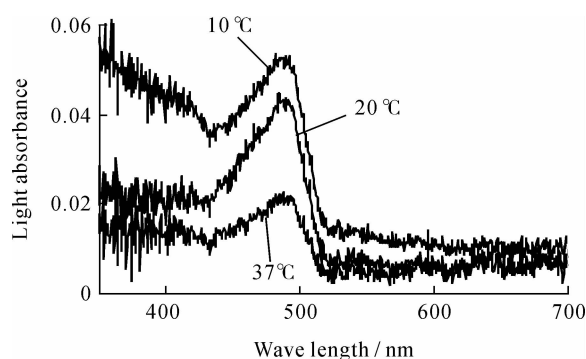
The non-fluorescent protein expression under different temperatures was first tested. Western blot using anti-6xHis antibody demonstrated that when recombinant His-MIM-I-BAR is expressed in BL21(DE3) under different temperatures, expression efficiency can be varied. At 37 °C, the expression level is the highest, the quantification

of which is about 25 fold than those at 10 °C. However, for GFP-labeled MIM-I-BAR proteins, the expression efficiency is quite different from non-GFP MIM-I-BAR towards temperature alternation. Western blot results in an opposite expression-efficiency trends compared to non-fluorescent MIM-I-BAR. At 10 °C, cell lysate contains significant larger amounts of His-MIM-I-BAR-GFP protein that develops a strong and clear band. But protein quantity decreased when the cells are cultured at 20 °C, and the expression is almost fully compromised at 37 °C (see Fig. 3). Samples from the left lane to right lane represent the standard molecular weight marker, cells cultured at 10 °C, cells cultured at 20 °C, and cells cultured at 37 °C, respectively. The molecular weight of the protein bands kilo Daltons (kDa) are confirmed by comparing them with the standard marker (about 28 kDa for His-MIM-I-BAR and about 54 kDa for His-MIM-I-BAR-GFP).



**Fig. 3** Temperature analysis for protein production. (a) Protein without GFP label; (b) Protein with GFP label

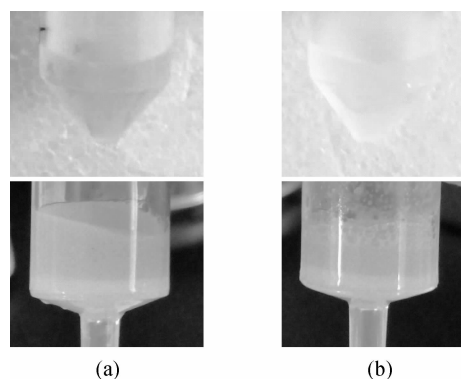
The results can also be directly visualized by the clear fluorescent from the *E. coli* itself, owing to the advantage of the GFP protein probe. After being precipitated by centrifuging, pellets of the 10 °C cultured cells system show a much brighter green color than those cultured at higher temperatures. Cells develop fluorescence under ultraviolet light, and the fluorescent level decreases remarkably when the incubation temperature goes up. The different light absorbance of the fluorescent protein was implemented using a spectrophotometer. The light absorption of the cell lysates features single peak around 485 nm due to the conjugation to a GFP-tag (see Fig. 4), the



**Fig. 4** The cellular GFP absorbance

intensity of which can be regarded as a measurement of the quantity of the protein. In accordance with the Western blot result, MIM-I-BAR-GFP recombinant protein produced at a low temperature gains higher light absorption. Considering that the absorbance is basically proportional to the concentration of the GFP-labeled protein, it confirms that 10 °C should be the optimal expression for MIM-I-BAR-GFP recombinant protein preparation.

After the bio-synthesis feature was characterized, a midi-preparation was carried out under the optimized condition to validate the productive consistency of the enlarged preparing system and the Ni-NTA binding ability of the His-tag product. After the cell culture volume was raised from 3 to 150 mL, the expression yield of the MIM-I-BAR-GFP protein was still considerably high. These protein products are capable of being immobilized/collected using Ni-NTA beads (see Fig. 5).



**Fig. 5** Midi-preparation of the proteins. (a) MIM-I-BAR-GFP protein; (b) MIM-I-BAR protein

The beads binding proteins can be stored stably at 4 °C in light-proof containers for a certain time. Immobilizing the proteins also prevents them from degrading. Moreover, immobilized proteins can be directly used in pull-down experiments. If beads-free protein is needed, an elution using imidazole and a dialysis against proper buffer should be carried out, and an HPLC process is recommended for further purification.

### 3 Conclusion

The missing in metastasis protein is a popular bio-macromolecule, particularly in the I-BAR domain, which is useful in scientific research and medical applications. We previously reported MIM-I-BAR recombinants construction using GST-tag<sup>[21–22]</sup>. However, GST is a relatively large protein tag that brings steric hindrance to a MIM-I-BAR structure, and thus usually requires complicated cleavage procedure. His-tag is small and has minor influence on the conjugated protein<sup>[20]</sup>, which is more suitable for MIM-I-BAR-GFP production. In this study, we amplified a MIM-I-BAR insert gene encoding a green fluorescent protein probe at the terminus. We found that although being cloned into the same vector pET-14b, genes

with or without a fluorescent probe encoding sequence displayed a direct opposite trend in expression efficiency with environmental temperature change. From 10 to 37 °C, MIM-I-BAR protein yields increase as expected. Interestingly, the MIM-I-BAR-GFP protein expression is most efficient at 10 °C, attenuated at 20 °C and mostly compromised at 37 °C, opposite to the temperature response of the non-GFP protein. This may be due to the toxic effects of GFP gene or its transcription products that potentially act intrinsically on the host BL21 (DE3) cells at the higher temperature. Moreover, it is known that GFP fluorescence initiates better at a relatively low temperature<sup>[23]</sup>. This may be another reason for the different production efficiency between MIM-I-BAR constructs with or without GFP.

With an optimal production temperature established, both proteins have been prepared in midi-scale and roughly purified on Ni-NTA columns, which are used to store for future protein interaction experiments or for the sophisticated purification of beads-free proteins. The GFP probe is advantageous because it favors the MIM-I-BAR protein with a visible marker, so that fast and accurate analysis applications are supported, such as fluorescent microscopy, flow cytometry and microplate system. Therefore, these proteins are useful for studies involving MIM-I-BAR/membrane interaction, biomaterial modification, I-BAR dimerization and targeting peptide/compound development.

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## 绿色荧光蛋白探针标记的 MIM-I-BAR 基因构建和表达

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**摘要:** 为制备可视化的转移消失蛋白(MIM)的 I-BAR 结构域重组体, 克隆了顺联绿色荧光蛋白(GFP)探针编码序列的 MIM-I-BAR 基因. 在 6xHis 标签原核表达质粒上成功构建了 DNA 序列. 同时, 实现了未标记荧光探针基因的 MIM-I-BAR 质粒的构建以作实验对照. 成功转染至 BL21(DE3) 大肠杆菌细胞后, GFP 偶联的 MIM-I-BAR (MIM-I-BAR-GFP) 蛋白表现出很强的可视荧光, 该表达产物可方便的通过目测、荧光显微镜、免疫印迹和紫外可见分光光度计等多种手段进行检测. 此外, 在考察不同条件下的蛋白表达效率过程中发现, 带有 GFP 探针的 MIM-I-BAR 重组蛋白在温度为 10 ℃ 时产率最高, 而并非 37 ℃. 这一特征与非荧光标记的 MIM-I-BAR 明显不同. 研究证实该最佳表达温度条件适用于重组蛋白产品中量制备. 所开发的带有荧光探针的 MIM-I-BAR 蛋白产品及其制备工艺在科学研究、生物医学应用以及药物开发过程中均有较高的应用价值.

**关键词:** 转移消失蛋白; inverse Bin-amphiphysin-Rvs; 绿色荧光蛋白; 基重组因; 表达; 纯化

**中图分类号:** Q816