

# High Efficient Expression of Bioactive Human BMP-14 in *E. coli* Using SUMO Fusion Partner

Jian Feng Li · Xian Wei Cui · Hai Yan Ji ·  
Ting Qiu · Xue Mei Ji · Ming Xian Du ·  
Hai Tao Wu · Xing Zhou Xu · Shuang Quan Zhang

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**Abstract** Bone morphogenetic proteins (BMPs) are cytokines from the TGF- $\beta$  superfamily, with important roles during embryonic development and in the induction of bone and cartilage tissue differentiation in the adult body. In this contribution, We report here the application of small ubiquitin-related modifier (SUMO) fusion technology to the expression and purification of human BMP-14. The fusion protein expressed in a soluble form was purified to a purity of 90% by Ni-IDA chromatography. After the SUMO-BMP14 fusion protein was cleaved by the SUMO protease at 30 °C for 1 h, the cleaved sample was re-applied to a Ni-IDA. Finally, about 45 mg recombinant hBMP-14 was obtained from 1 litre bacterial culture with no less than 95% purity. The purified hBMP-14 dimer was over 90% purity and could induce the expression of alkaline phosphatase activity in C2C12 cells in a dose-dependent manner. Thus the SUMO-mediated peptide expression and purification system potentially could be employed for the production of other homodimeric proteins.

**Keywords** Human bone morphogenetic protein-14 · *Escherichia coli* · C2C12 cells · Small ubiquitin-related modifier

## Abbreviations

hBMP-14 Human bone morphogenetic protein-14  
SUMO Small ubiquitin-related modifier  
IPTG Isopropyl- $\beta$ -D-1-thiogalactopyranoside  
ALP Alkaline phosphatase activity

## 1 Introduction

Bone morphogenetic proteins (BMPs) are a group of proteins involved in the development of many organs and tissues as well as in the establishment of the basic embryonic body plan [6]. Their crucial role in the latter sets the stage for their potential use in the regeneration of many types of tissues following injuries and diseases [7].

BMP-14 (synonyms: Cartilage-Derived Morphogenetic Protein 1, Growth differentiation factor-5) that belongs to the TGF- $\beta$  family, has received special attention because it plays a critical role in the early stages of development. BMP-14 is an important mediator for the growth and morphogenesis of various tissues and organs in mammals. The action of BMP-14 is more specially targeted to skeletal development. In vitro studies also show that the BMP-14 is expressed in early cartilage condensation, the perichondrium and the joint interzone [4, 5]. This effect on skeletal growth is corroborated by pathological conditions in mice and men involving mutations within the BMP-14 gene and resulting in loss of function [3]. Currently, most BMPs are obtained from mammalian cell cultures in low yields

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J. F. Li · X. W. Cui · H. Y. Ji · T. Qiu · X. M. Ji ·  
M. X. Du · H. T. Wu · X. Z. Xu · S. Q. Zhang  
Jiangsu Province Key Laboratory for Molecular  
and Medical Biotechnology, Life Science College,  
Nanjing Normal University, Nanjing 210046, Jiangsu,  
People's Republic of China

S. Q. Zhang (✉)  
Jiangsu Province Key Laboratory for Molecular and Medical  
Biotechnology, Jiangsu Engineering Research Center for  
Biomedical Function Materials, Jiangsu Key Laboratory  
for Supermolecular Medicinal Materials and Applications,  
Nanjing Normal University, Nanjing 210046,  
People's Republic of China  
e-mail: shuangquanz@yahoo.com

[1, 14] or from bacteria inclusion bodies after time-consuming refolding procedures [11–13]. The aim of the present work was to develop a highly productive and simple process for the production of human BMP-14 (hBMP-14).

In this paper, we described in detail the cloning of the hBMP-14 gene into pSUMO vector, the expression of SUMO-BMP14 in a soluble form, purification, and the biological assay of recombinant hBMP-14.

## 2 Materials and Methods

### 2.1 Bacterial Strains, Vectors and Enzymes

*Escherichia coli* DH5 $\alpha$  (maintained in our laboratory) was used for subcloning and plasmid amplification. *E. coli* BL21 (DE3) (Novagen, USA) was used as the expression host. The linearized pSUMO vector with *Bsa*I and *Bam*HI restriction sites and T7 promoter and kanamycin resistance was purchased from LifeSensors (LifeSensors, Malvern, PA, USA). All the restriction enzymes and T4 DNA ligase were purchased from Takara Biotech Co. Ltd. (Dalian, China).

### 2.2 Construction of Expression Vectors

The gene encoding mature peptide of hBMP-14 according to the amino acid sequence from the PeptoTech Inc. (<http://www.peprotech.com/product.asp?product%5Fid=120%2D01%28PeptoTech+Base+Catalog%29&category%5Fname=Display%28PeptoTech+Base+Catalog%29&search=GDF-5>) was amplified by PCR from cloning vector PMD18-T/BMP-14 obtained from the GenScript Corporation, China (<http://www.genscript.com.cn/index.html>) which contained the full-length hBMP-14 cDNA. The PCR fragments were separated by 1.5% gel electrophoresis, purified with a DNA gel extraction kit (AxyGEN Union, USA). The resulting PCR product was digested with *Bsa*I and *Bam*HI, and ligated into the pSUMO plasmid at the corresponding restriction sites. The ligation mixture was transformed into *E. coli* DH5 $\alpha$  cells for verification by sequencing.

### 2.3 Fusion Protein Expression

Plasmid pSUMO-BMP14 was transformed into competent *E. coli* BL21 (DE3). A single colony of *E. coli* BL21 (DE3) was picked and transferred to 3 mL Luria–Bertani (LB) medium containing 50  $\mu$ g/mL of kanamycin (LB-Kan). This culture was incubated overnight at 37 °C and 200 rpm. The overnight culture was diluted 1:100 in LB-Kan and incubated at 37 °C and 200 rpm until an OD<sub>600</sub> of 0.6–0.8 was reached. The culture was then cooled to

about 20 °C for 30 min and expression of hBMP-14 was induced by the addition of 0.2 mM IPTG. After a further incubation for 6 h at 20 °C and 200 rpm, bacteria were centrifuged (10 min, 6,000 rpm, 4 °C), washed once with PBS and frozen at –70 °C.

### 2.4 Purification of SUMO Fusion Protein

The pellet from 200 mL culture was resuspended in 15 mL binding buffer (20 mM Tris, 500 mM NaCl, 20 mM imidazole, and 10 mM PMSF, pH 8.0) and lysed on ice by sonication at 400 W for 100 cycles (4 s working, 8 s free). The supernatant of the cell lysate after centrifugation at 12,000 $\times$ g at 4 °C for 20 min was applied to a Ni<sup>2+</sup>-chelating column. After extensive washing with binding buffer, the fusion protein was eluted with five column volumes of elution buffer (20 mM Tris, 500 mM NaCl, and 250 mM imidazole, pH 8.0). The peak fractions containing the fusion protein were pooled and dialyzed overnight at 4 °C against phosphate-buffered saline (PBS).

### 2.5 Cleavage of the Fusion Proteins and Purification of Released hBMP-14

The SUMO-BMP14 protein (50  $\mu$ g) was reacted with 1 U of SUMO protease at 30 °C for 1 h. Since both SUMO and SUMO protease had 6 $\times$  His tags, but hBMP-14 did not, the cleaved SUMO-BMP-14 samples could be re-applied to the nickel column to obtain the purified hBMP-14 by subtracting the 6 $\times$  His-tagged proteins. Briefly, after the SUMO fusions were cleaved by the SUMO protease, the sample was loaded onto a nickel column with Ni-IDA resin. Most of the hBMP-14 without 6 $\times$  His tags was eluted in the flow-through (unbound) fractions, and the rest was recovered by washing the resin with binding buffer. The eluted and washed proteins appearing in fractions with high-UV values at OD<sub>280</sub> were pooled as the final purified sample. The purified proteins were checked by SDS-PAGE and the samples were stored at –80 °C for activity assay.

### 2.6 Reducing and Non-reducing SDS-PAGE

Electrophoresis was done using hand-cast 12% SDS-PAGE gels. Samples were mixed with either 2 $\times$  SDS Gel-loading buffer with DTT (reducing SDS-PAGE) or with 2 $\times$  SDS Gel-loading buffer without DTT (non-reducing SDS-PAGE) and boiled for 5 min at 95 °C before loading.

### 2.7 Biological Activity Assay of hBMP-14

The biological activity of hBMP-14 was tested by the induction of alkaline phosphatase activity (ALP) in C2C12 cells [10]. C2C12 cells were grown in MEM with 2 mM

L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and 10% fetal bovine serum (Invitrogen) at 37 °C and 10% CO<sub>2</sub>. One milliliter medium containing  $1 \times 10^5$  C2C12 cells was plated in a 24-well plate and the medium was replaced with 1 mL of fresh medium containing different concentrations of hBMP-14 after 24 h. After 4 days, cells were lysed in 0.2 mL buffer A (0.1 M glycerol, pH 9.6, 1% NP-40, 1 mM MgCl<sub>2</sub>, and 1 mM ZnCl<sub>2</sub>). Fifty microliters of cell lysates were mixed with 150  $\mu$ L of 0.3 mM *p*-nitrophenyl phosphate (Sigma) in buffer A and incubated in a 96-well plate at 37 °C for 30 min. The ALP was determined using VERSAmax tunable microplate reader (Molecular Devices) with absorbance set at 405 nm.

### 3 Results

#### 3.1 Plasmid Construction and the Expression of hBMP-14 Fusion Protein

Recombinant plasmid pSUMO-BMP14 sequence containing a His-tag for affinity purification was verified by DNA sequencing. Construct was transformed into the expression host *E. coli* BL21 (DE3), and subjected to a pilot expression test. As shown in Fig. 1a, there was an obvious protein band after IPTG induction, and colony 1 was chosen for the induction experiment.

#### 3.2 Purification of SUMO-BMP14 Fusion Protein

As described above, Ni-IDA resin was used for fusion protein purification. Most of the proteins without 6 $\times$  His tags were removed from the Ni-IDA resin using washing buffer containing 20 mM imidazole, and the 6 $\times$  His-

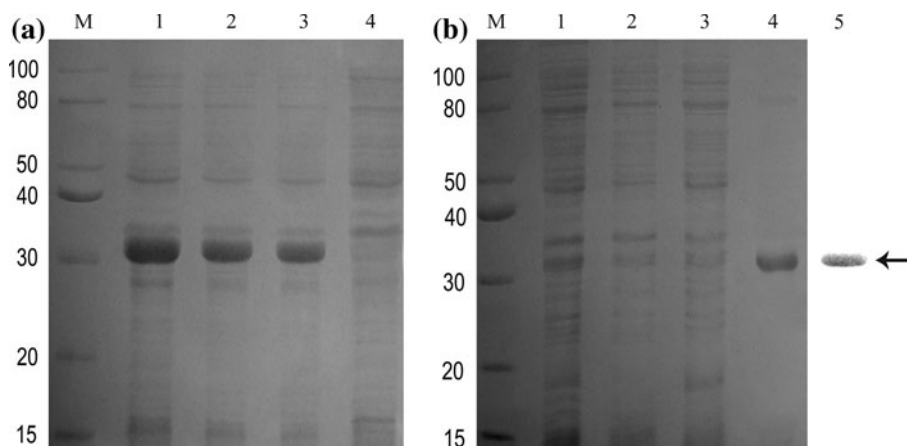
tagged SUMO-BMP14 (about 33 kDa) was eluted using elution buffer containing 250 mM imidazole with more than 90% purity (Fig. 1b). About 245 mg fusion protein can be obtained per liter of bacterial culture (Table 1).

#### 3.3 Cleavage of the Fusion Proteins and Purification of Recombinant hBMP-14

The SUMO-BMP14 protein (50  $\mu$ g) was competently cleaved with SUMO protease (1 U) at 30 °C for 1 h, confirmed by checking the proteins by a SDS-PAGE (Fig. 2a). After the cleaved sample was re-applied to a Ni-IDA column to subtract 6 $\times$  His-tagged SUMO and SUMO protease, final purified hBMP-14 was obtained with more than 95% purity (Fig. 2a). The flow-through was analyzed with SDS-PAGE gels under non-reducing (Fig. 2b, lane 1) or reducing (Fig. 2b, lane 2) conditions. The purified hBMP-14 (about 13.57 kDa) was filtered through 0.22  $\mu$ m and stored at -80 °C for activity assay. Table 2 shown the comparison of the SUMO fusion partner with other expression systems in production of recombinant hBMP-14. Finally, a purified recombinant hBMP-14 was produced at a yield of 45 mg/L (Table 1).

#### 3.4 Biological Activity Test

Bioactivity of purified hBMP-14 was tested in vitro bioassay. The purified hBMP-14 was diluted in Tris-EDTA buffer (100 mM Tris/HCl, 1 mM EDTA, pH 8.5) containing 1% BSA. The recombinant hBMP-14 could induce the expression of ALP in C2C12 cells in a dose-dependent manner compared to the negative control (serum-free medium or SUMO-BMP14). As a positive control, cells were incubated with 10% FBS (Fig. 3).



**Fig. 1** Expression and purification of hBMP-14 fusion protein. **a** Analysis of expressed fusion protein by SDS-PAGE. Lane M molecular mass marker, Lanes 1, 2, 3 colony 1, 2, 3 induced by 0.2 mM IPTG, Lane 4 negative control. **b** Purification of hBMP-14 fusion protein. Lane M molecular weight marker, Lane 1 supernatant

of cell lysate, Lane 2 flow-through, Lane 3 wash, Lane 4 elution. Lane 5 Western blot analysis of purified SUMO-BMP14 using mAb against His6-tag; the arrow indicated the location of the recombinant fusion protein

**Table 1** Isolation of recombinant hBMP-14 from pSUMO-BMP14 fusion protein

Purification step	Total protein (mg)	Fusion protein (mg)	Recombinant hBMP-14 (mg)	Purity (%) <sup>d</sup>
Sonicated supernatant	1,635 <sup>a</sup>	325 <sup>b</sup>	133 <sup>c</sup>	NA
SUMO-BMP14 after affinity chromatography	NA	245 <sup>b</sup>	100 <sup>c</sup>	90
Recombinant hBMP-14 after affinity chromatography	80 <sup>a</sup>	NA	60 <sup>c</sup>	>95
Dialysis	NA	NA	45 <sup>a</sup>	>95

Estimations are based on 1 L of bacterial culture

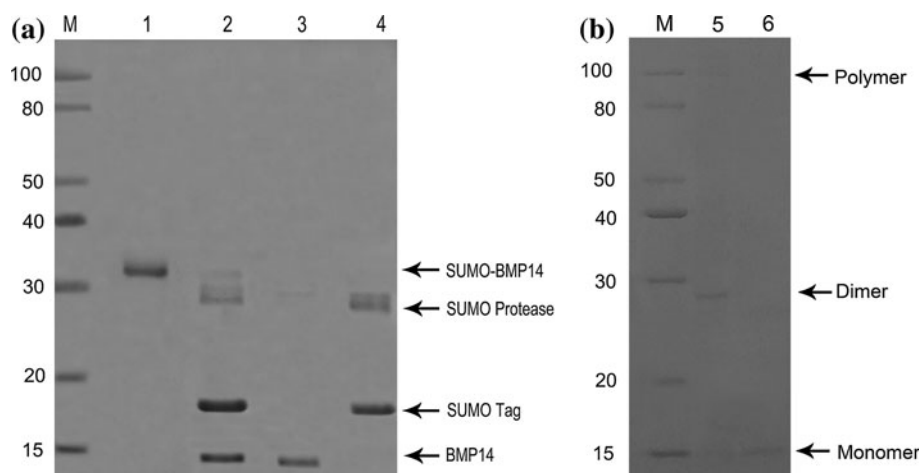
NA not applicable

<sup>a</sup> Determined using a Bradford assay

<sup>b</sup> Percent of SUMO-BMP14 fusion protein from total proteins was estimated by SDS gel scanning

<sup>c</sup> The amount of recombinant hBMP-14 was calculated as a fraction of SUMO-BMP14 fusion protein

<sup>d</sup> Purity of protein or peptide was estimated by SDS gels scanning as analyzed by the BandsScan software (BioMarin Pharmaceutical Inc, UK)



**Fig. 2** SDS-PAGE analyses for the recombinant hBMP-14 preparation. **a** fusion protein digestion by SUMO protease and recombinant hBMP-14 purification. Lane M molecular weight marker, Lane 1 purified fusion protein (33 kDa), Lane 2 mixture of fusion protein by SUMO protease digestion, Lane 3 purified recombinant hBMP-14 by

Ni-IDA (13.57 kDa), Lane 4 SUMO tag. **b** Reducing and non-reducing SDS-PAGE analyses for the purified hBMP-14. Lane 5 hBMP-14 (in the absence of DTT), Lane 6 hBMP-14 (in the presence of DTT)

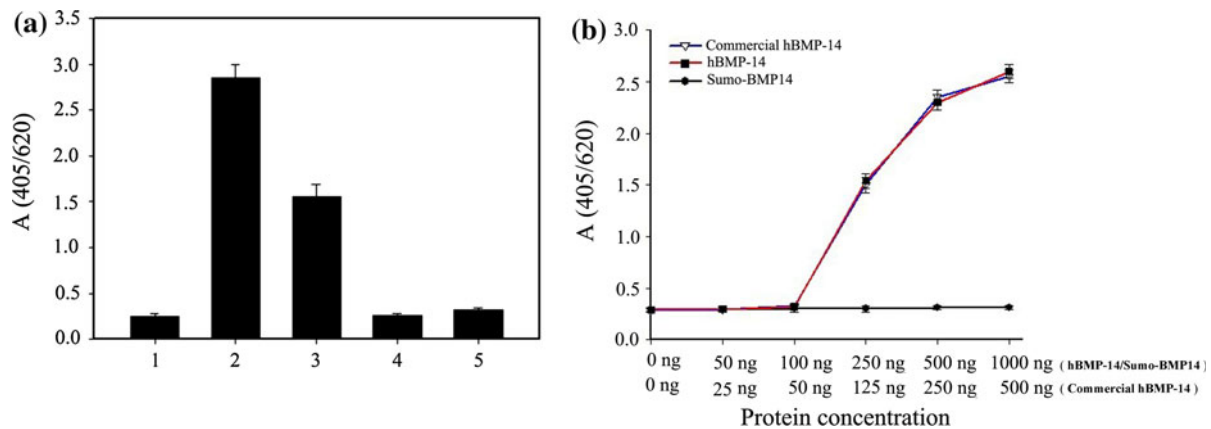
#### 4 Discussion

Expressing recombinant cytokine proteins, especially proteins with disulfide bridges, is quite an arduous task because they often fold incorrectly and aggregate, leading to either rapid degradation or to the accumulation of inclusion bodies when expressed in *E. coli*. Fortunately,

these problems are somewhat alleviated when expressed with a fusion partner. However, in the past, traditional fusion systems have given variable results of expression and have faced major problems attributed to either the inefficient cleavage of the fusion protein or cleavage within the target protein, both of which compound the difficulties of purification [15]. SUMO is superior to commonly used

**Table 2** Comparison of the SUMO expression systems with other various expression systems

Expression type	Approach	Key features	References
<i>E. coli</i> RBSIIP <sub>N25x/o</sub> vector inclusion bodies	Inclusion body refolding	NA	[9]
<i>E. coli</i> pET-25b vector soluble expression	Purified by affinity his-tag chromatography	Yield 35 mg/L, an extra 6× his-tag partner left at the N-terminus of rhBMP-14	[2]
<i>E. coli</i> pSUMO vector soluble expression	Purified by affinity his-tag chromatography, SUMO protease cleavage	Yield 45 mg/L, obtained intact and homogeneous native rhBMP-14	This paper



**Fig. 3** Biological activity of purified hBMP-14. **a** The induction of alkaline phosphatase (ALP) activity in C2C12 cells. *Lane 1* hBMP-14 dilution buffer, *Lane 2* recombinant hBMP-14 (500 ng), *Lane 3* positive control (medium + 10% FBS), *Lane 4* negative control

(serum-free medium), *Lane 5* SUMO-BMP14 (500 ng). **b** ALP activity under different concentration of recombinant hBMP-14, SUMO-BMP14 (negative control) and commercially available hBMP-14 produced in *E. coli* (positive control)

fusion tags in enhancing expression and solubility with the added advantage of generating recombinant protein with native sequences [8]. We hypothesized that the attachment of a highly stable and compact SUMO structure to the N-terminus of hBMP-14 would facilitate correct protein folding and enhance solubility and expression.

In previous reports, recombinant hBMP-14 production was achieved in bacterial inclusion bodies in fairly large amounts [9]. However, inclusion bodies require time-consuming steps for solubilization and refolding. Then soluble expression of recombinant hBMP-14 was achieved in *E. coli* using pET-25b vector and yield 35 mg/L. However, an extra 6× his-tag partner was left at the N-terminus of hBMP-14 [2]. In our approach, we expressed hBMP-14 as SUMO fusions in *E. coli* to evaluate the roles of SUMO and SUMO protease on the production of the cytokines. The SUMO fusion protein was successfully expressed in *E. coli* and high expression levels of soluble fusion protein were achieved. The fusion protein could be purified in one step with a purity of not less than 90% (Fig. 1b), and 245 mg SUMO-BMP14 was obtained per liter bacterial culture (Table 1). The fusion protein was then completely cleaved by SUMO protease, which is remarkably robust and highly specific. The hBMP-14 protein was recovered with 95% purity by purification with nickel affinity chromatography again, and 45 mg hBMP-14 was obtained per liter bacterial culture finally (Fig. 2a; Table 1). The biologically active hBMP-14 is a homodimeric protein. The purified hBMP-14 dimer was analyzed by SDS-PAGE with or without reducing reagent DTT. As shown in Fig. 2b, the purified hBMP-14 dimer was over 90% purity and could be further reduced to monomer. And the recombinant hBMP-14 could induce the expression of ALP in C2C12 cells in a dose-dependent manner (Fig. 3b).

In summary, the SUMO fusion system and customized expression and purification protocol described here have greatly improved the efficiency and lowered the costs of producing hBMP-14. There are many proteins that have structures similar to BMP-14, such as the TGF- $\beta$  superfamily, the SUMO fusion technology could be also widely applied to the production of other homodimeric proteins in *E. coli*.

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## References

- Bessa PC, Casal M, Reis RL (2008) *J Tissue Eng Regen Med* 2:1–13
- Bessa PC, Cerqueira MT, Rada T, Gomes ME, Neves NM, Nobre A, Reis RL, Casal M (2009) *Protein Expr Purif* 63:89–94
- Buxton P, Edwards C, Archer CW, Francis-West P (2001) *J Bone Joint Surg Am* 83-A(Suppl):S23–S30
- Coleman CM, Tuan RS (2003) *Dev Dyn* 228:208–216
- Hatakeyama Y, Tuan RS, Shum L (2004) *J Cell Biochem* 91:1204–1217
- Hogan BL (1996) *Genes Dev* 10:1580–1594
- Kirker-Head CA (2000) *Adv Drug Deliv Rev* 43:65–92
- Li JF, Zhang J, Zhang Z, Kang CT, Zhang SQ (2010) *Curr Microbiol* 62:296–300
- Nickel J, Kotsch A, Sebald W, Mueller TD (2005) *J Mol Biol* 349:933–947
- Ohte S, Shin M, Sasanuma H, Yoneyama K, Akita M, Ikebuchi K, Jimi E, Maruki Y, Matsuoka M, Namba A, Tomoda H, Okazaki Y, Ohtake A, Oda H, Owan I, Yoda T, Furuya H, Kamizono J, Kitoh H, Nakashima Y, Susami T, Haga N, Komori T, Katagiri T (2011) *Biochem Biophys Res Commun* 407: 213–218

11. Vallejo LF, Brokelmann M, Marten S, Trappe S, Cabrera-Crespo J, Hoffmann A, Gross G, Weich HA, Rinas U (2002) *J Biotechnol* 94:185–194
12. Vallejo LF, Rinas U (2004) *Biotechnol Bioeng* 85:601–609
13. von Einem S, Schwarz E, Rudolph R (2010) *Protein Expr Purif* 73:65–69
14. Wang EA, Rosen V, D'Alessandro JS, Bauduy M, Cordes P, Harada T, Israel DI, Hewick RM, Kerns KM, LaPan P, Luxenberg D, Mcquaid D, Moutsatsos IK, Nove J, Wozney JM (1990) *Proc Natl Acad Sci USA* 87:2220–2224
15. Zuo X, Li S, Hall J, Mattern MR, Tran H, Shoo J, Tan R, Weiss SR, Butt TR (2005) *J Struct Funct Genom* 6:103–111