Video Article

GST-His purification: A Two-step Affinity Purification Protocol Yielding Full-length Purified Proteins

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Abstract

Key assays in enzymology for the biochemical characterization of proteins *in vitro* necessitate high concentrations of the purified protein of interest. Protein purification protocols should combine efficiency, simplicity and cost effectiveness[1]. Here, we describe the GST-His method as a new small-scale affinity purification system for recombinant proteins, based on a N-terminal Glutathione Sepharose Tag (GST)[2,3] and a C-terminal 10xHis tag[4], which are both fused to the protein of interest. The latter construct is used to generate baculoviruses, for infection of Sf9 infected cells for protein expression[5]. GST is a rather long tag (29 kDa) which serves to ensure purification efficiency. However, it might influence physiological properties of the protein. Hence, it is subsequently cleaved off the protein using the PreScission enzyme[6]. In order to ensure maximum purity and to remove the cleaved GST, we added a second affinity purification step based on the comparatively small His-Tag. Importantly, our technique is based on two different tags flanking the two ends of the protein, which is an efficient tool to remove degraded proteins and, therefore, enriches full-length proteins. The method presented here does not require an expensive instrumental setup, such as FPLC. Additionally, we incorporated $MgCl_2$ and ATP washes to remove heat shock protein impurities and nuclease treatment to abolish contaminating nucleic acids. In summary, the combination of two different tags flanking the N- and the C-terminal and the capability to cleave off one of the tags, guaranties the recovery of a highly purified and full-length protein of interest.

Video Link

The video component of this article can be found at http://www.jove.com/video/50320/

Introduction

The purification of recombinant proteins is crucial to address fundamental questions in biochemistry. Conventional ways of protein purification like ion exchange chromatography and size exclusion chromatography rely on physical properties of the target protein such as its isoelectric point and charge or size, respectively. The latter protein characteristics are shared by a variety of proteins, which increases considerably the chance of contaminating proteins in conventional protein purification strategies. This problem may be circumvented with the use of multiple purification columns, which is time consuming. At the same time, the latter chromatography methods demand expensive experimental setup. Affinity-tag purification strongly increases target-specificity, as in most of the cases the tag will be unique to the protein of interest. In recent studies, Flag- or HA-affinity purification has been widely used.

In contrast to existing recombinant protein purification protocols in which single tags are used, we established the unique combination of two tags. Our method involves the fusion of a GST-tag at the N-terminus and a His-tag at the C-terminus of the protein of interest, for an optimal ratio between quantity and purity of the desired protein. The GST is a long tag (29 kDa), which is highly efficient for purification on glutathione Sepharose beads. Furthermore, using GST guarantees cost-effectiveness of our method[1]. The possibility to cleave off the GST with the PreScission enzyme (with recognition sequence LeuGluValLeuPheGln/GlyPro, resulting in the addition of only two amino acids) has many advantages, for instance, this strategy avoids alterations of the physiological protein functions due to allosteric hindrance. The small His-tag fused to the other protein extremity serves in a second purification step to increase protein purity by washing off the cleaved GST, as well as degraded proteins and other contaminants. Additionally, the protocol does not require a dialysis step when switching from the GST to the His-purification step column (TALON resin). Common contaminants in such purification processes are Heat Shock Proteins (HSP). The addition of an incubation step with $MgCl_2$ and ATP allows the removal of these contaminants (**Figure 1**).

Fast Protein Liquid Chromatography (FPLC) and High Performance Liquid Chromatography (HPLC) are common techniques that depend on expensive instrumentation to generate high yield of the purified protein of interest. The batch purification protocol we present here, in contrast, is manual and does not require an expensive instrumental setup. A high yield of protein can be reached by scaling up the protocol. At the same time, with the batch purification protocol, the elution volume can be adjusted in order to enhance protein concentration, which is not given with FPLC or HPLC.

Also, with the batch GST-His purification protocol, proteins with a size-range of ~10-300 kDa can be purified. One of the biggest advantages is given by the fact that one can purify several proteins at the same time, for instance, both a wild-type and its mutant protein. The success of the presented protocol solely depends on the expression level and solubility of the protein of interest.

Protocol

1. Production of Recombinant Baculovirus

Baculoviruses are generated using the Bac-to-Bac Baculovirus Expression System of Invitrogen mainly in accordance with the manufacturer's protocol with only slight modifications:

1. A modified pFastBac1 vector (Gibco, life) was created containing the GST and His-tags (**Figure 2**). The MultiCloning Site (MCS) of a pET-52b(+) vector (Novagen), containing a 10xHis-tag, was inserted into the MCS of a pFastBac1 vector to generate pFastBac1-Strep-10xHis. The pET-52b(+) MCS sequence was PCR amplified between the XbaI and BlpI restriction sites, adding a BglII restriction site at the 5' extremity and a HindIII restriction site at the 3' extremity. The PCR product was then cloned between BamHI and HindIII restriction site of pFastBac1, using the compatibility of BamHI and BglII restriction sequences. Since the aim was to generate a pFastBac1 allowing the expression of recombinant protein with streptavidin- and 10xHis-tags, the BamHI restriction site of the pET-52b(+) MCS sequence was not used. Also, the XbaI restriction site was not used to avoid the redundancy of restriction sites occurring between the sites already in the MCS of pFastBac1 and these inserted with the MCS of pET-52b(+). The GST coding sequence with the PreScission Protease recognition site (LeuGluValLeuPheGln/GlyPro) was then inserted into pFastBac1-Strep-10xHis. The GST cDNA sequence from pGEX-6P-2 (GE Healthcare) was PCR amplified with primers containing NcoI and KpnI restriction sites at the 5' and 3' extremities, respectively. The NcoI site allows the addition of a start codon but also an Alanine to the GST. The PCR product was then cloned into pFastBac1-Strep-10xHis between the NcoI and KpnI restriction sites, resulting in the deletion of the Streptavidin-tag. The new fusion vector thus obtained was named pFastBac1-GST-10xHis.
2. Clone the cDNA coding for the protein of interest into the pFastBac-GST-10xHis vector between the GST (N-terminal) and the His-tag (C-terminal). Be careful to remove the cDNA stop codon to allow the fusion with the C-terminal His-tag.
3. Transform *E. coli* DH10Bac with the recombinant pFastBac (obtained in step 1.2) to generate the bacmid. Allow colonies to grow overnight at 37 °C and several hours at 30 °C on selection plates containing Bluo-gal and IPTG (10 µg/ml tetracyclin, 50 µg/ml kanamycin, 7 µg/ml gentamycin, 100 µg/ml Bluo-Gal, 40 µg/ml IPTG).
4. Pick and restreak 2 white colonies (from step 1.3) on selection plates to confirm the white phenotype.
5. Inoculate 2 ml of LB containing 10 µg/ml tetracyclin, 50 µg/ml kanamycin, 10 µg/ml gentamycin with the two white colonies from step 1.4 and let them grow overnight under agitation (250 rpm) at 37 °C.
6. In order to purify the bacmid DNA, pellet the bacteria from step 1.5, resuspend the cells in 300 µl of solution I and add 300 µl of solution II (Maxiprep kit from Qiagen). Incubate for 5 min at room temperature, add 300 µl of 3 M KOAc pH 5.5 and incubate for 10 min on ice. Centrifuge the samples at 4 °C, maximum speed for 10 min. Add 800 µl of isopropanol to the supernatants and incubate for 10 min on ice. Centrifuge the samples for 15 min at 4 °C and wash the pellet with 500 µl of 70% ethanol. Let the pellet dry and resuspend it under sterile conditions in 40 µl of Tris-EDTA pH 8.0. The bacmid DNA should be stored at 4 °C.
7. Generate baculoviruses as described in the manufacturer's protocol. Baculoviruses can be stored at 4 °C protected from light.

2. Recombinant Protein Expression

1. In order to choose the most efficient baculovirus for purification and to monitor what time the peak of protein expression is reached, perform a mini-infection assay as follows: Infect 2 x 10^7 of Sf9 infected cells cultured at 27 °C in Grace's media (complemented with 10% FBS and 1% P/S) with 133 µl (1/150) of baculovirus. Collect aliquots of 1.5 ml at 0, 24, 48, and 72 hr post-infection. Pellet the cells and analyze the expression level of the protein of interest by western blotting using anti-tag antibodies.
2. Use the most efficient baculovirus from step 2.1 to infect a spinner containing 1.0 x 10^6 Sf9 cells per ml in 500 ml of media with a ratio of 1/150 between virus and media. Let the infected cells grow in suspension at 27 °C and harvest the cells when the maximum protein expression (as determined in step 2.1 above) is reached. Pelleted cells can be stored at -80 °C until further usage.

3. Preparation of Soluble Cell Lysate

All of the following incubation steps are carried out at 4 °C under mild rotation.

1. Lyse Sf9 cells expressing your protein of interest in ~20 ml of GST binding buffer (150 mM NaCl, 1 mM EDTA, 0.05% Triton-X-100, 1 mM DTT in PBS1X for a final concentration of NaCl of 250 mM, and protease inhibitor cocktail (Roche). In an ice water bath, dounce the solution 20x with a dounce homogenizer, sonicate 3x 30 seconds each (70% output), and dounce again 20x.
2. (Optional). Incubate the total cell lysate with 1 mM $MgCl_2$ and benzonase[7] nuclease (2.5 U/ml) for 30 min at 4 °C to remove DNA or RNA contamination.
3. Centrifuge at 18,000 rpm for 30 min at 4 °C. Keep the supernatant.
4. (Optional). Centrifuge the supernatant again for 30 min at 18,000 rpm at 4 °C to get a clear soluble lysate. Pass the supernatant through a 0.2 or 0.45 µm filter to avoid clogging.

4. Binding of Protein on GST Beads

1. Incubate the soluble cell lysate with 1 ml of GST beads (prewashed two times with 10 ml of GST binding buffer) for 1 hr at 4 °C under gentle rotation.

Comment: The incubation time needs to be optimized according to the stability of the protein and binding efficiency.

2. Quick spin at 700 rpm and remove supernatant containing unbound proteins (this can be kept for further analysis). Wash the GST-bound proteins (**Figure 3B**, lane 1) with GST washing buffer (GST binding buffer with 350 mM NaCl final).
3. Repeat step 4.2 2x. At the last wash, remove as much supernatant as possible.
4. Incubate the beads with 5 mM ATP and 15 mM $MgCl_2$ (in 10 ml GST binding buffer) for 1 hr at 4 °C to avoid nonspecific binding of heat-shock proteins[8]. Wash the beads three times with GST washing buffer.

5. PreScission Cleavage of the GST

1. Centrifuge the GST beads, remove the supernatant and wash the GST beads with P5 buffer (50 mM $NaHPO_4$ pH 7.0, 500 mM NaCl, 10% glycerol, 0.05% Triton-X-100, 5 mM imidazole).
2. Incubate the GST-beads with PreScission enzyme in P5 buffer from 3 hr to overnight at 4 °C. (Divide the GST beads in fractions of 100 µl and add 4-8 units (2-4 µl) of enzyme diluted in 100 µl of P5 buffer).

Comment: The incubation time of the PreScission step needs to be optimized according to the molecular weight as well as the stability of the protein. If degradation is observed, this incubation time can be decreased to a minimum of 2 hr instead of overnight.

3. Quick spin and collect the supernatant. Repeat this step two times after adding 100 µl of P5 buffer to the beads and pool all fractions (**Figure 3B**, lane 2).

Comment: The remaining beads can be used for analysis of cleavage efficiency (**Figure 3B**, lane 3). Usually, 70-80% of the protein is cleaved.

6. Protein-binding to TALON Metal Affinity Resin

1. Divide the elution from above into 3 fractions of 1 ml and incubate each with 100 µl Talon metal affinity resin dry beads (prewashed two times with P5 buffer) for 1 hr under rotation.

Comment: Dividing the elution in several fractions increases binding/washes efficiency.

2. Wash the resin 5 min with P30 buffer (P5 buffer with a final concentration of 30 mM imidazole).
3. Repeat step 6.2 2x and pool the beads in a single tube. Before the last wash, remove as much supernatant as possible.

Comment: This corresponds to the TALON bound sample (**Figure 3B**, lane 4).

7. Elution of the Purified Protein

1. Incubate the protein bound TALON resin for 5 min under rotation with P500 buffer (P5 buffer with a final concentration of 500 mM imidazole). Use a ratio between buffer and beads of 1:1 v/v. (For example, if you had taken 200 µl of dry beads, you will have to add 200 µl of P500). Repeat this step two times and keep each eluted fraction separately (named elution 1 (E1), elution 2 (E2) and elution 3 (E3); **Figure 3B**, lanes 5-7).

Comment: The remaining beads can be used for analysis of elution efficiency (**Figure 3B**, lane 8). Typically, 60-80% of the protein is eluted in total.

2. Analyze the quantity and quality of your purified protein by loading approximately 20-40 µl of each fraction with a standard BSA concentration on a SDS-PAGE and stain with Coomassie blue (**Figure 3B**) or SYPRO protein stain for visualization.

Comment: The protein of interest can also be detected throughout the purification procedure using anti-GST and anti-His antibodies (**Figure 3C**).

8. Storage of the Purified Protein

1. Dialyze the purified protein against an appropriate storage buffer (*e.g.* 20 mM Tris Acetate pH 8.0, 200 mM KAc, 10% Glycerol, 1 mM EDTA, 0.5 mM DTT) at 4 °C. It is important to check for precipitation of the purified protein during the dialysis. We usually dialyze 2x for 1 hr at 4 °C under agitation rotation.
2. Make small aliquots of the purified protein (10-20 µl) and freeze on dry ice for 30 min. Store the aliquots at -80 °C and avoid many thaw/freezing cycles.

Representative Results

In order to illustrate the efficiency of the GST-His purification protocol, we purified Rec14, a *S. pombe* protein of 32.9 kDa. The Rec14 cDNA was cloned in our modified pFastBac1 vector allowing the additions of GST- and His-tags at the N- and C- termini, respectively (**Figure 1A**). Recombinant baculovirus were then prepared and used to infect SF9 infected cells for protein expression. The soluble cell lysates were incubated with GST beads and bound-proteins were eluted by cleaving the GST with PreScission protease. The resulting Rec14-His protein was affinity purified on TALON resin and bound-proteins were eluted with TALON buffer containing 500 mM imidazole. Purified Rec14-His was dialyzed in storage buffer and stored at -80 °C (**Figure 1B**).

The soluble and total cell lysates from SF9 cells infected with the Rec14 recombinant baculovirus, or mock-infected as control, were analyzed by Coomassie blue staining, allowing us to confirm the expression of GST-Rec14-His protein (**Figure 3A**). During the purification process, many samples were analyzed to follow the efficiency of the method. Analysis of these samples by Coomassie blue staining (**Figure 3B**) shows that during the first step of purification, GST-Rec14 was correctly bound to GST beads, with an apparent molecular weight of ~60 kDa due to the fusion of Rec14 (32.9 kDa) with GST (29 kDa) (lane 1). After PreScission cleavage of the GST, GST-free Rec14-His migrates around 37 kDa (lane 2) while the cleaved GST can be visualized on the GST beads (lane 3, around 25 kDa). Despite a portion of GST-free Rec14 that still remained bound to GST beads (lane 3), notable enrichment in Rec14-His was achieved (lane 2, with no contaminant visible on Coomassie blue staining). This step could be improved by increasing the incubation time of the protein with PreScission. Analysis of Rec14-His bound to TALON beads (after step 7.1, lane 4) compared to the proteins eluted after GST purification step (lane 2), shows that a large fraction of Rec14-His is bound to TALON beads. After several washes, Rec14-His was eluted and collected. Three elutions had been performed. The analysis revealed a high purity of Rec14-His (no contaminant visible by Coomassie staining, lanes 5 to 7), and the greatest concentration of Rec14-His in the first elution. Comparison of eluted fractions (lanes 5 to 7) to the TALON beads after elution (lane 8) illustrates the efficiency of the elution, since only few Rec14-His can be detected as bound on beads.

Samples used in **Figure 3B** were subjected to western blot analysis with anti-GST and anti-His antibodies in order to follow Rec14 throughout the procedure (**Figure 3C**). The anti-GST blot shows that some GST-Rec14 and GST alone, were present in the eluted proteins from the GST purification step (lane 2), and that contaminants were removed by the His-tag affinity purification step. This blot allows us also to monitor that GST had been efficiently removed from Rec14 by the PreScission treatment and the His-tag purification step (lanes 4 to 8). The anti-His blot aims to detect Rec14. We can thus confirm that GST-Rec14-His and Rec14-His were not completely cleaved and removed from the GST beads (lane 11). Moreover, at a high concentration, Rec14 seems to aggregate (lane 13). In summary, the results presented demonstrate the efficiency of the GST-His purification for the purification of *S. pombe* Rec14.

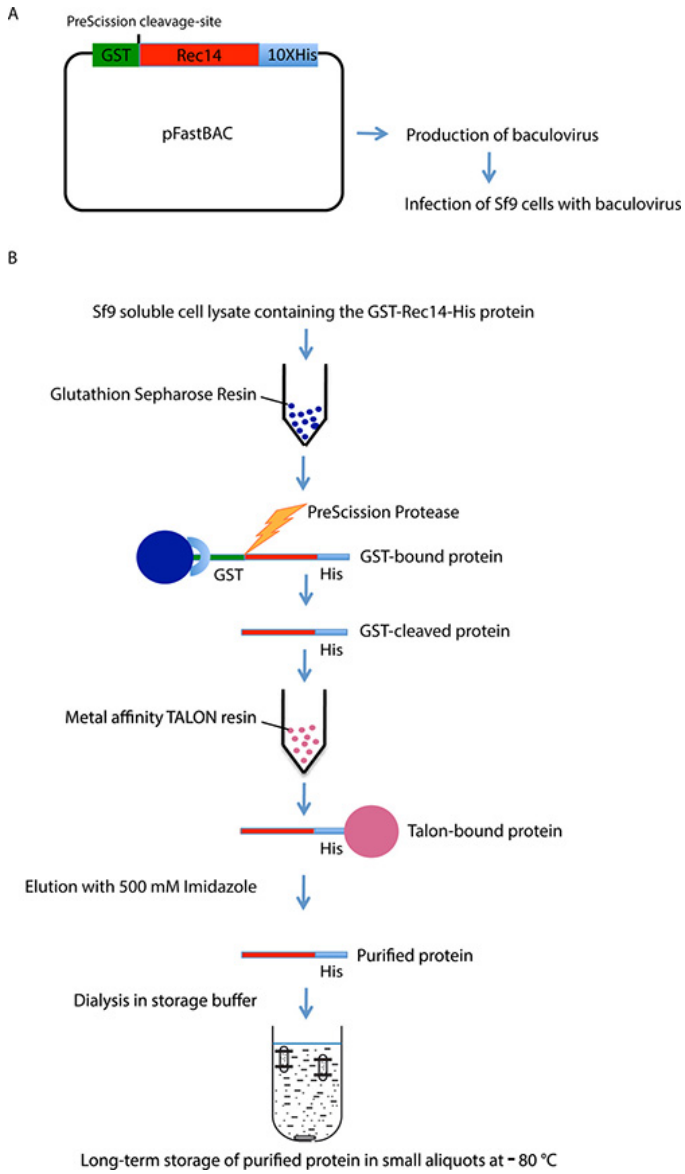
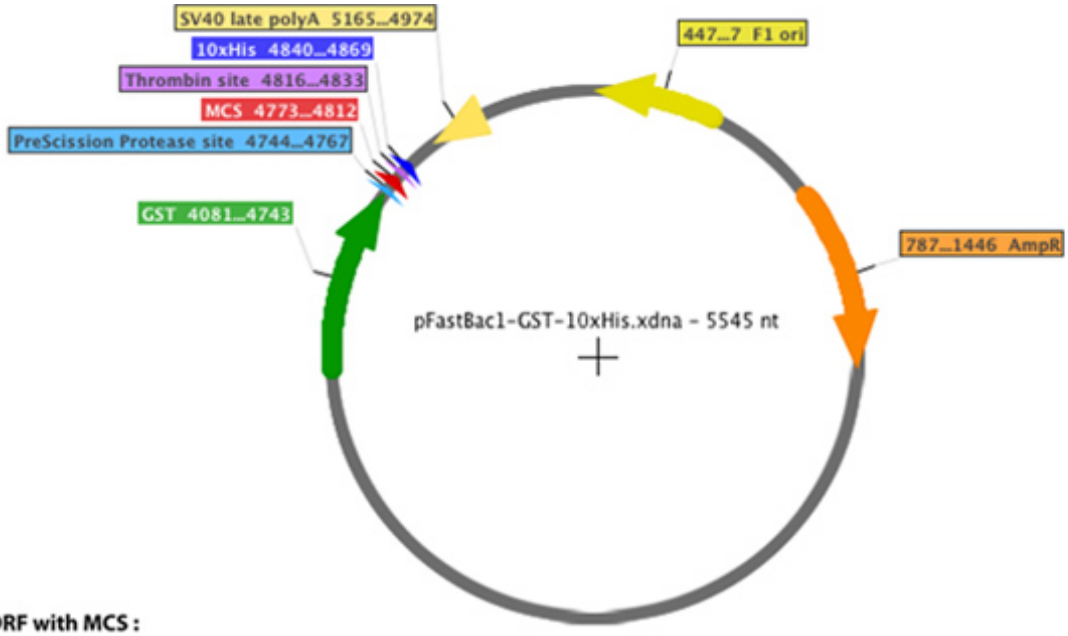


Figure 1. Schematic outline of the GST-His two-step affinity purification method. A. GST-Rec14-His construct cloned into pFastBac (Baculovirus expression vector). **B.** Two-step affinity purification of GST-Rec14-His from Sf9 soluble cell lysate: GST-binding, followed by PreScission cleavage of the GST, TALON-binding and elution with imidazole. Click here to view larger figure.



ORF with MCS :

BamHI/BglII *NcoI*

5'...GC Ggatct cctctagaaataattttgtttaactttaagaaggagatatacc **ATG** GCG TCC CCT ATA CTA GGT TAT TGG AAA ATT AAG GGC
CTT GTG CAA CCC ACT CGA CTT CTT TTG GAA TAT CTT GAA GAA AAA TAT GAA GAG CAT TTG TAT GAG CGC GAT GAA GGT
GAT AAA TGG CGA AAC AAA AAG TTT GAA TTG GGT TTG GAG TTT CCC AAT CTT CCT TAT TAT ATT GAT GGT GAT GTT AAA
TTA ACA CAG TCT ATG GCC ATC ATA CGT TAT ATA GCT GAC AAG CAC AAC ATG TTG GGT GGT TGT CCA AAA GAG CGT GCA
GAG ATT TCA ATG CTT GAA GGA GCG GTT TTG GAT ATT AGA TAC GGT GTT TCG AGA ATT GCA TAT AGT AAA GAC TTT GAA
ACT CTC AAA GTT GAT TTT CTT AGC AAG CTA CCT GAA ATG CTG AAA ATG TTC GAA GAT CGT TTA TGT CAT AAA ACA TAT
TTA AAT GGT GAT CAT GTA ACC CAT CCT GAC TTC ATG TTG TAT GAC GCT CTT GAT GTT GTT TTA TAC ATG GAC CCA ATG
TGC CTG GAT GCG TTC CCA AAA TTA GTT TGT TTT AAA AAA CGT ATT GAA GCT ATC CCA CAA ATT GAT AAG TAC TTG AAA
TCC AGC AAG TAT ATA GCA TGG CCT TTG CAG GGC TGG CAA GCC ACG TTT GGT GGT GGC GAC CAT CCT CCA AAA TCG GAT

KpnI *BamHI* *SalI* *NotI* *SacI*

CTG GAA GTT CTG TTC CAG GGG CCC CTG Ggg tac cag gat cct gta caa gtc gac gcg gcc gca gag ctc gct ctg gtg cca cgc ggt

HindIII

agt tcc gct catcaccaccatcatcaccatcaccaccac**TAA**ttaacctaggctgctgccaccgctgagcaataa aagctT GTC...3'

Protein :

MASPILGYWKIKGLVQPTRLLLEYLEEKYEEHLYERDEGDKWRNKKFELGLEFPNLPYYIDGDVKLTQSMAIIRYIADKHNMLGGCPKERA
EISMLEGAVLDIRYGVSRIAYSKDFETLKVDFLSKLPEMLKMFEDRLCHKTYLNGDHVTHPDFMLYDALDVVLYMDPMCLDAFPKLVCF
KKRIEAIPQIDKYLKSSKYIAWPLQGWQATFGGGDHPPKSDLEVLFQGPLGYQDPVQVDAAAELALVPRGSSAHHHHHHHHHH

Figure 2. Schematic representation of pFastBac1-GST-10xHis. GST (green), PreScission protease site (light blue), the multicloning site (red), and the 10-His tag (dark blue) are shown. In lower case are the nucleotide sequences originating from pET-52b(+). Click here to view larger figure.

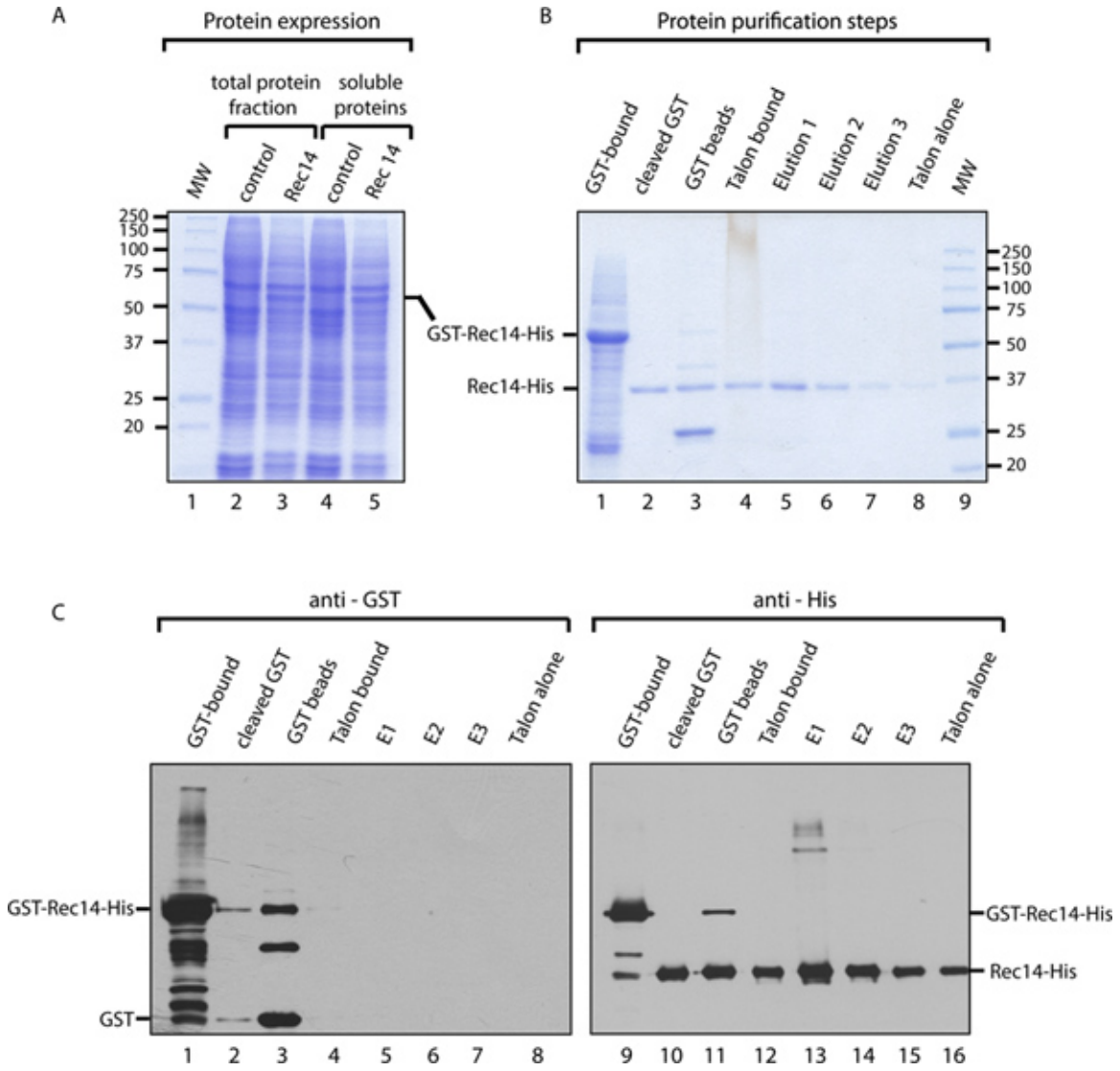


Figure 3. Exemplary result of GST-His purification of Rec14. **A**. Coomassie staining of total protein fraction (lane 2 & 3) and soluble cell lysate (lane 4 & 5) from mock-treated Sf9 cells (lane 2 & 4) or infected with GST-Rec14-His baculovirus (lane 3 & 5). Rec14-His and GST possess a molecular weight of approximately 32.9 kDa and 29 kDa, respectively, and the GST-Rec14-His fusion protein has a molecular weight of approximately 61.9 kDa. **B.** Coomassie staining demonstrating each step of protein purification method (for detailed explanation see PROCEDURE) **C.** Western blot of the fractions following the purification procedure with anti-GST (lane 1 to 8) and anti-His (lane 9 to 16) antibodies. Click here to view larger figure.

Discussion

The GST-His purification protocol presented here is suitable for the purification of a broad range of sizes of recombinant proteins: We successfully purified *Li*RAD51 (41kDa), piBRCA2 (120kDa), PALB2 (130kDa), and an unstable high molecular weight protein of *Leishmania infantum*: *Li*BRCA2 (125kDa)[6,9]. Moreover, the proteins purified with this protocol were biochemically active[6]. The success of this method solely depends on the expression, solubility and stability of the desired protein.

Molecular biology vectors for expression in other hosts, such as *E. coli* or yeast, can be easily modified using the widely available GST and His tags. This highlights the applicability and cost-effectiveness of the present technique for most molecular biology labs. A variety of advantages influenced our decision to choose infected cells over bacterial or mammalian cells for recombinant protein expression and purification. For example, posttranslational modifications such as methylation, phosphorylation and ubiquitinylation can strongly influence the enzymatic function of a protein[10]. Hence, to study the physiological properties of a protein, it is favorable to use a system that allows for posttranslational modification, such as Sf9 infected cells. Another advantage of using Sf9 over mammalian cells, for instance, is of economical nature, as the infected cell system requires considerably fewer cells and no costly transfection method compared to the mammalian system.

The simplicity of the presented method will help researchers to obtain a protein or protein complex in a highly purified form with standard lab equipment, which is advantageous for a lab with minimal equipment.

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