Protein Expression and Purification Kit Cat. No. L00208

Technical Manual No. TM0186 **Version 07112010**

Index

1. Product Description

GenScript Protein Expression and Purification Kit provide all the major reagents and materials (Table 1) for the cloning, expression and purification of target protein. The target protein is first expressed as a GST fusion protein in *E. coli* using the GenScript expression vector pGS-21a. The GST fusion protein is bound to a column packed with GenScript Glutathione Resin (L00206) and then eluted with reduced glutathione. Finally, to obtain the tag-free target protein, the purified GST fusion protein is digested with recombinant porcine Enterokinase (EK) to remove GST tag.

Table 1. Components of Protein Expression and Purification Kit

Components	Characteristics
Glutathione Resin (L00206, 10 ml)	20 ml 50% slurry with 10 ml 4% cross-linked agarose (average particle size: 90 µm) coupled with glutathione. The total binding capacity is 6 mg horse liver GST/ml settled resin.
pGS-21a (SD0121, 10 µg)	A bacteria expression vector. The fusion protein contains both the 6×His tag and GST tag, and can be purified by using either Ni-Charged Resin or Glutathione Resin or both for higher purity. Both tags can be removed by EK cleavage.
Columns (5)	5 disposable plastic columns with a capacity of about 12 ml for each column.
Glutathione (5 x 0.154 g)	L-Glutathione (reduced), 0.154 g Glutathione in each tube can make 50 ml of 10 mM elution solution if dissolved in 50 ml of 50 mM Tris-HCl, pH 8.0.
Enterokinase (2 x 100 IU)	Recombinant Porcine Enterokinase with a 6×His tag at the N-terminal for easy removal of EK after fusion protein cleavage.

2. Operation

Buffer Preparation

Water and chemicals used for buffer preparation should be of high purity. It is recommended filtering the buffers by passing them through a 0.45 µm filter before use.

Binding/Wash Buffer (1×PBS): 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 140 mM NaCl, 2.7 mM KCl, pH 7.3

Elution Buffer: 10 mM glutathione , pH 8.0 [0.154 g of reduced glutathione dissolved in 50 ml of 50 mM Tris-HCl (pH 8.0), and adjust the final pH to 8.0]

3. Protocols

A. Subcloning of Target Gene into Expression Vector pGS-21a

pGS-21a is provided as a lyophilized powder, centrifuge the powder to the tube bottom then add 20 µl distilled deionized water (ddH_2O) to dissolve it before use. Vortex and make sure all the DNA is dissolved. If needed, incubate at 50°C for 10 min. The dissolved DNA can be used directly if only less than 10 µg of the vector are needed. For large amount of vector, take 1 µl of the DNA solution and transform competent DH5α or TOP10 cells for a Maxiprep using Qiagen Maxiprep kit. For more information about this vector, visit GenScript website: http://www.genscript.com/cgi-bin/products/marker.cgi?code=SD0121.

1. Choose the appropriate restriction sites on pGS-21a. Subclone the gene of target protein into pGS-21a using the chosen restriction sites, use appropriate competent cells for protein expression.
2. Choose 10 clones and grow them. Prepare Minipreps from culture using GenScript Miniprep kit.
3. Cut the plasmids with the chosen restriction enzymes. Run an agarose gel to check whether the plasmids have the inserts and select positive clones.
4. Sequence the positive clones to verify the sequence of the insert.

B. Expression of the Fusion Protein and Preparation of Cell Extract

1. Inoculate 1 L LB containing 100 µg/ml Ampicillin with 250 µl mini culture and incubate at 37°C with shaking at 250 rpm.
2. Periodically check the OD_{600} of the culture until the OD_{600} reaches 0.5-0.6.
3. Set aside 1 ml of the culture as the un-induced control. Induce the culture with 1 ml 0.4 M IPTG (final concentration 0.4 mM, the IPTG concentration needs to be optimized in some cases).
4. Grow the culture at 37°C for 3 hours or as the time optimized.
5. Harvest cells by centrifugation at 3,000 g at 4°C for 10 min, remove and discard the supernatant. Resuspend the cell pellet in 3 ml ice-cold PBS buffer per 50 ml culture and centrifuge at 3,000 g at 4°C for 10 min. Remove and discard the supernatant.
6. Freeze the cell pellet at -80°C for 1 hour (This is also a convenient point to stop and one can continue the procedure later).
7. Thaw cell pellet on ice and re-suspend cells in 3 ml of ice-cold PBS buffer per 50 ml culture. If desired, add appropriate additives, such as non-ionic detergents (NP-40) or protease inhibitors (PMSF).
8. Break the cells by brief pulses of sonication on ice until the sample is no longer viscous.
9. Centrifuge at 12,000 g at 4°C for 10 min and carefully transfer the supernatant (soluble fraction) to a clean and pre-chilled tube and resuspend pellet (insoluble fraction) with 3 ml of ice-cold 1 x PBS Buffer per 50 ml of *E. coli* culture.

10. Aliquot 10 µl samples from both soluble and insoluble fractions for SDS-PAGE Analysis (by adding equal volume of 2 x SDS sample loading buffer, boil for 5 min and run SDS-PAGE to determine the amount and solubility of the GST-fusion protein).

Note:

1. The binding of GST or GST-fusion protein to High-Affinity GST Resin is not affected by 1% Triton X-100, 1% Tween-20, 1% CTAB, 10 mM DTT, 0.03% SDS, or 0.1% NP-40. These chemicals can be used to reduce non-specific binding.
2. If the target GST-fusion protein forms inclusion body (insoluble protein), inclusion body has to be properly solubilized and refolded prior to purification.

C. Purification of Target GST Fusion Protein

Sample Preparation

The sample should be centrifuged and/or filtered through a filter before it is applied onto the column. If the sample is too viscous, dilute it with Binding/Wash Buffer to prevent clogging the column. It is not necessary to filter the sample before performing batch purification. For detailed procedures, refer to Technical Manual of Glutathione Resin (L00206).

Packing of Column

1. Shake gently the bottle containing the Glutathione Resin until all the resin is completely in suspension.
2. Transfer an appropriate amount of slurry to an empty column. Usually 1 ml settled resin (2 ml 50% slurry) can bind 6 mg of GST protein.
3. Equilibrate the Glutathione Resin with 10 bed volumes of Binding/Wash Buffer.

Column Purification

1. Apply clear solution (sonicate, *etc*) containing GST-fusion protein prepared in 1×PBS to the equilibrated column with the flow rate of 0.5-1 ml/min.
2. Add Binding/Wash Buffer to wash the column just after all the sample solution get into the column, use 20 bed volumes of Binding/Wash buffer for wash. Protease inhibitors such as PMSF are better added to the Binding/Wash solution to inhibit protease activity.
3. Elute the target GST fusion protein with 10-15 bed volumes of freshly made Elution Buffer. Monitor protein content by measuring absorbance at 280 nm.
4. Aliquot 10-20 µl of original sample solution, flow through, wash and eluate respectively, and analyze all the samples by SDS-PAGE to confirm the presence of the target protein.
5. Pool eluate containing target protein. Remove free glutathione by dialysis at 4°C against a buffer of choice or by using a G15 Sephadex desalt column.

Regeneration of Column

The Glutathione Resin can be reused to purify the same protein three times without regeneration. If the target GST-fusion protein is different, however, the Glutathione Resin must be regenerated using the following protocol:

1. Wash the column with 2 bed volumes of 0.1 M Tris HCl + 0.5 M NaCl, pH 8.5.
2. Wash the column with 2 bed volumes of 0.1 M sodium acetate + 0.5 M NaCl, pH 4.5.
3. Re-equilibrate the column with 3-5 bed volumes of 1×PBS.
4. For long-term storage, the resin should be stored in 1× PBS containing 20% ethanol at 2 - 8°C.

D. Cleavage of Recombinant GST-Fusion Protein with Enterokinase

1. Measure the concentration of the purified GST-fusion protein. For efficient cleavage, the protein concentration should be higher than 2.5 µM. If the protein concentration is lower than 2.5 µM, concentrate the protein.
2. The rPorcine Enterokinase works well in 50 mM Tris-HCl, pH 8.0 (1×Reaction Buffer). The purified GST-fusion protein in 50 mM Tris-HCl, pH 8.0 can be cleaved by simple EK addition. Add 1 IU of EK (0.5 µl) to up to 50 µg of GST-fusion protein and incubate at 16-25°C for 16 hrs to completely cleave the GST tag from the target protein.
3. If the fusion protein is not in 50 mM Tris-HCl, pH 8.0, set-up the cleavage reaction as following:

   10× Reaction Buffer: 5 µl
   Fusion protein: up to 50 µg
   rPorcine Enterokinase: 1 IU
   ddH_2O: to 50 µl

   Incubate at 16-25°C for 16 hrs to completely cleave the GST tag from the target protein.
4. Analyze the cleavage reaction by SDS-PAGE.

Note: Enterokinase can be removed using High Affinity Ni-Charged Resin (L00223). For detailed procedures, refer to the technical manual of this product.

4. Troubleshooting

Problem	Probable Cause	Solution
The yield of the purified fusion protein is low or undetectable	The fusion protein forms inclusion body.	Grow bacteria at low temperature (20-30°C), or reduce final concentration of IPTG to 0.1 mM for protein induction, or reduce the induction time.
		Properly dissolve and refold the inclusion body prior to the purification.
	The fusion protein does not bind to Glutathione Resin efficiently.	Use batch method for purification. Incubate clear solution (sonicate, etc) containing GST-fusion protein with Glutathione Resin for 2 hours or longer (such as overnight) and then load the mixture onto the column.
	The fusion protein does not contain active GST.	Use mild sonication condition or other lysis method, such as lysozyme so that GST is not denatured.
	The fusion protein is degraded by protease.	Add appropriate protease inhibitors such as PMSF in lysis buffer and Binding/Wash Buffer.
	The fusion protein is not efficiently eluted from Glutathione Resin.	Increase elution time or Increase the concentration of glutathione to 15 mM or higher in the Elution Buffer.
		Adjust the pH of Elution buffer to 8.0-9.0 without increasing the glutathione concentration.
		Add Triton X-100 (0.1%, final concentration) or Noctylglucoside (2%, final concentration) or NaCl (0.1-0.2M, final concentration) to the Elution Buffer.
Multiple bands observed in the eluted protein	The fusion protein is degraded by protease.	Add appropriate protease inhibitors (or inhibitor cocktails) such as PMSF in the lysis buffer and Binding/Wash Buffer.

	Some host proteins, such as chaperones, may interact with the fusion protein.	Add DTT (5 mM, final concentration) in the Binding/Wash buffer. Incubate the recombinant protein solution in chaperonin buffer (2 mM ATP, 10 mM $MgSO_4$, 50 mM Tris-HCl) at 37°C for 10 min prior to the purification.
	Over-sonication will cause some protein to bind to the fusion protein.	Use milder sonication condition or another lysis method.
	Some protein will bind to the fusion protein or beads non-specifically.	Optimizing the wash conditions. Detergents such as 1% Triton X-100, 1% Tween-20, 0.03% SDS, or 0.1% NP-40 may be used to reduce non-specific binding. Salt concentration in the Binding/Wash Buffer can also be optimized to reduce non-specific binding.
EK does not cleave the fusion protein efficiently	EK activity is inhibited by high concentration of salts.	EK is inhibited by 1 mM PMSF, 250 mM of NaCl or Imidazole. Dilute the protein solution or change the buffer by dialysis or using desalt columns. EK works well in 50 mM Tris-HCl, pH 8.0.
Secondary cleavage is observed	As little as 0.0625% SDS can cause significant secondary cleavage.	Do not use SDS to reduce non-specific binding. Up to 1% of Triton X-100 can be used to reduce non-specific binding.

5. Related Products

Cat. No.	Product Name
L00210	Protein A Resin
L00400	Ultra Protein A Resin
L00209	Protein G Resin
L00239	Protein L Resin
L00405	Chicken IgY Precipitating Resin
L00223	High Affinity Ni-Charged Resin
L00206	Glutathione Resin
L00353	Streptavidin Resin
L00272	IminoBiotin Resin
L00207	GST Fusion Protein Purification Kit
L00403	High-Affinity Iodoacetyl Resin
L00404	High-Affinity Antibody Purification Kit

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