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I. DESCRIPTION

Protein Expression and Purification Kit (L00208) provides all the major reagents and materials (in bold) for the cloning, expression and purification of target proteins. 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 **High-Affinity GST Resin** and then eluted with **glutathione** (reduced). Finally, the purified GST fusion protein is digested with recombinant porcine **enterokinase (EK)** to remove the GST tag to obtain the target protein.

II. SPECIFICATIONS

GST Resin (10 ml)	50% slurry with glutathione coupled to cross-linked agarose (average particle size: 90 µm). The capacity is > 6 mg horse liver GST/ml packed resin.
pGS-21a (10 µg)	A bacteria expression vector. The fusion protein contains both the (His) ₆ tag and GST tag, and can be purified by using either Ni-column or GST column 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 (His) ₆ tag at the N-terminal for easy removal of EK after fusion protein cleavage.

III. KEY FEATURES

- Kit (L00208) provides all the major reagents and materials for the cloning, expression and purification of target proteins.
- pGS-21a vector is included for easy cloning, high-level expression and convenient purification of proteins fused with both (His)₆ tag and GST tag.
- Both the (His)₆ tag and GST tag can be removed by EK.
- GST Resin, empty columns and elution reagent are all provided in the kit for convenient purification of the fusion proteins.



IV. RELATED PRODUCTS

SD0121 pGS-21a Expression Vector

Z01004 rPorcine Enterokinase

L00206 High-Affinity GST Resin

L00207 GST Fusion Protein Purification Kit

Components	L00206	L00207	L00208
GST Resin	10 ml	10 ml	10 ml
Columns		5 empty columns	5 empty columns
Glutathione, reduced		5 x 0.154 g	5 x 0.154 g
Enterokinase			2 x 100 IU
Expression vector			pGS-21a
Manual	TM0185	TM0185	TM0186

V. STORAGE

Store empty columns at room temperature.

Store High-Affinity GST Resin and Glutathione at 4°C, do not freeze.

Store pGS-21a, rPorcine Enterokinase and 10X buffer at -20°C.

VI. GST-FUSED PROTEIN PURIFICATION PROTOCOL

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

Generate large amount of expression vector pGS-21a. pGS-21a is delivered as a lyophilized powder, add 20 μ l water to dissolve it before use. Please vortex diligently and make sure all the DNA is dissolved. If needed, incubate at 50°C for 10 min. One can use it directly if you only need less than 10 μ g of the vector. For large amount of vector, take 1 μ l of the solution and transform competent DH5a or TOP10 cells for a Maxiprep using Qiagen Maxiprep kit. For more information about this vector, log on to GenScript website:

<http://www.genscript.com/cgi-bin/products/marker.cgi?code=SD0121>.

1. Choose the appropriate restriction sites from 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 μ g/ml of Ampicillin with 250 μ l of mini culture and incubate at 37°C with shaking at 250 rpm.
2. Periodically check the OD₆₀₀ of the culture until the OD₆₀₀ reaches 0.5-0.6.



3. Set aside 1 ml of the culture as the un-induced control. Induce the culture with 1 ml of 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 long as 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 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:

- a. 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 may be used to reduce non-specific binding.
- b. 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 Recombinant GST-Fusion Protein

1. Shake gently the bottle containing the High-Affinity GST Resin until all the resin is completely in suspension.
2. Transfer an appropriate amount of resin (50% slurry) to a disposable column (included in Kit L00207 and L00208) using a pipet. Usually 1 ml of resin (from 2 ml of 50% slurry) can bind more than 6 mg of GST protein.

Note: Gentle pressure with a gloved thumb or using the top cap over the top of the column may be required to start the flow of liquid.

3. Wash the GST resin with 10 bed volumes of cold (4°C) PBS.
4. Apply clear solution (sonicate, etc) containing GST-fusion protein in PBS to the equilibrated column with the flow rate at 10-15 cm/h.



5. Add PBS to wash the column just after all the protein solution get into the column, use 20 bed volumes of PBS for wash. Protease inhibitors such as PMSF are better added to the wash solution to inhibit protease activity.
6. Elute the fusion protein with 10-15 bed volumes of freshly made 10 mM glutathione elution buffer (0.154 g of reduced glutathione dissolved in 50 ml of 50 mM Tris-HCl, pH 8.0.).
7. Monitor elution of the fusion protein using absorbance readings at 280 nm.
8. Aliquot 10-20 μ L of supernatant containing GST-fusion protein, flow through, wash and the eluted protein, respectively, and analyze all the samples by running SDS-PAGE to confirm the presence of the target protein. An example was shown in Figure 1.
9. Pool eluted fractions containing target protein. Remove free glutathione by dialysis at 4°C against a buffer of choice or by using a G15 Sephadex desalt column. If the eluted GST-fusion protein will be cleaved with EK, the elute is better dialyzed against 50 mM Tris-HCl, pH 8.0 or passed through a G15 Sephadex desalt column pre-equilibrated with 50 mM Tris-HCl, pH 8.0.

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 μ M. If the protein concentration is lower than 2.5 μ M, concentrate the protein.
2. The rPorcine Enterokinase works well in 50 mM Tris-HCl, pH 8.0. 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 μ l) to up to 50 μ g of GST-fusion protein and incubate at 16-25°C for 16 hrs to completely cleave the 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 follows:

10X reaction buffer:	5 μ l
Fusion protein:	up to 50 μ g
rPorcine Enterokinase:	1 IU
ddH ₂ O:	to 50 μ l

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

E. Regeneration and Storage of High-Affinity GST Resin

GenScript High-Affinity GST Resin can be reused to purify the same protein three times without regeneration. If the target GST-fusion protein is different, however, High-Affinity GST 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 1X PBS.
4. For long-term storage, the resin should be stored in 20% ethanol at 4°C.

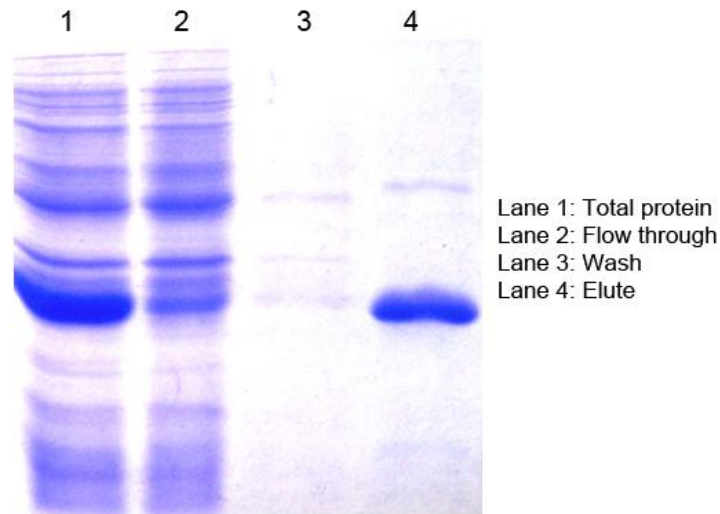


Fig.1. Purification profile using High-Affinity GST Resin

VII. TROUBLESHOOTING

The table below is guideline for 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 GST Resin efficiently.	Use batch method for purification. Incubate clear solution (sonicate, etc) containing GST-fusion protein with GST 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 the lysis solution and wash solution.
The fusion protein is not efficiently eluted from High-Affinity GST Resin.		Increase elution time or Increase the concentration of glutathione to 15 mM or higher in the elution buffer.
		Adjust the pH of the 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 (0.1-0.2 M, 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 solution and wash solution.
	Some host proteins, such as chaperonins, may interact with the fusion protein.	Add DTT (5 mM, final concentration) in the 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 wash solution 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.

VIII. ORDER INFORMATION

Protein Expression and Purification Kit Cat. No. L00208

For Research Use Only.

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