



## SuperGlu Agarose Resin User Guide

Protein Ark's SuperGlu resin is designed for rapid one-step purification, and is ideal for preparative purification and contaminant removal.

The SuperGlu Agarose resin provides high binding capacity with high stability, chemical compatibility and reuse.

Compatible with all common liquid chromatography instruments (including ÄKTA™ FPLC's), peristaltic pumps and syringes.



### Features of the SuperGlu Agarose Affinity Resin:

- Fast and reliable affinity purification.
- Highly stable 7.5% cross linked Agarose with coupled Glutathione ligand provides high buffer stability and broad compatibility.
- High binding capacity for Glutathione S-transferase (GST) tagged recombinant proteins.
- Simple bind-wash-elute procedure

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### Specification:

|                           |  |
|---------------------------|--|
| Item:                     | SuperGlu1A<br>SuperGlu10A<br>SuperGlu25A<br>SuperGlu100A |
| Volume:                   | 1 ml, 10 ml, 25 ml, 100 ml                               |
| Resin:                    | SuperGlu Agarose   |
| Base Matrix:              | 7.5% cross-linked Agarose                                |
| Coupled ligand:           | Glutathione  |
| Typical Binding Capacity: | 10 mg  |
| Mean Bead Size:           | 40 µm  |
| Recommended flow rate:    | 1 ml/min   |
| Maximum flow rate:        | 6 ml/min   |
| Max. operating pressure:  | 0.5-0.8 MPa  |
| Storage (2-8°C):          | 20% Ethanol  |

## Chemical compatibility:

| <b>Buffer compatibility</b>              |                                  |
|--|----------------------------------|
| Standard buffers:                        | Common aqueous buffers and salts |
| pH range:                                | 3.0-12.0                         |
| <b>Chelating agents</b>                  |                                  |
| EDTA:                                    | 1 mM                             |
| <b>Sulfhydryl reagents</b>               |                                  |
| $\beta$ -mercaptoethanol:                | 5 mM                             |
| DTT:                                     | 10 mM                            |
| <b>Denaturants</b>                       |                                  |
| Urea:                                    | 4 M                              |
| Guanidinium hydrochloride:               | 3 M                              |
| <b>Detergents</b>                        |                                  |
| DDM (n-Dodecyl- $\beta$ -Dmaltoside):    | 0.1%                             |
| OG (n-Octyl- $\beta$ -Dglucopyranoside): | 5%                               |
| Triton <sup>®</sup> X-100:               | Up to 2 %                        |
| Tween <sup>®</sup> 20:                   | Up to 2%                         |
| NP-40:                                   | 0.2%                             |
| SDS:                                     | Up to 0.03% (w/v)                |
| Cetyltrimethylammonium bromide (CTAB):   | UP to 1%                         |
| C12E8:                                   | 0.05%                            |
| Brij 35:                                 | 0.1%                             |
| Cholate:                                 | 5%                               |
| Deoxycholate:                            | 2%                               |
| CHAPS :                                  | 1%                               |
| <b>Other additives</b>                   |                                  |
| NaCl                                     | 1 M                              |
| NaOH                                     | 0.1 M                            |
| HCl                                      | 0.1 M                            |
| Ethanol:                                 | 70% (v/v)                        |

## **Principles of GST affinity chromatography:**

Glutathione S-transferase (GST) is a 26 kDa protein whose DNA sequence is frequently integrated into expression vectors as a terminal tag for the production of recombinant proteins. The expression of which produces a GST-tagged protein in which the functional GST protein is fused to the N or C-terminus of the recombinant protein. GST rapidly folds into a stable and highly soluble protein helping to promote greater expression, solubility, and folding of the recombinant proteins. In addition, the GST enzyme/tag can be detected and affinity purified by binding to its substrate glutathione (a Glu-Cys-Gly tripeptide). The simplicity of GST affinity purification is extremely attractive as the immobilised glutathione substrate lends itself to a simple bind-wash-elute mode of operation using lysate samples without any prior treatment (e.g. buffer exchange steps) providing the appropriate buffer formulations are used.

### **GST affinity resin:**

Reduced glutathione tripeptide (Glu-Cys-Gly) covalently immobilised onto 7.5% cross-linked agarose beads via an 11 atom spacer through its sulfhydryl group.

### **Application drivers for GST chromatography:**

Screening expression clones for high levels of GST-tagged proteins.  
Purification of recombinant proteins for raising antibodies.  
Purification of recombinant proteins for activity and/or structural studies.  
Removal of cleaved Glutathione S-transferase tag from protein samples.

### **General considerations for selecting optimal binding conditions for the GST resin:**

The key parameters affecting the binding of the GST-Tagged recombinant protein to the affinity resin are the flow rate over the column and the structural integrity of the Glutathione-S-transferase enzyme tag. Glutathione-S-transferase shows slow kinetics for its Glutathione substrate and it is important to maintain a low flow rate over the resin during loading of the lysate to achieve the maximum binding capacity. Binding is also dependent upon preserving the native structure and function of the Glutathione S-transferase enzyme. Binding is most effective under physiologic conditions (or neutral buffers) such as Tris-buffered saline (TBS) pH 7.5 and is not compatible with protein denaturants.

If the binding efficiency is found to be poor and the lysis buffer differs significantly from the pre-equilibration buffer, it is recommended that the lysate be dialysed, titrated with a concentrated stock solution, or buffer exchanged using an ultrafiltration device with a more appropriate pre-equilibration buffer.

It is imperative that the lysate is completely clear prior to loading on the column as any particulate matter (e.g. cell debris) may partially foul and clog-up the resin resulting in an increased back pressure and reduced flow rates. This will significantly increase the binding, washing and elution times and effect the final purify of the eluted protein. It is recommended that the cleared lysate be filter just prior to loading even if it has been previously filtered several days before. Ideally samples should be processed rapidly and, if the need arises, the protein purified at 4°C. It is also recommended that number of freeze/thaw cycles be minimised during storage to reduce the amount of aggregation/precipitation of the proteins.

Optimal buffer conditions for binding the target molecule to a resin are critical for successful purification of the protein. If the binding conditions are not optimal with respect to pH, salt concentration, detergent ...etc, purification can be adversely affected.

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## Protein purification conditions:

This protocol describes the purification of recombinant glutathione-S-transferase (GST) tagged proteins from an *E. coli* cell pellet under native conditions using Protein Ark's SuperGlu resin. Reagent amounts given apply to IPTG-induced bacterial culture of a well-expressed protein (approximately 10-50 mg/l). Cells are lysed with lysozyme because it is an inexpensive and efficient method for cells that have been frozen. However, other lysis methods based on physical disruption (e.g. sonication or homogenization) or detergents (e.g. CHAPS) can also be used. The GST-tagged protein is then purified from the cleared lysate under native conditions in a bind-wash-elute procedure. This method is most efficient when the GST-tag is available, correctly folded and accessible. It is recommended that the bind-wash-elute conditions be tested and optimised in order to achieve the optimum conditions for purification.

**Example:** Buffers for purifications using reduced glutathione elution.

Lysis buffer:

125 mM Tris-HCl, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 1% Triton X-100, pH 7.4

Wash buffer:

125 mM Tris-HCl, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, pH 7.4

ATP wash buffer:

50 mM Tris-HCl, 2 mM \*ATP, 10 mM MgSO<sub>4</sub>, pH 7.4

Elution buffer:

125 mM Tris-HCl, 150 mM NaCl, 1 mM DTT, 1% Triton X-100, 50 mM reduced glutathione, pH 7.4

NOTE: Optimal buffer conditions may vary depending on the protein of interest. Proteins may require addition of protease inhibitor cocktail, EDTA, 1-5 mM DTT, 1% BSA, or detergents such as 0.5-1% Igepal CA-630 (Nonindet P-40) or 0.5-1% Tween-20. Note also that Triton X-100 is optional.

\*NOTE: Add ATP to the 'ATP wash buffer' immediately before use.

## Procedure

1. Thaw the *E. coli* cell pellet on ice.
2. Resuspend the cell pellet in 'Lysis buffer' (50 ml / litre cell media) supplemented with 1 mg/ml Lysozyme.
3. Incubate at room temperature for >30 min (or > 1 hour at 4°C) on an end-over-end shaker.
4. Centrifuge the lysate for 30 min at 4°C and 10,000 x g. Collect the supernatant.
5. Wash the resin with 3-5 CVs of distilled water to remove the 20% ethanol before equilibrating with 10 CVs of 'Wash buffer'.
6. Filter the cleared lysate through a 0.2 µm syringe filter directly before loading on to the column at the recommended flow rate.
7. After loading wash the column with 'Wash buffer' until the measured absorbance (OD=280nm) reaches a stable baseline.
8. To remove chaperone contaminants wash the column with 3-5 CV's of 'ATP wash buffer'.
9. Elute the GST-tagged protein with 3-5 CV's of 'Elution buffer' or until the measured absorbance (OD=280nm) reaches a stable baseline.
10. Analyze all fractions by SDS-PAGE.

NOTE: Freezing the cell pellet at -20°C for 30 min prior to incubation at room temperature improves lysis by lysozyme.

NOTE: Benzonase® is recommended to reduce the viscosity caused by the nucleic acid (3 U/ml bacterial culture) if required.

NOTE: Add 1 protease inhibitor table to reduce protease activity

NOTE: Mechanical lysis can also be used by repeated freeze/thaw, vortexing, homogenization, sonication or french press if required.

NOTE: If the supernatant remains cloudy then repeat step 4.

NOTE: For optimal binding reduce the flow rate over the column to maximise the interaction time with the resin.  
1 ml HiFliQ GST column= flow rate 0.2-1 ml/min  
5 ml HiFliQ GST column= flow rate 1-5 ml/min

NOTE: This will typically take 10-15 CV's.

NOTE: Keep the wash fractions for SDS-PAGE analysis if required.

NOTE: ATP shows high absorbance at 280nm preventing monitoring of the ATP wash.

NOTE: Retain the ATP wash fractions for SDS-PAGE analysis.

NOTE: Additional elution's with increased reduced glutathione concentrations may be required.

NOTE: Collect the eluate in separate tubes for SDS PAGE analysis and protein concentration determination.

NOTE: Western Blot experiment using an anti-His antibody can be performed if required.

NOTE: Volumes and buffer conditions are protein depended and may require scouting and optimising. Tris and PBS both work well. We do recommend DTT and minimum 150 mM NaCl for binding, washing and elution steps. We observe better yields with pH 7.4 than pH 8.0. Increasing the reduced glutathione concentration from 10mM to 50 mM is also recommended.

NOTE: It is recommended that each column is re-used for identical protein samples to avoid cross-contamination.

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## Desalting and concentrating the purified protein:

Reduced glutathione, EDTA, or detergents should be removed by diafiltration using ultrafiltration concentrators or rapid dialysis against an appropriate buffer for your downstream application.

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## Column washing and regeneration conditions:

Protein Ark's SuperGlu resin should be carefully washed with PBS (wash buffer) and stored in 20% ethanol after each run. If the column shows increased back pressure or loss of binding capacity then this may be due to the accumulation of precipitated, denatured, or non-specifically bound substances and proteins. This protocol delineates washing and regeneration procedures for the SuperGlu column to remove these substances and proteins. All volumes are given in column bed volume (CV).

**Example:** Buffers washing and regeneration.

Regeneration buffer #1:

6M Guanidine hydrochloride

Regeneration buffer #2:

70% Ethanol

Regeneration buffer #3:

1% Triton X-100

Wash buffer:

10-100 mM Sodium phosphate, 2.7 mM potassium chloride, 0.137 M NaCl, pH 7.4 (PBS)

Storage buffer:

20% Ethanol

**Wash and regeneration procedure 1:** removal of precipitated and denatured substances and proteins.

1. Wash the SuperGlu resin with 5 CVs of 'Wash buffer'.
2. Wash the column with 2 CVs 'Regeneration buffer #1'.
3. Immediately wash the column again with 5 CVs of 'Wash buffer'.
4. Wash the column with 3-5 CVs of 'storage buffer' for long term storage at 4°C.

NOTE: The column is now ready for re-use if required.

NOTE: If wash procedure 1 does not fully resolve the problem then repeat or proceed to wash procedure 2.

**Wash and regeneration procedure 2:** removal of hydrophobic substances and proteins.

1. Wash the SuperGlu resin with 5 CVs of 'Wash buffer'.
2. Wash the column with 3-4 CVs of 'Regeneration buffer #2' (or 2 CVs of 'Regeneration buffer #3').
3. Immediately wash the column again with 5 CVs of 'Wash buffer'.
4. Wash the column with 3-5 CVs of 'storage buffer' for long term storage at 4°C.

NOTE: The column is now ready for re-use if required.

NOTE: If wash procedure 2 does not fully resolve the problem then repeat the procedure with the alternative 'Regeneration buffer'.

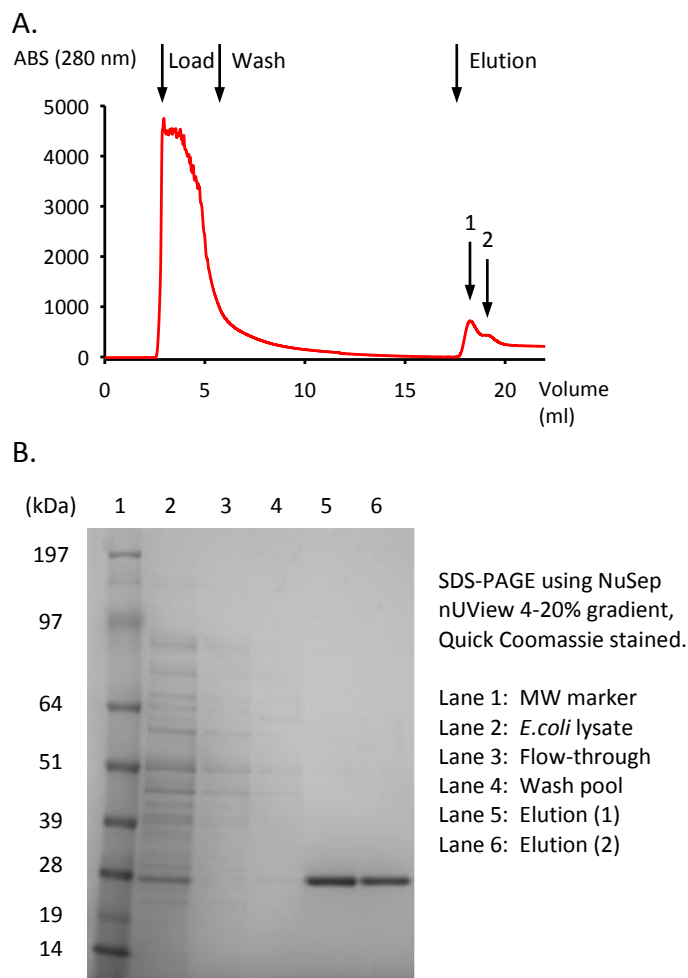
## Performance data:

### Purification of GST-tagged protein from *E.coli* lysate.

Glutathione affinity purification of recombinant GST-tagged protein from 1 ml cleared *E.coli* lysate using a 1 ml HiFliQ GST FPLC column on an FPLC. The eluted fraction contained >95% pure protein according to SDS-PAGE analysis (Figure 1B).

|                 |   |
|-----------------|---|
| Sample:         | 1 ml <i>E.coli</i> lysate                   |
| Column:         | 1 ml HiFliQ GST FPLC Column                 |
| Instrument:     | FPLC  |
| Flow rate:      | 1 ml/min                                    |
| Binding buffer: | PBS pH 7.5                                  |
| Elution Buffer: | 50 mM Tris, 10mM reduced glutathione, pH8.0 |
| Eluted Protein: | 0.7 mg                                      |

Figure 1. Purification of GST-tagged protein from *E.coli* lysate on a 1 ml HiFliQ GST FPLC column. (A) FPLC chromatogram (B) SDS-PAGE analysis.



## Storage conditions:

|                     |                                  |
|---------------------|----------------------------------|
| Item:               | SuperGlu Resin                   |
| Shipping:           | 20 % Ethanol at room temperature |
| Short-term storage: | Equilibration buffer             |
| Long-term storage:  | 20% Ethanol at 4°C               |

**NOTE:** Do not freeze or store the resin in buffer or water for long periods. The resin is stored in 20 % ethanol and reusable for up to 2 years from the date of resin manufacture. Please see the label for the recorded expiry date.



## Questions and answers:

1. What is the shelf-life of the SuperGlu resin?

The resin is guaranteed for 2 years after the date of manufacture provided they are stored at 2-8°C.

2. Do I need to filter the buffers prepared in my laboratory?

It is good laboratory practice to filter all buffers.

3. How should I prepare my sample for GST affinity separation?

Many chromatographic procedures demand that the sample is pre-conditioned prior to loading. We recommend that all samples are filtered to at least 0.45 µm (preferably 0.20 µm) pore size. High viscosity is mostly attributed to contaminating DNA or RNA. The intrinsic viscosity of a lysate can be reduced by either drawing it through a syringe needle several times or by adding appropriate amounts of DNase and/or RNase (5-10 µg/ml) to the lysis buffer and incubating the mix on ice for 15 mins.

4. Should I add DTT (or β-mercaptoethanol) to the lysis buffer?

Concentrations less than or equal to 10 mM DTT (or 5 mM β-mercaptoethanol) can be used with the SuperGlu resin and may significantly increase binding of some GST-tagged proteins to the column.

5. How can I regenerate the SuperGlu Resin?

We recommend that you wash the column with PBS and store in 20% ethanol between each run. If column performance and binding capacity becomes reduced then we recommend washing with harsher conditions. See 'column washing and regeneration conditions' for further details.

6. Should I be concerned if the column partially dries out during the chromatographic steps?

The resin is robust although we recommend flushing out as much air as possible from the column before continuing. Partially dried resin rehydrates rapidly however the performance of the column (binding capacity and running pressure) may be affected.

7. Can I load purified protein immediately on to an SDS-gel?

Proteins purified from the SuperGlu resin under the recommended conditions can be loaded on to an SDS-polyacrylamide gel.

8. Do I need to remove the GST-tag from the recombinant protein after purification?

Due to its size, the GST-tag can affect the activity, stability, or structure determination. If required, a protease cleavage site (e.g. Factor Xa Protease, TEV, or enterokinase) can be engineered between the GST-tag and the target protein.

The tag can then be cleaved off and the protein re-purified by passing it back through the SuperGlu resin or the HiFlIQ GST FPLC column in order to remove the digested tag and undigested GST-tagged protein.

9. *Under what circumstances can I re-use the resin?*

The SuperGlu resin is designed for re-use. We recommend regular washing and cleaning between purifications in order to maintain performance. Should you observe a slowdown in flow rate or increase in back pressure then we recommend washing and regenerating the column prior to further use. See 'Column washing and regeneration conditions' for further details.

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## Troubleshooting assistance:

Bubbles or cracks appear in the resin bed:

- The resin has been stored at a cool temperature and then rapidly warmed up. The resin should be warmed slowly to room temperature before use.

The sample does not flow easily through the column:

- The resin is clogged with particulates. Pre-filter the sample just before loading it on to the column.
- If the column is not stored at 2-8°C, or it has been used more than once and stored in the absence of a bacteriostat, microbial growth may restrict flow through the column.

No binding or elution of the target protein is observed from the column:

- Extensive sonication can denature the GST-tagged protein resulting in loss of the GST-tag's enzyme activity and preventing the protein from binding to the column.
- Adding 1-10 mM DTT to the 'Lysis buffer' can significantly increase binding of the GST-tagged protein to the column.
- Check the pH of the 'Lysis buffer'. If the pH is not within the range of 6-8 then binding of the GST-tag to the SuperGlu resin will be affected. If required, dialyse, titrate with a concentrated stock solution, or buffer exchanged the lysate to within the correct pH range.
- Test the binding conditions and SuperGlu resin are working correctly using just the expressed GST-tag sample for bind-wash-elution.
- The retention time may not be sufficient for binding. Reduce the flow rate of the loading stage down to 0.2-0.5 ml/min (1 ml HiFlIQ GST column) or 1-3 ml/min (5 ml HiFlIQ GST column).
- The column may contain a build-up of precipitated, denatured, or hydrophobic substances and proteins which may impede binding. See 'Column washing and regenerating conditions' for details of washing and regeneration protocols or use a new SuperGlu column.

The recovery of target protein is low:

- Increase the elution time and volume of the 'Elution buffer'.
- The Elution buffer may not be strong enough for elution. Try increasing the pH from 7.4 to 8-9, increasing the salt concentration to 0.2 M NaCl, or increasing the amount of reduced glutathione in the 'Elution buffer'.
- Hydrophobic interactions from the fused protein maybe affecting elution. These may require disrupting using 0.1% Triton X-100 or 2% N-octylglucoside to improve elution of some GST-tagged proteins.

Poor resolution of the target protein:

- Multiple proteins maybe visible on the SDS-PAGE showing co-purification of the GST-tagged protein with another protein. This may be DnaK (70 kDa) or another chaperone protein involved in protein folding. These maybe removed by including the 'ATP wash buffer' step in the purification procedure (see 'protein purification conditions' for details).
- This maybe the result of partial degradation of the GST-tagged protein. Try including protease inhibitor tablets in the 'Lysis buffer'.
- In order to minimize degradation try reducing the expression, lysis and purification times.
- Co-express and purify the GST-tagged protein with a molecular chaperone to increase stability and reduce degradation. For example: DnaK, DnaJ, GroEL, or GroES (the vectors for which and readily available).
- The procedure may require performing at 4°C to reduce degradation and stabilise the GST-tagged recombinant protein.
- Target the GST-tagged protein to the periplasm region of the *E.coli* cell during expression using a signal peptide sequence (for example pelB, OmpA, DsbA, TolB and MalE). This may aid folding and stability whilst reducing degradation. This will however reduce the expression levels considerably.

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## Glossary:

*affinity chromatography* - chromatographic separation based on a specific interaction between an immobilized ligand and a binding site on a macromolecule.

*chaperone protein* - protein which assists with the folding or stability of another.

*chaotropic agent* - a molecule which interferes with hydro-phobic interactions by disrupting the ordered structure of water molecules. Examples include urea and guanidine hydrochloride.

*chelating agent* - a compound such as EDTA or EGTA that is able to combine with a metal ion to form a structure with one or more rings.

*cleared lysate* - the soluble cell extract after the cell debris and other particulates have been removed by centrifugation.

*column bed volume (CV)* - the total volume occupied by the chromatographic packed bed. It is also referred to as the column volume or CV.

*DL-Dithiothreitol (DTT)* – reducing agent used to break disulphide bonds.

*expression vector* – a cloning vector intended for the foreign gene to be expressed in the host organism.

*french pressure cell* – a device that uses high shear forces to rupture microbial cells. The suspension is poured into a chamber, which is closed at one end by a needle valve and at the other end by a piston. Pressures of up to 16,000 lb/in<sup>2</sup> are applied by a hydraulic press against a closed needle valve. When the desired pressure is attained, the needle valve is fractionally opened to marginally relieve the pressure. The cells subsequently expand and rupture, thereby releasing the cellular components through the fractionally open valve.

*freeze-thawing* – a method that is sometimes used to break open cells by successive periods of slow freezing and thawing. Ice crystals are generated during the freezing stage, which disrupt the cells when they melt during thawing. The method, however, is slow and releases a limited amount of subcellular components.

Glutathione S-transferase (GST) - 26kDa protein whose DNA sequence is frequently integrated into expression vectors as a terminal tag for the production of recombinant proteins.

*immobilized* - bound to a surface, usually through covalent linkages.

*inclusion bodies* – quite a lot of proteins form insoluble crystalline aggregates known as inclusion bodies when they are expressed at high levels inside bacteria. The proteins can be solubilized using denaturants such as 8 M urea or 6 M guanidine hydrochloride.

*ion exchange chromatography* - chromatographic separation based on different charge properties of macromolecules.

*isoelectric point* - the pH at which the protein has no net charge.

*lysozyme* – an enzyme that hydrolyzes  $\beta$ -1,4-linkages between N-acetylmuramic acid and 2-acetamido-2-deoxy-D-glucose in peptidoglycan heteropolymers of prokaryotic cell walls. An example is egg white lysozyme and this enzyme is used to disrupt cells in order to liberate expressed proteins. 1 mg/ml lysozyme is normally added to *E.coli* cells in lysis buffer and incubated for 30 min to aid cell disruption. The pH optimum for lysozyme is pH 9.2 (Davies *et al* 1969).

*recombinant protein* – a protein coded for by a cloned gene which has often been modified to increase the expression of that protein or to alter the properties of the protein.

*sonication* – this technique uses ultrasonic energy to generate high transient pressures that are believed to disrupt the cells.

*tris(2-chloroethyl) phosphate (TCEP)* – strong irreversible reducing agent used to break disulphide bonds.

*truncate* - terminate prematurely or to shorten by cutting.

## Literature:

Affinity Separations: A Practical Approach 1997 (Matejtschuk, P Ed.) IRL PRESS at Oxford University Press. ISBN: 0-19-963550-1.

Biochemistry, 2nd Edition 1996 (Mathews, CK & van Holde, KE) Benjamin/Cummings Publ. Co. ISBN: 0-8053-3931-0.

Cameron, I. R *et al* (1989). *Trends in Biotechnology* 7, 66-70.

Cuatrecasas, P., Wilcheck, M. and Anfinsen, C.B. (1968) *Proc. Natl. Acad. Sci. U.S.A.* 61, 636.

Davies, A., Neuberger, A. and Wilson, B. (1969). *Biochim. Biophys. Acta* 178, 294.

Protein Purification Applications: A Practical Approach 1995 (E.L.V. Harris and S. Angal Eds.) IRL PRESS at Oxford University Press. ISBN: 0-19-963023-2.

Recombinant Protein Protocols 1997 (RS Tuan Ed.) Humana Press, ISBN: 0-89603-400-3.

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## Ordering information:

| Product                         | Volume | Order Code   |
|---------------------------------|--------|--------------|
| SuperGlu Agarose Resin (1 ml)   | 1ml    | SuperGlu1A   |
| SuperGlu Agarose Resin (10 ml)  | 10ml   | SuperGlu10A  |
| SuperGlu Agarose Resin (25 ml)  | 25 ml  | SuperGlu25A  |
| SuperGlu Agarose Resin (100 ml) | 100 ml | SuperGlu100A |

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## Technical support:

Contact the Protein Ark technical support and sales centre for assistance:

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- This product is for research use only and is not intended for use in clinical diagnosis. No claims beyond replacement of unacceptable material or refund of purchase price shall be allowed.