



All SH3 Domain Phager

**Rapid Screening of Human SH3 Ligands with a
Ready-to-use Phage Display Library**

Instruction Manual

1 Introduction	04
1.1 Phage Display	04
1.2 SH3 Domains	05
1.3 Limited Use License	05
2 Product Info	06
2.1 Library Content	06
2.2 Additional Material Required not Included in the Kit	06
2.3 Library Construction Information	06
2.4 Important Notes	06
2.5 References	06
3 SH3 Phage Display	06
Day 1	06
3.1 Production of Over Night Culture of TGI Cells on M9 Medium Plates	06
Day 2	06
3.2 Coating of Epoxy Dynabeads®	07
3.3 Production of Over Night Culture of TGI Cells in M9 Medium	07
Day 3	07
3.4 Production of TGI Cells in LB Medium for Phage Amplification	07
3.5 Blocking of Protein Coated Beads	07
3.6 Preparation of Phage Solution	07
3.7 Phage Adsorption	07
3.8 Phage Elution	08
3.9 Phage Titration and <i>E. coli</i> Transduction	08
Day 4	08
3.10 Plasmid Preparation and Sequencing	08
4 References	09
5 Appendix	10
5.1 Phagemid Map of pG8JH-SA-261	10
5.2 Cloning Site of pG8JH-SA-261	10
5.3 Media and Solutions	11
6 Contact	12

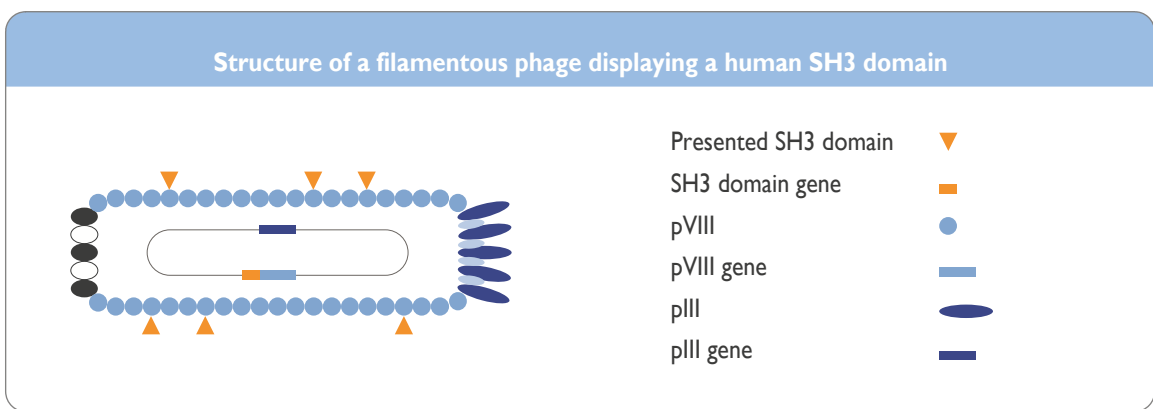
I. Introduction

I.1 Phage Display

Phage display is a powerful tool for the discovery of protein interaction partners from a variety of target molecules (Scott and Smith, 1990; Uchiyama *et al.*, 2005). This technology utilizes the ability to express foreign proteins on the outside of phage envelope as fusions to the phage coat protein, while the DNA encoding the fusion resides within the phage (Watters and Baker, 2004). Filamentous phages such as M13 have approximately five copies of the gene III coat protein on their surface; thus, a foreign DNA sequence inserted

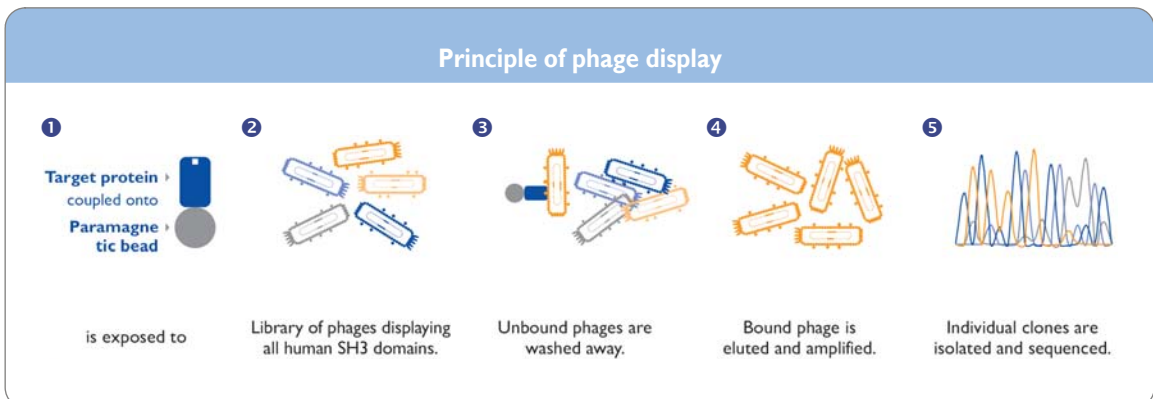
into this gene results in multiple copies of the fusion protein displayed by the phage. Similarly, the major coat protein encoded by the gene VIII can also display a foreign insert. The gene VIII allows up to 2700 copies of the insert per phage (Phizicky and Fields, 1995).

In contrast to virulent bacteriophages that complete their life cycle by lysing the bacterial cell, filamentous phages do not kill their host. Multiple copies of phages displaying a particular insert are secreted without breaking the cell envelope (Cesareni, 1992).



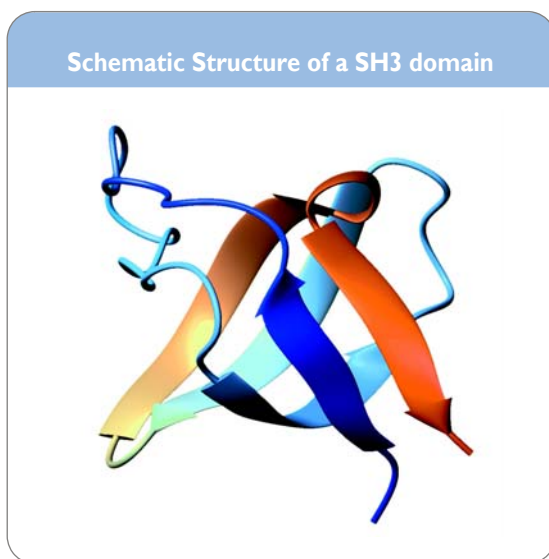
The ultimate aim of phage display is the selection of phages, which bind to the target molecule of interest with high affinity from large excess or phage clones that do not bind or do so with lower affinity (Smith *et al.*, 2005). This will be achieved, because phages that bind to an immobilized target molecule can be eluted and then amplified by infecting fresh bacterial host cells, so that each individual phage in the subpopulation is represented by millions of copies in the amplified stock

(Smith and Petrenko, 1997). This so called “panning” can be repeated several times until a population of best binders is enriched. The sequence of a binding peptide is determined by sequencing the part of the phage genome that encodes the peptide. Finally, the insert can be reproduced as a recombinant or synthetic peptide. By this methodology, specific and selective ligands to target molecules can be found (Koivunen *et al.*, 1999).



1.2 SH3 Domains

One of the central ambitions of molecular and cellular biologists is to identify how signals are formed and transduced within the cell. Intracellular signal transduction is mainly achieved through networks of protein-protein interactions, which are often mediated by peptide-binding modular domains. One such domain is the Src-homology 3 (SH3) domain, which is 50-70 amino acids long and was originally identified as a sequence present in the cytoplasmic tyrosine kinase c-Src (Yang *et al.*, 1994). The common structural motif of SH3 domains consists of five antiparallel β -strands and a short 3_{10} -helix. The β -strands form two β -sheets that are almost perpendicular to each other in a sandwich structure (Robertsson, *et al.*, 2005).



The target proteins of SH3 domains are bound by recognizing proline-rich ligands that usually contain a short general consensus sequence Pro-Xaa-Xaa-Pro (PXXP) (Ferraro, *et al.*, 2005; Rickles *et al.*, 1995; Winters and Pryciak, 2005). The PXXP motif is about ten amino acids long and forms a left-handed canonical type II polyproline helix (Bauer *et al.*, 2007). The majority of SH3 ligands were departed into two classes according to their binding orientation: N-terminal to C-terminal (class I) or C-terminal to N-terminal (class II) (Hou, *et al.*, 2006). The orientation of the peptide is dictated by the location of a positively charged residue, relative to the PXXP core, which forms a salt bridge with an acidic residue in the SH3 domain (Kay, *et al.*, 2000). Secondary, these residues such as arginine and lysine provide additional binding energy through electrostatic interactions with residues in the specificity pocket and are responsible for the binding specificity of a specific SH3 domain (Cheadle, *et al.*, 1994; Li, 2005).

SH3 domains participate in the regulation of cell growth and differentiation, they are involved in pathogenesis of diseases such as cancer, and microbial pathogens, such as human immunodeficiency virus (HIV), also exploit cellular SH3-mediated processes (Kärkkäinen, *et al.*, 2006). Although SH3 domains have been the focus of numerous studies since their discovery in the year 1988, our understanding of the mechanisms of ligand recognition and signaling by this family of PRD (proline recognition domain) is far from complete (Mayer, *et al.*, 1988; Li, 2005).

With this present phage display library we provide a powerful and unbiased system for simultaneous assaying of the complete human SH3 proteome for the strongest binding to target proteins of interest, without the limitations posed by short linear peptide ligands or confounding variables of more indirect methods for protein interaction screening (Kärkkäinen, *et al.*, 2006).

1.3 Limited Use License

With the purchase of the **All SH3 Domain Phager** ("Product") the buyer acquires the non-transferable right to use the purchased amount and components of the product for internal research conducted by the buyer only. The buyer cannot use this Product or components of this Product or peptides and proteins discovered with this Product for commercial purposes. Commercial purposes means any activity performed by a party for consideration and may include, but is not limited to (1) use of the Product or its components to provide a service, information or data, (2) resale of the Product or its components, whether or not such Product or components are resold for research only, (3) use or incorporation of a specific SH3 domain or other peptide, protein and DNA sequence coding for any such protein or peptide identified with or derived from the Product into a product that will be commercialized for uses as a therapeutic, *in vitro* or *in vivo* diagnostic, imaging, purification, separation, or industrial enzyme products, or for any other commercial purposes.

For clarification, the buyer of the Product cannot use any peptide, protein and DNA sequence coding for any such protein identified with the Product for any commercial purpose.

For information on purchasing a license to use this Product or peptides, proteins identified with this Product for commercial purposes please contact:

GENEART AG
Business Development
Im Gewerbepark B35
93059 Regensburg
Germany
Phone: +49 (0)941 942 76 - 0

2. Product Info

2.1 Library Content

- Quantity: 400 µl in 2x YT-medium supplemented with Ampicilin and Kanamycin
- Titer: >10¹¹ pfu/ml

Package size is sufficient for up to 4 primary library pannings.

Store all components at -20°C.

2.2 Additional Material Required not Included in the Kit

- Dynabeads® M-270 Epoxy (Invitrogen, catalog number 143-01)
- Magnetic bead separator
- *E. coli* TGI cells (Stratagene, catalog number 200123)

2.3 Library Construction Information

The library contains more than 300 different phages (refer to the list supplied) carrying the open reading frame for 305 individual human SH3 domains, optimized for expression in *E. coli*. The reading frames are fused to M13 surface protein p8 for multivalent display. Subcloning of the inserts was performed via BssHII and NotI in phagemid pG8JH, a derivative of pG8H6 (Jacobsson and Frykberg, 1996).

For library production, *E. coli* TGI cells were transformed individually with 305 distinct phagemid constructs. Single colonies of each transformation were pooled and grown together in liquid culture. After infection with helper phage, amplification of the SH3 domain phages was conducted for five hours in order to minimize the variation in phage distribution due to

different growth and production rates among the clones. Subsequently, the phage supernatant was filter sterilized and titrated.

Quality control

Sequencing of ten randomly selected clones from a control infection of TGI cells yielded ten different clones (P-DLG (16), Nck2 #1 (55), Homology to FISH (87), FISH #3 (88), Homology to FISH (94), Intersectin 1 #2 (113), Intersectin 2 #4 (145), CIN85 #1 (165), Homology to PRAX1 #3 (201), Dbs (287)) indicating an approximately equal distribution.

Please note that reamplification of supplied library to propagate phages results in loss of library diversity and utility.

2.4 Important Notes

The product is not to be used for human diagnostic or drug purposes or to be administered to humans. All due care and attention should be exercised in the handling of many of the materials described in this text.

2.5 References

Kesti, T., Ruppelt, A., Wang, J.-H., Liss, M., Wagner, R., Taskén, K. and Saksela, K. 2007. Reciprocal Regulation of SH3 and SH2 Domain Binding via Tyrosine Phosphorylation of a Common Site in CD3ε. *J Immunol* 179: 878-885

Kärkkäinen, S., Hiipakka, M., Wang, J.-H., Kleino, I., Vähä-Jaakkola, M., Renkema, G.H., Liss, M., Wagner, R. & Saksela, K. 2006. Identification of preferred protein interactions by phage-display of the human Src homology-3 proteome. *EMBO R* 7 (2): 186-191

3. SH3 Phage Display

Please note that the Manual is structured in day stages because every day stage ends with an over night incubation. So it is easier for you to plan the phage display. Recipes of buffers, solutions and reagents are described in the Appendix.

Day I

3.1 Production of Over Night Culture of TGI Cells on M9 Medium Plates

Material and reagents required

- M9 medium plates
- *E. coli* TGI cells (Stratagene, catalog number 200123)

Protocol

- Streak out the *E. coli* TGI cells on M9 plates and incubate at 37°C over night.

Day 2

3.2 Coating of Epoxy Dynabeads®

Material and reagents required

- Dynabeads® M-270 Epoxy (Invitrogen, catalog number 143.01 or 143.02)
- DMSO
- Sodium phosphate buffer (0.1 M, pH 7.4)
- Ammonium sulfate solution (3 M)
- Target protein, 10 µg
- Protein for negative control (We recommend to use GST if panning against a GST-tagged protein of interest.), 10 µg
- Magnetic bead separator

Protocol

- To make it easy to withdraw samples from the vial prepare bead suspension in DMSO as described in Invitrogen product manual and store at 2-8°C. Add 2 ml DMSO to the 143.01-vial or 10 ml to the 143.02-vial.
- Thaw the Epoxy Dynabeads® solution and resuspend the beads well by vortexing for 1-2 minutes.
- For every protein you wish to analyze transfer 100 µl beads to a single microcentrifuge tube and place the tube in the magnetic bead separator for 4 minutes.
- Pipette off and discard the supernatant, leaving beads undisturbed.
- Wash beads 3 times as follows □ □ □:
 - Resuspend the beads in 100 µl sodium phosphate buffer.
 - Vortex to mix properly.
 - Place in the magnetic bead separator for 2 min, and carefully remove and discard the supernatant.
- Dilute 10 µg of every target protein in 100 µl sodium phosphate buffer.
- Add protein solution to the washed beads and vortex.
- Add 50 µl ammonium sulfate solution and vortex.
- Incubate at 4°C over night with slow tilt rotation. Do not let the beads settle during the incubation period.

3.3 Production of Over Night Culture of TGI Cells in M9 Medium

Material and reagents required

- M9 plus medium
- M9 Plate with over night culture of *E. coli* TGI cells (Day1, Point 3.1)

Protocol

- Inoculate 5 ml M9 plus medium with a single colony and shake at 37°C over night.

Day 3

3.4 Production of TGI Cells in LB Medium for Phage Amplification

Material and reagents required

- Over night culture of TGI cells in M9 medium
- LB medium

Protocol

- Inoculate 50 ml LB medium with the equivalent volume of $OD_{600} = 0.05$ of the over night culture.
- Shake at 37°C at 220 rpm until OD_{600} is between 0.4 and 0.5 (approx. time between 1.5 and 3 hours).
- Use fresh or store on ice for max. 24 h (storage leads to decrease of transduction efficiency).

3.5 Blocking of Protein Coated Beads

Material and reagents required

- Epoxy Dynabeads®, once coated with target protein and once coated with negative-control protein (e.g. GST)
- Blocking buffer
- PBS (pH 7.4)

Protocol

- Place the tube containing protein/beads on a magnetic separator for 4 minutes and carefully remove the supernatant.
- Add 100 µl blocking buffer and shake gently at room temperature for 1 h.
- (We recommend using this incubation time to prepare the phages; see 3.6).
- Place the tube in a magnetic separator and remove the supernatant.
- Wash beads 3 times as follows □ □ □:
 - Resuspend the beads in 1 ml PBS and vortex.
 - Place the tube in a magnetic separator and remove the supernatant.

3.6 Preparation of Phage Solution

Material and reagents required

- All SH3 Domain Phager
- Blocking buffer

Protocol

- Dilute 100 µl SH3 Phage Display Library (6×10^{10} pfu/ml) in 100 µl blocking buffer.
- Incubate at room temperature for 15 min.

3.7 Phage Adsorption

Material and reagents required

- Phage solution (200 µl)
- Washed beads with coupled protein
- Washing buffer

Protocol

- Add 200 µl of phage solution to the washed beads, vortex briefly and incubate at room temperature for 1 h with gentle mixing.
- Place the tube in a magnetic separator, remove and discard the supernatant.
- Wash the beads 10 times as follows □ □ □ □ □ □ □ □ □ □:
 - Resuspend the beads in 1 ml washing buffer.
 - Place the tube in a magnetic separator and remove the supernatant.

Note: The Tween20 concentration can be varied to obtain optimal results. Higher concentrations will reduce background binding but may also reduce the number of specific binders depending on the affinity of the SH3 domain to the protein of interest. We recommend to start with a concentration of 0.05 % (v/v) and to increase the concentration in steps of 0.05 if very large numbers of different clones are obtained.

3.8 Phage Elution

Material and reagents required

- Elution buffer
- Tris (1 M, pH 9.0)
- pH paper
- Washed beads

Protocol

- Add 100 µl elution buffer to the beads, vortex briefly and incubate at room temperature for 10 min with gentle mixing.
- Place the tube in the magnetic bead separator and transfer the supernatant into a new tube. Discard the beads.
- Neutralize the supernatant by adding 30 µl Tris. Test pH by pipetting 1 µl of the solution to pH paper. If necessary, add more aliquots of 10 µl Tris until the solution is neutral.

3.9 Phage Titration and E. coli Transduction

Material and reagents required

- Phage eluate (target protein and negative control)
- LB medium
- Over night culture of TGI cells
- SOBAG medium plates

Protocol

- Create a dilution series from the phage eluates.

It is not easy to predict the optimal dilution range as this depends on the affinity of the SH3 domains to your protein of interest and the conditions under which the experiment was performed. As a starting range we recommend dilutions ranging from undiluted to 1:1,000 in steps of 10. Prepare the dilutions subsequently by mixing 10 µl of the eluate and preceding dilutions with 90 µl LB medium.

- Add 500 µl TGI cells for every dilution to a single microcentrifuge tube.
- Shake at 220 rpm at 37°C for 10 min.
- Add 5 µl of the respective diluted phages and vortex briefly.
- Incubate at 37°C for 30 min without shaking.
- Plate 100 µl of the suspension onto a SOBAG plate.
- Incubate the plates in an inverted position at 37°C over night.

Day 4

3.10 Plasmid Preparation and Sequencing

- To compare the obtained values count the colonies and calculate the titer of the eluate as follows:

$$\text{number of colonies} \times \text{dilution factor} \times 1000 = \text{titer in pfu/ml}$$

Ideally, a factor of 100 or more between the eluate from your protein of interest and the negative control should be obtained. However, depending on your protein and the experimental conditions this difference may not be that clear. Therefore, it is recommended to proceed with the identification of the clones in any case.

- To identify the SH3 domains which were selected during the panning round, isolate the phagemids from the obtained colonies by performing mini preps.
- Sequence the DNA using primer J-55 (forward) or H-301 (reverse) (see map below) and identify the clones by comparison with the list of protein sequences supplied.

It is difficult to predict the number of colonies to be analyzed as this depends mainly on the protein of interest. Ideally, if only one SH3 domain binds to the protein, all sequenced colonies should contain the same phagemid. However, unspecific binding usually occurs to a certain degree and there may be different SH3 domains that bind to the protein. We recommend to start by sequencing 10 clones and depending on the results to increase the number as necessary. Colonies from the negative control (e.g. GST when panning against a GST tagged protein of interest) should also be sequenced to rule out false-positive results due to unspecific binding of certain SH3 domains to the negative control protein or the magnetic beads.

4. References

- Bauer, F., Schweimer, K., Meiselbach, H., Hoffmann, S., Rösch, P. and Sticht, H. (2007) Structural characterization of Lyn-SH3 domain in complex with a herpesviral protein reveals an extended recognition motif that enhances binding affinity. *Prot Sci.* 14, 2487-2498
- Cesareni, G. (1992) Peptide display on filamentous phage capsids. *FEBS.* 307 (1), 66-70
- Cheadle, C., Ivashchenko, Y., South, V., Searfoss, G.H., French, S., Howk, R., Ricca, G.A. and Jaye, M. (1994) Identification of a Src SH3 domain binding motif by screening a random phage display library. *JBC.* 269 (39), 24034-24039
- Ferraro, E., Via, A., Ausiello, G. and Helmer-Gitterich, M. (2005) A neural strategy for the interference of SH3 domain-peptide interaction specificity. *BMC Bioinformatics.* 6 (Suppl 4), S13
- Hou, T., Chen, K., McLaughlin, W.A., Lu, B. and Wang, W. (2006) Computational analysis and prediction of the binding motif and protein interacting partners of the Abl SH3 domain. *PLoS Comput Biol.* 2 (1), 46-55
- Jacobsson, K. and Frykberg, L. (1996) Phage display shot-gun cloning of ligand-binding domains of prokaryotic receptors approaches 100% correct clones. *Biotechniques.* 20, 1070-1076
- Kärkkäinen, S., Hiipakka, M., Wang, J., Kleino, I., Vähä-Jaakkola, M., Renkema, G.H., Liss, M., Wagner, R. and Saksela, K. (2006) Identification of preferred protein interactions by phage display of the human Src homology-3 proteome. *EMBO R.* 7 (2), 186-191
- Kay, B.K., Williamson, M.P. and Sudol, M. (2000) The importance of being proline: the interaction of proline-rich motifs in signaling proteins with their cognate domains. *FASEB.* 14, 231-241
- Koivunen, E., Arap, W., Rajotte, D., Lahdenranta, J. and Pasqualini, R. (1999) Identification of receptor ligands with phage display peptide libraries. *J Nucl Med.* 40, 883-888
- Li, S.S. (2005) Specificity and versatility of SH3 and other proline-recognition domains: structural basis and implications for cellular signal transduction. *Biochem J.* 390, 641-653
- Mayer, B.J. (2001) SH3 domains: complexity in moderation. *J Cell Sci.* 114 (7), 1253-1263
- Mayer, B.J., Hamaguchi, M. and Hanafusa, H. (1988) A novel viral oncogene with structural similarity to phospholipase C. *Nature (London).* 332, 272-275
- Phizicky, E.M. and Fields, S. (1995) Protein-protein interactions: Methods for detection and analysis. *Microbiol Rev.* 59 (1), 94-123
- Rickles, R.J., Botfield, M.C., Zhou, X., Henry, P.A., Brugge, J.S. and Zoller, M.J. (1995) Phage display selection of ligand residues important for Src homology 3 domain binding specificity. *PNAS.* 92, 10909-10913
- Robertsson, J., Petzold, K., Löfvenberg, L. and Backman, L. (2005) Folding of spectrin's SH3 domain in the presence of spectrin repeats. *Cell Mol Biol Lett.* 10, 595-612
- Scott, J.K. and Smith, G.P. (1990) Searching for peptide ligands with an epitope library. *Science.* 249, 386-390
- Smith, G.P. and Petrenko, V.A. (1997) Phage display. *Cem Rev.* 97, 391-410
- Smith, J., Kontermann, R.E., Embleton, J. and Kumar, S. (2005) Antibody phage display technologies with special reference to angiogenesis. *FASEB* 19, 331-341
- Uchiyama, F., Tanaka, Y., Minari, Y. and Tokui, N. (2005) Designing Scaffolds of peptides for phage display libraries. *J Biosci Bioeng.* 99 (5), 448-456
- Watters, A.L. and Baker, D. (2004) Searching for folded proteins *in vitro* and *in silico*. *Eur J Biochem.* 271, 1615-1622
- Winters, M.J. and Pryciak, P.M. (2005) Interaction with the SH3 domain protein Bem1 regulates signaling by the *Saccharomyces cerevisiae* p21-activated kinase Ste20. *Mol Cell Biol.* 25 (6), 2177-2190
- Yang, Y.S., Garbay, C., Duchesne, M., Cornille, F., Julian, N., Fromage, N., Tocque, B. and Roques, B.P. (1994) Solution structure of GAP SH3 domain by ¹H NMR and spatial arrangement of essential Ras signaling-involved sequence. *EMBO J.* 13 (6), 1270-1279

5.3 Media, Buffer and Solutions

Name	Ingredients	Amount
Sodium phosphate buffer (0.1 M)	NaH ₂ PO ₄ • H ₂ O Na ₂ HPO ₄ • 2 H ₂ O	2.62 g/l 14.42 g/l pH 7.4
Ammonium sulfate solution	Ammonium sulfate	39.6 g Dissolve in 0.1 M sodium phosphate buffer (pH 7.4)
Blocking buffer (Prepare fresh before use)	BSA	5% (w/v) in washing buffer
Washing buffer	PBS with added Tween20	Depending on target protein between 0.05 and 0.5% (v/v)
PBS	NaH ₂ PO ₄ • H ₂ O Na ₂ HPO ₄ • 2 H ₂ O NaCl	0.26 g/l 1.44 g/l 8.78 g/l pH 7.4
Elution buffer	Glycine	200 mM pH 2.2
Tris		1 M pH 9.0
SOBAG Medium	Tryptone Yeast extract NaCl MgCl ₂ Glucose Ampicillin Agar for plates	20 g/l 5 g/l 0.5 g/l 2.5 mM 100 mM 100 µg/l 15 g/l
5x M9 salt solution (autoclaved)	Na ₂ HPO ₄ • 7 H ₂ O KH ₂ PO ₄ NaCl NH ₄ Cl	64 g/l 15 g/l 2.5 g/l 5.0 g/l
M9 medium	ddH ₂ O (sterile) 5x M9 salt solution 1 M MgSO ₄ (autoclaved) 1 M CaCl ₂ (autoclaved) 20% (w/v) Glucose (sterile) 1% Thiamine (sterile) Agar for plates	750 ml 200 ml 2 ml 0.1 ml 20 ml 10 ml 15 g/l
M9 plus medium	M9 medium 20% (w/v) Glucose	980 ml 20 ml
LB medium	Tryptone Yeast extract NaCl	10 g/l 5 g/l 10 g/l <i>Adjust to pH 7.0 with NaOH</i>

Europe

GENEART AG
Josef-Engert-Str. 11
93053 Regensburg
Germany
SH3-domain@geneart.com
Phone: +49 (0)941 94 27 6-0
Fax: +49 (0)941 94 27 6-711

North America

GENEART, Inc.
1243 Islington Ave, Suite 705
Toronto, ON, M8X 1Y9
Canada
SH3-domain@geneart.com
Phone: +1 (416) 233-0666
Fax: +1 (416) 233-0854

West America

GENEART, Inc.
1350 Old Bayshore Highway, Suite 480
Burlingame, CA 94010
USA
SH3-domain@geneart.com
Phone: +1 (650) 343-4363
Fax: +1 (650) 343-4355