TAGZyme™ Handbook

For
Exoproteolytic cleavage of N-terminal His tags

March 2003

QIAGEN

QIAGEN Worldwide

QIAGEN Companies

Australia	**QIAGEN Pty Ltd** ABN 75 072 382 944	PO Box 25 • Clifton Hill • Victoria 3068 Orders 03-9489-3666 • Fax 03-9489-3888 • Technical 1-800-243-066
Canada	**QIAGEN Inc.**	2800 Argentia Road • Unit 7 • Mississauga • Ontario • L5N 8L2 Orders 800-572-9613 • Fax 800-713-5951 • Technical 800-DNA-PREP (800-362-7737)
France	**QIAGEN S.A.**	3 avenue du Canada • LP 809 • 91974 COURTABOEUF CEDEX Orders 01-60-920-920 • Fax 01-60-920-925 • Technical 01-60-920-930
Germany	**QIAGEN GmbH**	QIAGEN Strasse 1 • 40724 Hilden Orders 02103-29-12000 • Fax 02103-29-22000 • Technical 02103-29-12400
Italy	**QIAGEN S.p.A.**	Via Grosio, 10/10 • 20151 Milano Orders 02-33430411 • Fax 02-33430426 • Technical 02-33430414
Japan www.qiagen.co.jp	**QIAGEN K.K.**	Forefront Tower II • 13-1, Kachidoki 3 Chome • Chuo-ku, Tokyo 104-0054 Telephone 03-5547-0811 • Fax 03-5547-0818 • Technical 03-5547-0811
Switzerland	**QIAGEN AG**	Auf dem Wolf 39 • 4052 Basel Orders 061-319-30-30 • Fax 061-319-30-33 • Technical 061-319-30-31
UK and Ireland	**QIAGEN Ltd.**	Boundary Court • Gatwick Road • Crawley • West Sussex, RH10 9AX Orders 01293-422-911 • Fax 01293-422-922 • Technical 01293-422-999
USA	**QIAGEN Inc.**	28159 Avenue Stanford • Valencia • CA 91355 Orders 800-426-8157 • Fax 800-718-2056 • Technical 800-DNA-PREP (800-362-7737)

www.qiagen.com

QIAGEN Distributors

Please see the last page for contact information for your local QIAGEN distributor.

Contents

Kit Contents **5**

Storage and Stability **6**

Safety Information **6**

Product Use Limitations **7**

Product Warranty and Satisfaction Guarantee **7**

Technical Assistance **7**

Introduction **8**

The TAGZyme Principle **9**

Proteins with intrinsic DAPase stop points 9

Proteins without intrinsic DAPase stop points 10

Applications for the TAGZyme System **12**

TAGZyme pQE Vectors **13**

QIA*express* pQE vectors 13

Regulation of expression – pREP4 plasmid 14

E. coli host strains 15

The Cloning Procedure **17**

Cleavage Protocols **20**

Notes Before Starting **20**

His tag design 20

Expression, Ni-NTA purification, and desalting of His-tagged proteins 20

Buffers and enzyme activity 21

DAPase Enzyme 21

Qcyclase Enzyme 21

pGAPase Enzyme 22

Cysteamine activation 22

Stepwise Use of the TAGZyme System **22**

Analysis during the TAGZyme procedure 23

Cleavage of N-terminal 6xHis tags using DAPase Enzyme **25**

Buffer preparation 25

Desalting 25

Protocol 1. DAPase digestion (small-scale pilot experiment) for proteins containing intrinsic DAPase stop points **26**

Protocol 2. Preparative DAPase digestion for proteins containing intrinsic DAPase stop points 28
Protocol 3. Removal of DAPase Enzyme by subtractive IMAC 29
Cleavage of N-terminal His tags using DAPase, Qcyclase, and pGAPase Enzymes 30
Buffer preparation 30
Desalting 30
Protocol 4. DAPase – Qcyclase digestion (small-scale pilot experiment) 31
Protocol 5. Preparative DAPase – Qcyclase digestion 33
Protocol 6. Removal of DAPase and Qcyclase Enzymes by subtractive IMAC 34
Protocol 7. pGAPase Digestion 35
Protocol 8. Preparative pyroglutamate removal using pGAPase Enzyme 37
Protocol 9. Removal of pGAPase Enzyme by subtractive IMAC 38
Troubleshooting Guide 39
Appendix 41
Enzyme specifications 41
Solutions required 44
Use of blunt-end–cutting restriction enzymes to introduce various amino acids at the N-terminus of the mature protein 44
Recommended PCR cloning strategy for use of the vector pQE-TriSystem in combination with TAGZyme cleavage 48
His tags suitable for exoproteolytic cleavage by the TAGZyme system 50
Small-scale subtractive IMAC 51
Recommendations for subtractive IMAC scale-up 51
References 52
Ordering Information 54
QIAGEN Distributors 55

Kit Contents

TAGZyme™ Kit — for processing of approx. 10 mg of tagged protein **Catalog No.**	**34300**
DAPase™ Enzyme (10 U/ml)	0.5 units (50 µl)
Qcyclase™ Enzyme (50 U/ml)	30 units (600 µl)
pGAPase™ Enzyme (25 U/ml)	10 units (400 µl)
Cysteamine·HCl (20 mM)	1000 µl
Ni-NTA Agarose	10 ml
Disposable columns (empty)	20
Handbook	1
Product sheets DAPase Enzyme and Qcyclase/pGAPase Enzymes	each 1

TAGZyme pQE Vector Set **Catalog No.**	**32932**
Vectors TAGZyme pQE-1 and -2	each 25 µg
Handbook	1

TAGZyme DAPase Enzyme (2.5 U) — for processing of approx. 50 mg of tagged protein **Catalog No.**	**34362**
DAPase Enzyme (10 U/ml)	2.5 units (250 µl)
Cysteamine·HCl (20 mM)	1000 µl
Handbook	1
Product sheet DAPase Enzyme	1

TAGZyme Qcyclase/pGAPase Enzymes (150 U/50 U) — for processing of approx. 50 mg of tagged protein **Catalog No.**	**34342**
Qcyclase Enzyme (50 U/ml)	150 units (5 x 600 µl)
pGAPase Enzyme (25 U/ml)	50 units (5 x 400 µl)
Handbook	1
Product sheet Qcyclase/pGAPase Enzymes	1

TAGZyme DAPase Enzyme (50 U)* — for processing of approx. 1 g of tagged protein	
Catalog No.	**34366**
DAPase Enzyme (10 U/ml)	50 units (5 ml)
Cysteamine·HCl (20 mM)	25 ml
Handbook	1
Product sheet DAPase Enzyme	1

TAGZyme Qcyclase/pGAPase Enzymes (3000 U/1000 U)* — for processing of approx. 1 g of tagged protein	
Catalog No.	**34346**
Qcyclase Enzyme (50 U/ml)	3000 units (5 x 12.5 ml)
pGAPase Enzyme (25 U/ml)	1000 units (5 x 8 ml)
Handbook	1
Product sheet Qcyclase/pGAPase Enzymes	1

* *Bulk enzyme quantities are customized products; delivery may take up to 6 weeks. Enzymes are also available in GMP-grade. Please inquire.*

Storage and Stability

DAPase™, Qcyclase™, and pGAPase™ Enzymes, Cysteamine·HCl, and vectors TAGZyme pQE-1 and -2 should be stored at –20°C.

Ni-NTA Agarose should be stored at 2–8°C. Ni-NTA matrices should not be frozen.

Disposable columns can be stored at room temperature.

See Appendix for enzyme stability information.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs). These are available online in convenient and compact PDF format at www.qiagen.com/ts/msds.asp where you can find, view, and print the MSDS for each QIAGEN kit and kit component.

Ni-NTA Agarose

Contains nickel-nitrilotriacetic acid. Harmful, sensitizer, and flammable. Risk and safety phrases*: R10-22-40-42/43. S13-26-36-46.

* *R10: Flammable. R22: Harmful if swallowed. R40: Possible risks of irreversible effects. R42/43: May cause sensitization by inhalation and skin contact. S13: Keep away from food, drink, and animal feedingstuffs. S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. S36: Wear suitable protective clothing. S46: If swallowed, seek medical advice immediately and show the container or label.*

Product Use Limitations

The TAGZyme™ System is developed, designed, and sold for research purposes only. It is not to be used for human diagnostic or drug purposes or to be administered to humans unless expressly cleared for that purpose by the Food and Drug Administration in the USA or the appropriate regulatory authorities in the country of use. All due care and attention should be exercised in the handling of many of the materials described in this text.

Product Warranty and Satisfaction Guarantee

QIAGEN guarantees the performance of all products in the manner described in our product literature. The purchaser must determine the suitability of the product for its particular use. Should any product fail to perform satisfactorily due to any reason other than misuse, QIAGEN will replace it free of charge or refund the purchase price. We reserve the right to change, alter, or modify any product to enhance its performance and design. If a QIAGEN product does not meet your expectations, simply call your local Technical Service Department or distributor. We will credit your account or exchange the product — as you wish.

A copy of QIAGEN terms and conditions can be obtained on request, and is also provided on the back of our invoices. If you have questions about product specifications or performance, please call QIAGEN Technical Services or your local distributor (see inside front cover).

Technical Assistance

At QIAGEN we pride ourselves on the quality and availability of our technical support. Our Technical Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in molecular biology and the use of QIAGEN products. If you have any questions or experience any difficulties regarding the TAGZyme System or QIAGEN products in general, please do not hesitate to contact us.

QIAGEN customers are a major source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at QIAGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

For technical assistance and more information please call one of the QIAGEN Technical Service Departments or local distributors (see inside front cover).

Introduction

The TAGZyme System is an efficient and specific solution for the complete removal of small N-terminal His tags and other amino acid tags by the use of exopeptidases. The method is based on the use of dipeptidyl aminopeptidase I (DAPase Enzyme) alone or in combination with glutamine cyclotransferase (Qcyclase Enzyme) and pyroglutamyl aminopeptidase (pGAPase Enzyme). These recombinant enzymes contain a C-terminal His tag and can therefore be bound to Ni-NTA matrices. This allows their removal from the reaction solution by Immobilized-Metal Affinity Chromatography (IMAC). This feature has been utilized in the design of a simple process consisting of aminopeptidase cleavage followed by subtractive IMAC. The system can form the backbone of a combined TAGZyme–IMAC strategy for the efficient production of highly purified and homogeneous recombinant proteins (see Figure 1). As the TAGZyme enzymes carry a His tag at their C-terminus, the tag is not digested by the N-terminal exopeptidase DAPase Enzyme.

The use of exopeptidases in the TAGZyme System eliminates the risk of endoproteolytic cleavage within the body of the protein, and the rapid rate of reaction means that sensitive proteins can be processed even at 4°C in a matter of hours.

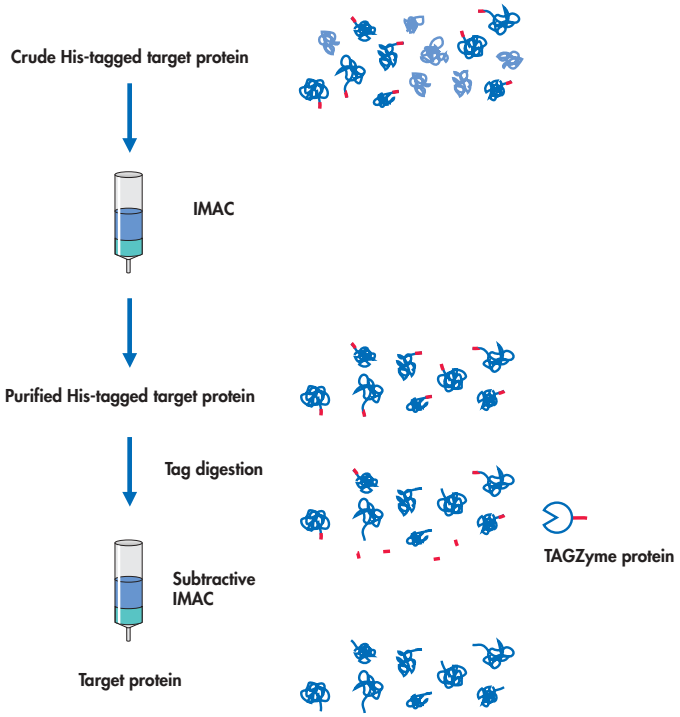


Figure 1. *Schematic summary of the TAGZyme procedure.*

The TAGZyme Principle

The TAGZyme System makes use of a highly specific and processive exoproteolytic cleavage of N-terminal amino acids for the removal of small affinity tags from proteins (e.g., 6xHis tags). After completion of the digestion, the exoproteolytic enzymes — which carry His tags at the C-terminus — are removed by subtractive IMAC. The efficiency of the total procedure is supplemented by the use of a series of *E. coli* expression vectors, TAGZyme pQE-1 and -2. These expression vectors combine the high-level expression of 6xHis-tagged proteins delivered by the pQE vector series with specially designed His tag-coding and multiple cloning site sequences that allow complete and convenient removal of N-terminal His tags. The two vectors have been optimized for use with the TAGZyme System. Occasionally, problems may be encountered when processing other tags. For an overview of the suitability of other tags for TAGZyme processing, please consult Table 2 (page 17). N-terminal amino acids are cleaved off as dipeptides by dipeptidyl aminopeptidase I (DAPase Enzyme, Figure 2A). After removal of the first N-terminal dipeptide the enzyme progressively cleaves off dipeptides until a stop point is encountered. Stop points are certain amino acids in defined positions within a dipeptide (see Table 1). As the His tags of TAGZyme enzymes appear at the C-terminus they are not subject to digestion.

Table 1. DAPase stop points

Amino acid	DAPase stop point (↓) sequence*
Lysine (Lys, K)	Xaa-Xaa…Xaa-Xaa ↓ Lys-Xaa Xaa-Xaa…
Arginine (Arg, R)	Xaa-Xaa…Xaa-Xaa ↓ Arg-Xaa Xaa-Xaa…
Proline (Pro, P)	Xaa-Xaa…Xaa-Xaa ↓ Xaa-Xaa Pro-Xaa…
Proline (Pro, P)	Xaa-Xaa…Xaa-Xaa ↓ Xaa-Pro Xaa-Xaa…
Glutamine (Gln, Q)	Xaa-Xaa…Xaa-Xaa ↓ Gln†-Xaa Xaa-Xaa…

* *Natural DAPase stop points (↓) are the following amino acids in the given position within a dipeptide (dipeptides that are cleaved off are underlined).*

† *In the presence of excess Qcyclase Enzyme.*

Proteins with intrinsic DAPase stop points

A range of mature proteins have natural DAPase Enzyme stop points (Table 1) causing the enzyme to release its substrate, i.e., the detagged mature protein, as soon as this amino acid occurs in the N-terminal position. Accordingly, N-terminal His tags containing an even number of amino acid residues can be completely and specifically removed by treatment with DAPase Enzyme alone (Figure 2A). After digestion is complete, the C-terminally His-tagged DAPase Enzyme, residual undigested target protein, and copurified contaminants are removed by subtractive IMAC . The entire process is typically completed within 45 minutes.

Proteins without intrinsic DAPase stop points

cDNA coding for proteins that do not contain a natural stop point can be cloned into a TAGZyme pQE vector in such a way that a glutamine (Gln, Q) residue is introduced into the position between the last cleavable dipeptide and the first authentic amino acid of the target protein. DAPase cleavage is then performed in the presence of a second enzyme, glutamine cyclotransferase (Qcyclase Enzyme), which catalyzes the cyclization of an N-terminal glutamine residue to pyroglutamate (Figure 2B). Presence of excess Qcyclase Enzyme ensures immediate cyclization of the inserted glutamine residue when the His tag has been digested and the glutamine appears in the N-terminal position. A protein possessing an N-terminal pyroglutamate residue cannot serve as a substrate for DAPase Enzyme and is thus protected against further DAPase digestion. Following the coincubation, both enzymes are removed from the reaction solution by subtractive Ni-NTA IMAC. The target protein is obtained by cleaving off the N-terminal pyroglutamyl residue through the action of a third enzyme, pyroglutamyl aminopeptidase (pGAPase Enzyme) (Figures 2B). This process is typically completed within 120 minutes. A glutamine DAPase stop point can be introduced by cloning the protein coding sequence into the expression vector TAGZyme pQE-2. Here, any uneven amino acid position can be chosen for the glutamine residue, and the first amino acid of the target protein must immediately follow. Alternatively, TAGZyme pQE-1 is the vector of choice whenever the sequence of the protein allows cloning into the blunt-ended *Pvu*II restriction site, which links the first amino acid of the target protein to the glutamine stop point (Figure 4, page 16).

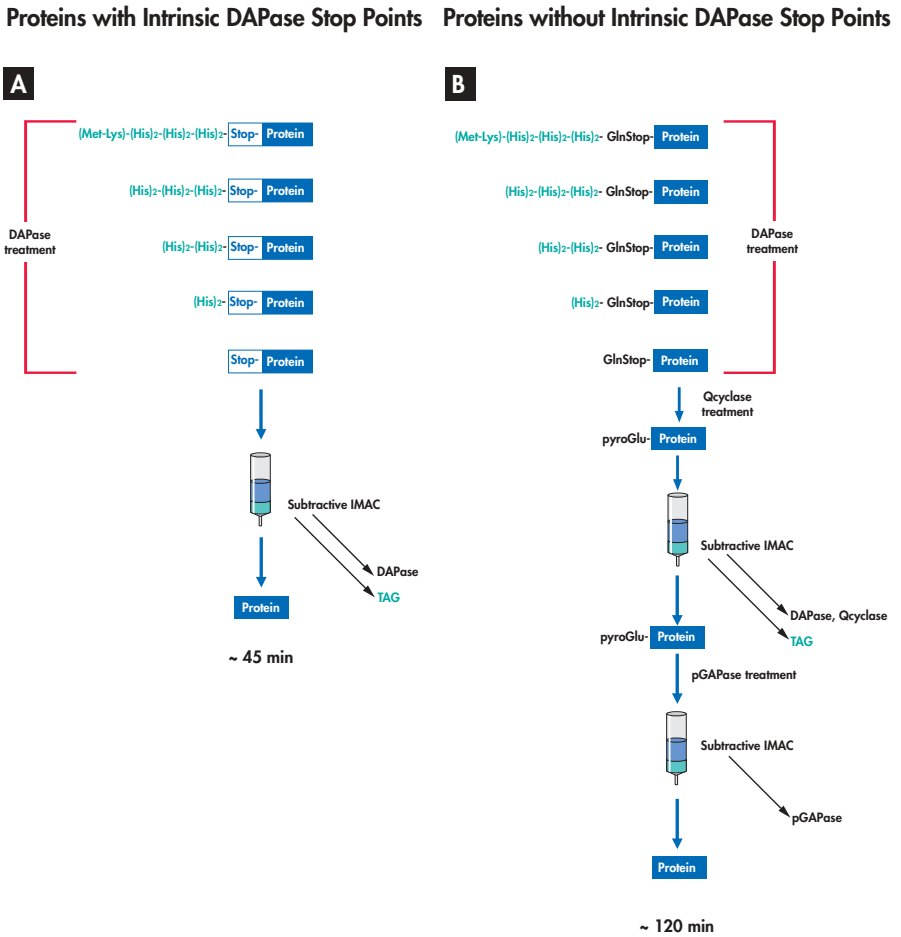


Figure 2. *Combined cleavage and purification strategy.* **A** *Procedure for proteins having an intrinsic DAPase stop point* **B** *Procedures for proteins with an introduced glutamine DAPase stop point.*

Applications for the TAGZyme System

The TAGZyme System is suitable for cleavage of every N-terminal His tag that contains certain sequence characteristics that are described in this handbook.

Therapeutic proteins

In the production of therapeutic proteins for use in humans or animals, it is often desirable to remove any artificial additions such as His tags.

The specificity and the speed of action of the recombinant enzymes in the TAGZyme System make it an ideal tool for production of recombinant proteinaceous therapeutics, where precisely defined cleavage characteristics are required. Due to the independence of this system from sequence-determined cleavage sites, the TAGZyme System provides the highest level of specificity of protein cleavage and target protein integrity. TAGZyme System components are available in various formats including bulk quantities of single enzymes.

Moreover, the TAGZyme System can be combined with Ni-NTA–based IMAC protein purification strategies for which Drug Master Files (Type II) are available for support in regulatory affairs. The subtractive Ni-NTA IMAC processing steps within the TAGZyme procedure can contribute to an increase in the purity of the target protein.

For production where the highest safety standards are required, GMP-quality TAGZyme enzymes are available. Please contact QIAGEN for details (see inside front cover).

Protein crystallography and structure determination

Structure determination of protein crystals has become more and more important in the context of drug screening and rational design of small molecules for protein ligands. In order to elucidate a protein's structure it is often desirable to crystallize the protein in the native state, i. e., without affinity tag additions.

Screening for crystallization conditions is often done in a high-throughput format. QIAGEN offers an automated solution for the production of large amounts of His-tagged protein for this purpose in 96-well format. TAGZyme is an ideal supplementary tool for this automated protein purification. Please contact QIAGEN for further details.

Protein microarrays

Depending on the chemistry of coupling of a purified protein to the surface of a chip and on the chip's intended application, it may be desirable to remove His tags from proteins prior to immobilization.

In combination with an automated solution for the purification of His-tagged proteins in a high-throughput format, TAGZyme is the method of choice to produce a large number of authentic, detagged proteins in a convenient and timesaving procedure.

Please contact QIAGEN for details of available solutions in automated high-throughput protein purification and downstream processing.

Basic research

Because the TAGZyme System is available in a small kit size and protocols are provided for the processing of a large range of proteins, the system is equally suited to His-tag removal from proteins used in small assays in basic research.

TAGZyme pQE Vectors

The vectors TAGZyme pQE-1 and -2 are based on the pQE-30 and pQE-80 vectors respectively, and are used for expression of N-terminally 6xHis-tagged proteins in *E. coli*. The His tag has been modified to encode the epitope Met-Lys-His_6 (MKH_6) a short His tag and the N-terminus Met-Lys motif that delivers a high expression level and lowest level of methionine processing in *E. coli* (1). Furthermore, in the rare case of methionine processing in *E. coli* the N-terminal lysine residue would act as a DAPase stop point (see Table 1, page 9) and prevent DAPase from out-of-frame digestion. The presence of this DAPase stop point leaves the protein intact allowing removal of the protein by subtractive IMAC.

QIA*express* pQE vectors

High-level expression of 6xHis-tagged proteins in *E. coli* using the QIA*express* pQE vectors is based on the T5 promoter transcription-translation system. pQE plasmids belong to the pDS family of plasmids (2) and were derived from plasmids pDS56/RBSII and pDS781/RBSII-DHFRS (3). These low-copy plasmids (Figure 3) have the following features:

- Optimized promoter-operator element consisting of phage T5 promoter (recognized by the *E. coli* RNA polymerase) and two *lac* operator sequences which increase *lac* repressor binding and ensure efficient repression of the powerful T5 promoter
- Synthetic ribosomal binding site, RBSII, for high translation rates
- 6xHis-tag coding sequence either 5' or 3' to the cloning region
- Multiple cloning site and translational stop codons in all reading frames for convenient preparation of expression constructs
- Two strong transcriptional terminators: t_0 from phage lambda (4), and T1 from the *rrnB* operon of *E. coli*, to prevent read-through transcription and ensure stability of the expression construct
- β-lactamase gene (*bla*) confers resistance to ampicillin (5) at 100 µg/ml (the chloramphenicol acetyl transferase gene (CAT) present between t_0 and T1 has no promoter and is not normally expressed)
- ColE1 origin of replication (5)

Restriction maps and sequences for the cloning regions of QIA*express* vectors are presented in Figure 5. The entire sequence information is available at www.qiagen.com. A strategy for the use of the pQE-TriSystem vector for protein expression in insect and mammalian cells in combination with the TAGZyme System is outlined in the Appendix, page 48.

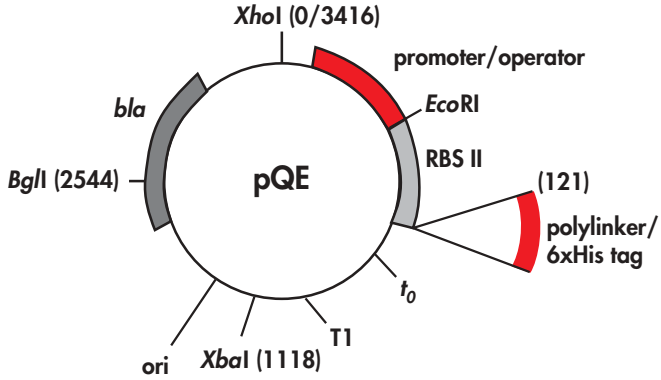


Figure 3. *pQE vectors.*

Regulation of expression — pREP4 plasmid

The extremely high transcription rate initiated at the T5 promoter can only be efficiently regulated and repressed by the presence of high levels of the *lac* repressor protein. *E. coli* host strains used in the QIA*express* System use a *lac* repressor gene in *trans* or *cis* to the gene to be expressed. In the *trans* system, the host strains contain the low-copy plasmid pREP4 which confers kanamycin resistance and constitutively expresses the *lac* repressor protein encoded by the *lac* I gene (6). The pREP4 plasmid is derived from pACYC and contains the p15A replicon. Multiple copies of pREP4 are present in the host cells that ensure the production of high levels of the *lac* repressor protein that binds to the operator sequences and tightly regulates recombinant protein expression. The pREP4 plasmid is compatible with all plasmids carrying the ColE1 origin of replication, and is maintained in *E. coli* in the presence of kanamycin at a concentration of 25 µg/ml. The *cis*-repressed vectors pQE-80L, -81L, and -82L contain the *lacI*^{q} gene and do not require the presence of pREP4.

Expression of recombinant proteins encoded by pQE vectors is rapidly induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) which binds to the *lac* repressor protein and inactivates it. Once the *lac* repressor is inactivated, the host cell's RNA polymerase can transcribe the sequences downstream from the promoter. The transcripts produced are then translated into the recombinant protein. The special "double operator" system in the pQE expression vectors, in combination with the high levels of the *lac* repressor protein generated by pREP4 or the *lacI*^{q} gene on pQE-80L, pQE-81L, or pQE-82L, ensure tight control at the transcriptional level. The pREP4 plasmid is already present in the QIA*express E. coli* strains M15[pREP4] and SG13009[pREP4]. Using pQE-80L, pQE-81L, or pQE-82L with the cis-*lacI*^{q} gene, expression rates are comparable with those obtained using pQE-30, pQE-31, or pQE-32 vectors in combination with pREP4.

E. coli host strains

Any *E. coli* host strain containing both the expression (pQE) and the repressor (pREP4) plasmids can be used for the production of recombinant proteins. The QIA*express* System uses *E. coli* strain M15[pREP4] which permits high-level expression and is easy to handle. Strain SG13009[pREP4] (*7*) is also supplied and may be useful for the production of proteins that are poorly expressed in M15[pREP4]. Both the M15 and SG13009 strains are derived from *E. coli* K12 and have the phenotype Nal^S, Str^S, Rif^S, Thi^-, Lac^-, Ara^+, Gal^+, Mtl^-, F^-, $RecA^+$, Uvr^+, Lon^+.

E. coli strains that harbor the *lacI*^{q} mutation, such as XL1 Blue, JM109, and TG1, produce enough *lac* repressor to efficiently block transcription, and are ideal for storing and propagating pQE plasmids. These strains can also be used as expression hosts for expressing nontoxic proteins, but they may be less efficient than the M15[pREP4] strain, and expression is regulated less tightly than in strains harboring the pREP4 plasmid. If the expressed protein is toxic to the cell, "leaky" expression before induction may result in poor culture growth or in the selection of deletion mutants that grow faster than bacteria containing the correct plasmid. Note that *E. coli* strains M15 and SG13009 do not harbor a chromosomal copy of the *lacI*^{q} mutation, so pREP4 must be maintained by selection for kanamycin resistance. The *cis*-repressed pQE-80L series of vectors can be easily used with any *E. coli* host strain and kanamycin selection is not necessary.

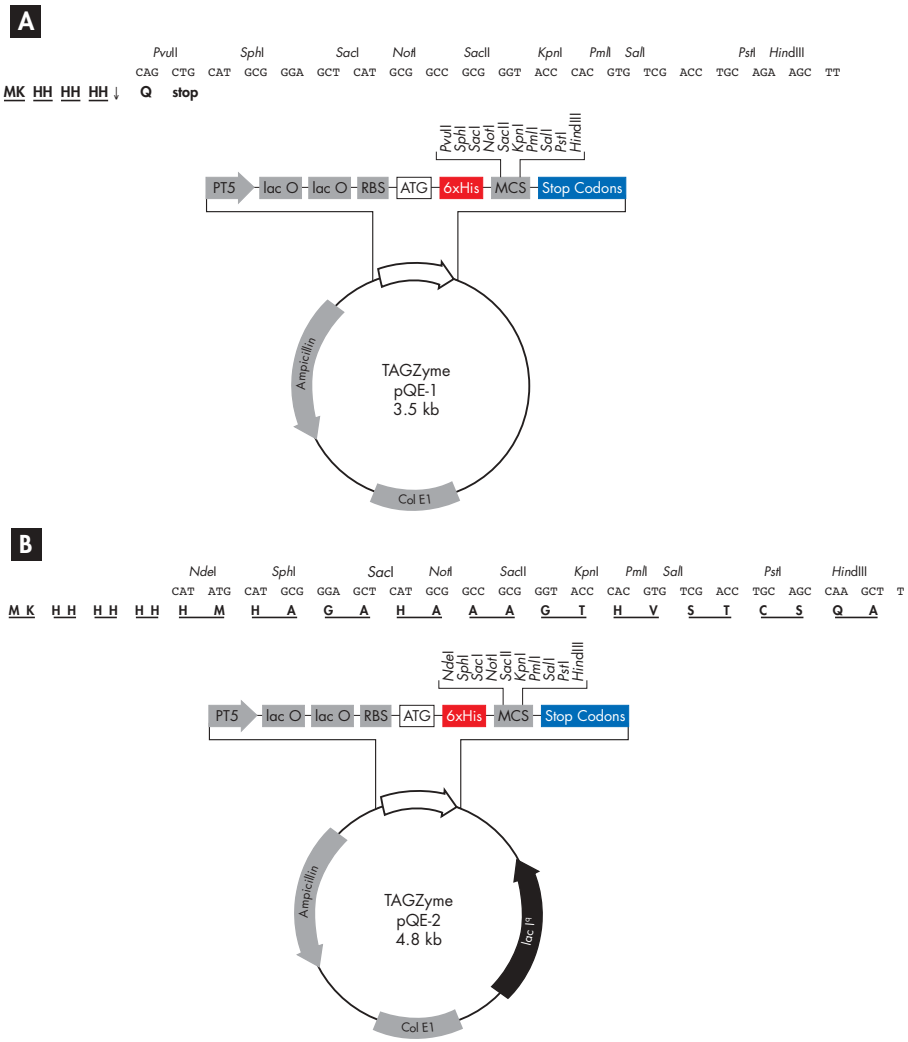


Figure 4. *TAGZyme pQE vectors for N-terminal His tag constructs which facilitate exoproteolytic removal of N-terminal residues by TAGZyme enzymes. Restriction sites within the multiple cloning sites, DNA sequences, and the corresponding N-terminal amino acid sequences are shown.* **A** *DAPase cleaves off the underlined dipeptides and DAPase digestion stops at the glutamine residue (Q) at position "↓" in the presence of excess Qcyclase.* **B** *DAPase Enzyme cleaves off the underlined dipeptides until an introduced stop point is reached.*

Refer to *The QIA*expressionist (8) or to www.qiagen.com for further details and sequences of the TAGZyme pQE-1 and -2 vectors.

The Cloning Procedure

The cleavage activity of DAPase Enzyme is dependent upon the amino acid composition of the His tag to be digested, i.e., of the dipeptides to be cleaved. The TAGZyme pQE *E. coli* expression vectors have been specially designed to provide reliable and efficient His tag cleavage.

The TAGZyme pQE-2 tag sequences allow removal of the complete N-terminal tag irrespective of the cloning site of the DNA insert. Vector TAGZyme pQE-1 can be used to insert a glutamine DAPase stop point. See Table 13 in the Appendix for a list of blunt-cutting restriction enzymes that can be used in conjunction with *Pvu*II for PCR cloning of expression inserts. After IMAC purification and subsequent treatment with DAPase Enzyme and excess Qcyclase Enzyme, the pyroglutamate residue generated can then be removed by the use of pGAPase Enzyme (see above).

Table 2. Examples of the cleavage rates of various dipeptides by DAPase Enzyme.

Rapid rate of cleavage	Medium rate of cleavage	Slow rate of cleavage	No cleavage = DAPase stop point
Xaa – Arg	Xaa – Asp*	Xaa – Phe†	Arg – Xaa
Xaa – Lys	Xaa – Glu*	Gly – Met	Lys – Xaa
His – Ala	Phe – Xaa†	Gly – Ser	Xaa – Pro
His – Gln	Ala – Ala	Ser – Met	Xaa – Xbb – Pro
His – Gly	Asp – Asp*,‡		Gln – Xaa§
His – His	Glu – Glu*,‡		
His – Met	Glu – His*		
Ala – His	Gly – Phe†		
Gly – His	Ser – Tyr		
Met – His	Ser – Thr		
His – Val	Gly – Ala		
	Gly – Thr		

* *Positively or negatively charged side chains inhibit DAPase Enzyme cleavage, and sequences containing aspartic acid (Asp) or glutamic acid (Glu) can only be digested by DAPase Enzyme at acidic pH while sequences containing histidine (His) residues require a pH above 6.0. Therefore, 6xHis tags containing Glu or Asp can only be digested by DAPase Enzyme at a pH between 6.0 and 6.5.*

† *With a few exceptions, dipeptides containing Phe, Ile, Leu, Tyr, and Trp in either of the two positions of the dipeptide, are subject to slow cleavage.*

‡ *Medium to slow cleavage rate.*

§ *Gln-Xaa serves as a stop point for DAPase Enzyme only in the presence of Qcyclase Enzyme, which converts the glutamine residue to pyroglutamate (which can be removed by the action of pGAPase Enzyme).*

The use of expression vectors other than TAGZyme pQE constructs in conjunction with TAGZyme enzymes is possible. However, since these vectors have not been specifically designed for use with the TAGZyme System, the amino acid sequence to be cleaved off should be carefully analyzed for efficiency of dipeptide cleavage using Table 2. It is also recommended to make use of Table 2 and the guidelines given below whenever a new N-terminal tag is designed for cleavage by DAPase Enzyme or in combination with Qcyclase Enzyme and pGAPase Enzyme. The TAGZyme System is equally suited for use with prokaryotic and eukaryotic expression systems.

In addition to considering the dipeptide composition within the N-terminus to be cleaved, the following guidelines should be followed in order to achieve successful removal of His tags from recombinant proteins using the TAGZyme System.

- The total number of amino acid residues in the tag to be cleaved off should not exceed 30.
- If the target protein contains an intrinsic DAPase stop point (see Table 1) which is to be utilized, the tag must be composed of an even number of residues. If the target protein does not contain a stop point the tag must be C-terminally extended with a glutamine residue (Q) in addition to an even number of residues N-terminal to the glutamine residue.
- Arginine (R) and lysine (K) residues must not be placed in odd-numbered positions of the tag.
- The tag sequence must not contain any proline (P) residues.
- The N-terminal sequence of the tag should be designed to minimize in vivo processing of the N-terminal methionine residue.

Processing of methionine leads to out-of-frame DAPase digestion and results in the accumulation of erroneously processed products. In *E. coli*, the residue adjacent to the initial methionine influences the degree of processing, and a bulky residue should be placed in this position. We recommend lysine (found in TAGZyme pQE-1 and -2 vectors), but arginine, glutamate, or aspartate are also effective as a penultimate residue that reduces methionine processing in *E. coli* (1). Please note that DAPase–Qcyclase Enzyme treatment must be carried out at pH 6.0–6.5 if aspartate or glutamate residues occur in the His-tag sequence to be cleaved (see Table 2 and Table 3).

Table 3. Characteristics of recommended His tag N-termini

N-terminus	Level of methionine processing in *E. coli**	Expression level in *E. coli*†	DAPase Enzyme cleavage rate	Other advantages
Met[1] Lys[2] – His tag	Very low	Very high	Rapid	DAPase Enzyme stop point for out-of-frame digestion‡
Met[1] Arg[2] – His tag	Low	Very high	Rapid	DAPase Enzyme stop point for out-of-frame digestion‡
Met[1] Glu[2] – His tag	Low	High	Medium (only at acidic pH)	
Met[1] Asp[2] – His tag	Low	High	Medium (only at acidic pH)	

* *See reference 1 and 11.*
† *See reference 1.*
‡ *See also Table 1.*

In general, the nucleotide sequence encoding the tag has a strong influence on the expression level and self-complimentary mRNA sequences close to the ATG start codon should be avoided. Furthermore, it has been observed that the amino acids lysine (codon AAA) and arginine (example codon CGT) significantly improve expression if placed next to the start methionine coded by ATG.

The tag sequence should be designed to avoid dipeptides that result in slow DAPase cleavage rates. The rate at which DAPase Enzyme cleaves off different dipeptides from the N-terminus depends on the amino acid residues in the dipeptide. Dipeptides containing an arginine or lysine residue in the C-terminal position are cleaved off very quickly, whereas dipeptides containing a hydrophobic amino acid residue (i.e, Phe, Ile, Leu, Tyr, Trp) are cleaved off relatively slowly. Examples of cleavage rates with respect to dipeptide composition are shown in Table 2, page 17.

Proteins expressed using other pQE vectors that encode N-terminal His tags are poorly processed as they encode the sequence Met-Arg-Gly-Ser. The second dipeptide, Gly-Ser is slowly cleaved by DAPase Enzyme (see Table 2). Furthermore, C-terminal His tags encoded by the pQE vectors pQE-60, -70, -16, and -TriSystem are not removed by the TAGZyme System, as the exoprotease cleaves dipeptides from the N-terminus of proteins.

In some cases, in vivo His-tag cleavage can occur, leading to erroneously processed products. Such processing may be observed when arginine residues appear in the middle of His-tag sequence or close to the target protein sequence. It is recommended that arginine residues only appear close to the N-terminus of the tag.

In response to out-of-frame digestion resulting from methionine processing or in vivo cleavage of the tag, incorporation of DAPase stop points in the tag is recommended (see above).

Erroneously processed products with two or more consecutive histidine residues remaining can be efficiently removed by subtractive Ni-NTA IMAC after DAPase digestion. Lysine, arginine, and alternating glutamine residues (in the presence of Qcyclase Enzyme) are functional stop points. It is recommended to place arginine in the N-terminal sequence of a tag (see above).

Cleavage Protocols

Notes Before Starting

His-tag design

Please note that the precise amino acid sequence of a polyhistidine tag and the nucleotide sequence selected to encode it are of great importance for the overall performance of the resulting construct during expression, post-translational processing, purification, and tag removal. For these reasons, we strongly recommend using the pQE-TAGZyme expression vectors that have been optimized for use with the TAGZyme System. If other vectors are used, please follow the guidelines given in this handbook regarding tag design and gene construction strategy.

Expression, Ni-NTA purification, and desalting of His-tagged proteins

For expression and purification of 6xHis-tagged proteins refer to *The QIA*expressionist, *a handbook for high-level expression and purification of 6xHis-tagged proteins* (8).

The TAGZyme procedure works well at pH 6.3–7.5 in the absence of imidazole. Because an addition of imidazole or a reduction in pH are usually used for elution of His-tagged proteins from Ni-NTA matrices, the purified target should be desalted before starting the TAGZyme procedure. Desalting can be performed using standard techniques such as gel filtration, ultrafiltration, hollow-fibre modules, or dialysis.

The presence of imidazole leads to a decrease in the cleavage rate, probably due to competitive inhibitory effect on DAPase catalysis. Simple dilution of IMAC elution fractions for direct use in the TAGZyme procedure without prior desalting may be possible but reaction parameters must be optimized in pilot experiments. Imidazole should be diluted to a concentration of 50 mM or lower. The final protein concentration should not be lower than 0.3 mg/ml.

Buffers and enzyme activity

Phosphate buffers are recommended for use with the TAGZyme System due to their low tendency to strip metal ions from IMAC matrices. However, other buffers may be used such as Tris, Bis-Tris, Bis-Tris·Propane, and MES. For further details on the compatibility of reagents with Ni-NTA IMAC matrices and procedures, refer to Table 4 in *The QIA*expressionist (8) or visit www.qiagen.com.

The optimal pH for TAGZyme catalyzed removal of tags, and also for removal of His-tagged contaminants using subtractive IMAC, is 6.7 to 7.5. All the TAGZyme enzymes work well between 4°C and 37°C as outlined in Table 4.

Table 4. Effect of reaction temperature on enzymatic activity

Reaction temperature	Relative activity of TAGZyme enzymes
37°C	100%
Room temperature (20–25°C)	50%
4–6°C	10%

DAPase Enzyme

DAPase Enzyme is inactive in the presence of denaturants (guanidine hydrochloride or urea), even at low concentrations. DAPase Enzyme is 85–100% active in the presence of up to 0.4% CHAPS, Tween® 20, Tween 80, and Triton® X-100 and 60–70% active in the presence of 1% of these detergents.

The enzyme has a broad pH optimum between pH 4 and 8. However, the activity depends on the dipeptides to be cleaved off (see Table 2 and Table 3). The optimal pH of DAPase-catalyzed removal of His tags is pH 6.7 to 7.2. The enzyme's activity is approximately 50% of maximum at pH 6.3 and 7.5 and approximately 10% at pH 5.8 and 8.0. DAPase Enzyme must be activated before use by the reducing agent cysteamine (see protocols).

Qcyclase Enzyme

Qcyclase Enzyme has a pH optimum of 8 and shows approximately 60–70% of maximum activity at pH 7 and pH 9.5 and approximately 40% at pH 6.2. Qcyclase Enzyme is inactive below pH 4.5. Qcyclase Enzyme is not inactivated by reducing agents such as cysteamine and DTT.

pGAPase Enzyme

pGAPase Enzyme is 100% stable in the presence of 1.6 M guanidine hydrochloride for 140 minutes and shows approximately 85% of maximum activity in the presence of 3.2 M guanidine hydrochloride for 140 minutes. Because pGAPase Enzyme is a cysteine peptidase, urea is not recommended for use with pGAPase Enzyme because the presence of low amounts of the chemical decomposition product cyanate will inactivate the enzyme.

The enzyme is 85–100% active in the presence of up to 0.4% CHAPS, Tween 20, Tween 80, and Triton X-100 and about 60% active in the presence of 1% of these detergents. pGAPase Enzyme is slightly activated by sarkosyl and shows 125% activity in the presence of 1% sarkosyl.

pGAPase Enzyme has a broad pH optimum between pH 6 and 10 with approximately 70% activity at pH 6 and 10. pGAPase Enzyme must be activated before use by the reducing agent cysteamine (see protocols).

TAGZyme Buffer (see below) provides optimal TAGZyme enzyme activity conditions and its use is recommended in TAGZyme procedures.

Cysteamine activation

Cysteamine is used as an activator for DAPase Enzyme and pGAPase Enzyme. It is thought that at a physiological pH, cysteamine, and its oxidized form cystamine, act as a hydrogen donor for the reduction of disulfides in the enzymes.

Stepwise Use of the TAGZyme System

Normally, by following the recommendations below, sufficient cleavage of the target protein will be obtained. However, the actual rate of cleavage of an N-terminal His tag may vary from protein to protein. This can be due to variations in steric accessibility of the tag or due to modifications of amino acid residues. If the optimal cleavage conditions have to be determined for a specific purification process, it is recommended that a pilot DAPase or DAPase–Qcyclase digestion reaction be carried out.

Therefore, the use of the TAGZyme System should include the following steps:

- Pilot digestion to establish optimum enzyme concentrations
- Preparative DAPase or DAPase–Qcyclase digestion
- Subtractive IMAC using Ni-NTA resin
- Digestion with pGAPase Enzyme and second subtractive IMAC (for proteins without intrinsic DAPase stop points)

Analysis during the TAGZyme procedure

We recommend that the efficiency of cleavage reactions is checked by subjecting samples from the different steps of the TAGZyme procedure to SDS-PAGE analysis. The pGAPase Enzyme-catalyzed removal of pyroglutamate cannot be observed directly but may be confirmed by subjecting an aliquot of the fully processed protein to a second round of DAPase treatment and subsequent analysis by SDS-PAGE. The result of efficient pGAPase Enzyme-catalyzed pyroglutamate removal will be quantitative DAPase Enzyme-catalyzed conversion of the deprotected target protein (i.e., without an N-terminal pyroglutamate residue) into truncated forms with increased electrophoretic mobility.

Figure 5, page 24 shows an SDS-PAGE analysis of a TAGZyme-cleaved His-tagged protein. His-tagged human tumor necrosis factor α (hTNFα) expressed in *E. coli* and purified by Ni-NTA IMAC, was subjected successively to DAPase–Qcyclase and pGAPase treatment and the authentic target protein hTNFα was recovered from the reaction solution by subtractive IMAC (lane 5). In addition to the TAGZyme enzymes and residual, unprocessed His-tagged TNFα, minor contaminants were efficiently removed in this step. The completeness of the pGAPase Enzyme-catalyzed removal of the pyroglutamate residue was checked by incubation of an aliquot of the fully processed TNFα with DAPase Enzyme. A quantitative DAPase Enzyme-catalyzed conversion of deprotected TNFα into a truncated form with increased electrophoretic mobility was observed (lane 6) confirming efficient pGAPase Enzyme-catalyzed removal of the pyroglutamate residue.

Figure 5. *Removal of the His tag from His-tagged hTNFα using the TAGZyme System.*

1*: Purified His-tagged hTNFα (the tag was an alternating tag as described in the Appendix, "His tags suitable for exoproteolytic cleavage by the TAGZyme System").*

2*: After incubation for 10 minutes with DAPase and Qcyclase Enzymes at 37°C.*

3*: After incubation for 20 minutes with DAPase and Qcyclase Enzymes at 37°C.*

4*: After incubation for 30 minutes with DAPase and Qcyclase Enzymes at 37°C.*

5*: After completion of the reaction untagged, pyroglutamyl-extended hTNFα was recovered by subtractive IMAC, subjected to pGAPase digestion, and mature hTNFα was recovered in the flow-through fraction of a second round of subtractive IMAC.*

6*: An aliquot of the processed protein was incubated with excess DAPase (0.125 U/mg hTNFα) for 2 hours in order to analyze the efficiency of the pGAPase-catalyzed removal of pyroglutamate. The two subunits of His-tagged DAPase Enzyme are visible.*

All samples were subjected to SDS-PAGE and the gel stained by Coomassie® Blue. ***M****: markers.*

Cleavage of N-terminal 6xHis tags using DAPase Enzyme

The data in Table 5 can be used as a rough guide to the amount of DAPase Enzyme necessary to completely digest the N-terminal tag of a recombinant protein. However, substantial differences in activity may be encountered from protein to protein (see above). Therefore, the figures in Table 5 should be considered as a starting point for the pilot experiments only, and not as a general rule for any protein and reaction scale.

Table 5. Times generally required for DAPase digestion at the given DAPase Enzyme-to-target protein ratio

Amount of target protein	Amount of DAPase Enzyme	Reaction temperature	Required reaction time
1 mg	50 mU	4°C	5 h
50 µg	2.5 mU	20–25°C	60 min
		37°C	30 min

The following section is for DAPase digestion of tagged proteins containing an intrinsic stop point (see above). For the combined DAPase–Qcyclase and pGAPase digestion see Protocols 4 and 5.

Buffer preparation

From a 10x stock solution (see Appendix) prepare enough 1x TAGZyme Buffer (20 mM sodium phosphate, pH 7.0; 150 mM NaCl) for desalting and the TAGZyme digestion. Approximately 50 ml 1x TAGZyme Buffer is sufficient for a small-scale experiment.

Note that tags containing glutamate or aspartate residues can only be efficiently cleaved at pH 6.0–6.5. In this case, prepare the TAGZyme Buffer at lower pH (20 mM sodium phosphate, pH 6.0–6.5; 150 mM NaCl).

Desalting

Desalt your His-tagged protein using gel filtration, ultrafiltration dialysis, hollow-fibre filtration, or the method best suited to your needs. It is recommended that proteins are exchanged into 1x TAGZyme Buffer.

Ni-NTA matrices show negligible leaching of nickel ions into the elution buffer (10). However, when using IMAC resins that bind metals more weakly, addition of 5–10 mM EDTA to the eluted protein fraction prior to the desalting procedure is recommended to remove nickel ions from the His tag.

Protocol 1. DAPase digestion (small-scale pilot experiment) for proteins containing intrinsic DAPase stop points

The amount of DAPase Enzyme and the length of incubation time required for complete digestion of the tag depend on the target protein and its concentration. Generally, digestion is complete after treatment for 30 minutes at 37°C with 50 mU DAPase Enzyme per mg protein but the optimal conditions for the processing of a His-tagged protein must be determined in pilot experiments. Table 6 gives guidelines for the conditions required for digestion of 50 µg target protein in TAGZyme buffer. Protein concentration should be at least 0.3 mg/ml. The reaction should take place in a volume of 50 to 300 µl. Larger reaction volumes require longer incubation times due to the dilution of the reactants.

Table 6. Recommendations for DAPase Enzyme digestion pilot experiments

Enzyme/reagent	For processing of 50 µg His-tagged target protein	
	Starting point	Titration range
DAPase Enzyme (1 U/ml)*	2.5 mU (2.5 µl)	0.5–5 mU (0.5–5 µl)
Cysteamine·HCl (2 mM)*	5 µl	1–10 µl

** Dilute an aliquot of the DAPase Enzyme and cysteamine stock solutions according to steps 1 and 2 of protocol 1.*

It is recommended that the progress of each digestion be analyzed by SDS-PAGE. Take 5 µl samples from each reaction every 10 minutes, add SDS sample buffer to inactivate the enzyme, and load samples onto an SDS gel including an undigested control. Alternatively, or in addition to varying the enzyme concentration, the incubation temperature can be varied.

Procedure

1. **Prepare a 1/10 dilution of DAPase Enzyme stock solution (10 U/ml) using 1x TAGZyme Buffer.**
2. **Prepare a 1/10 dilution of cysteamine·HCl stock solution (20 mM) using distilled water.**
3. **Mix 1 volume DAPase Enzyme solution (1 U/ml) with 2 volumes cysteamine·HCl (2 mM).**

   DAPase Enzyme is activated by cysteamine in this step.
4. **Incubate for 5 min at room temperature. The enzyme mixture must be used within 15 min of preparation.**

   During DAPase Enzyme preparation, bring the protein solution to the desired incubation temperature (4–37°C).
5. **Start the digestion by adding the enzyme mix to the temperature-adjusted protein solution.**
6. **Incubate for a length of time that should deliver complete digestion (see Table 5). If desired, take samples during the reaction for SDS-PAGE analysis.**

   If removal of DAPase Enzyme is required, please refer to the protocol for small-scale subtractive IMAC in the Appendix, page 51.

Protocol 2. Preparative DAPase digestion for proteins containing intrinsic DAPase stop points

The TAGZyme Kit has been designed to enable processing and purification of at least 10 mg of desalted and IMAC-purified tagged protein. Suitable protein concentrations in digestion reactions are between 0.3 and 5 mg/ml. The amount of DAPase Enzyme (mU) to be used for digestion per mg of desalted protein is established in the pilot DAPase digestion experiment. Table 7 lists the corresponding volumes to be used in a preparative digestion per mg of 6xHis-tagged protein.

Table 7. Recommendations for preparative DAPase digestion

Enzyme/reagent	For processing of 1 mg His-tagged target protein	
	Starting point	Titration range
DAPase Enzyme (10 U/ml)	50 mU (5 µl)	10–100 mU (1–10 µl)
Cysteamine·HCl (20 mM)	5 µl	1–10 µl

In most cases a linear scaling up of the conditions found to give complete digestion in the pilot experiment will give satisfactory results in a preparative digestion. If desired, a time course can be carried out to verify the optimal digestion conditions determined in the pilot experiments.

1. **Mix 1 volume DAPase Enzyme solution (10 U/ml) with 1 volume cysteamine·HCl (20 mM).**

   DAPase Enzyme is activated by cysteamine in this step.

2. **Incubate for 5 min at room temperature. The enzyme mixture must be used within 15 min of preparation.**

   During DAPase Enzyme preparation, bring the protein solution to the desired incubation temperature (4–37°C).

3. **Start the digestion by adding the enzyme mix to the temperature-adjusted protein solution.**

4. **Incubate for a length of time that was found to be sufficient for complete digestion in the pilot experiment. If desired, take samples during the reaction for SDS-PAGE analysis.**

Protocol 3. Removal of DAPase Enzyme by subtractive IMAC

This protocol applies to a cleavage reaction containing 1 mg target protein. Refer to Table 17, page 51 for Ni-NTA IMAC scale up recommendations.

1. **Resuspend the Ni-NTA Agarose suspension by shaking and pipet 1 ml of the 50% suspension (corresponding to 0.5 ml bed volume) into one of the disposable columns provided in the kit.**

   Alternatively, pack an LC or FPLC® column with 0.5 ml bed volume of Ni-NTA Agarose*.

2. **Equilibrate the column with 5 ml (10 column volumes) 1x TAGZyme Buffer.**

   Steps 1 and 2 can be performed during enzyme incubation. When using a chromatography system, do not exceed a flow rate of 0.5 ml/min to ensure quantitative binding of the His-tagged DAPase Enzyme.

3. **Pass the digestion reaction mixture through the column and collect the flow-through fraction.**

   Important! The flow-through fraction contains the processed native protein.

4. **Wash the column with 2 ml of 1x TAGZyme Buffer or until the flow-through no longer contains protein. Collect the fraction(s).**

   Important! The flow-through fractions contain the processed native protein.

5. **Add the flow-through fraction from step 3 to the protein-containing fractions from step 4. Discard the Ni-NTA resin used in this subtractive step.**

   The TAGZyme procedure is finished and the detagged native protein is ready for further applications.

** An HR 5/2 or HR 5/5 FPLC column (Amersham Pharmacia Biotech) is suitable for such a volume of matrix. For processing of larger amounts of protein please refer to the bed-volume size and flow-rate recommendations on page 51. In general, column height should be ≥2x column diameter.*

Cleavage of N-terminal His tags using DAPase, Qcyclase, and pGAPase Enzymes

This section is for the combined DAPase–Qcyclase and pGAPase Enzyme digestion. For the digestion of proteins with a natural stop point using DAPase Enzyme alone see Protocols 1 and 2.

The data in Table 8 can be used as a rough guide to the amounts of DAPase and Qcyclase Enzyme necessary to completely digest the N-terminal tag of a recombinant protein. However, substantial differences in activity may be encountered from protein to protein (see above). Therefore, the figures in Table 8 should be considered as a starting point for the pilot experiments only, and not as a general rule for any protein and reaction scale.

Table 8. Times generally required for DAPase–Qcyclase digestion at the given enzyme-to-target protein ratio

Amount of target protein	Amount of DAPase	Amount of Qcyclase	Reaction temperature	Required reaction time
1 mg	50 mU	3 U	4°C	5 h
50 µg	2.5 mU	150 mU	20–25°C	60 min
			37°C	30 min

Buffer preparation

From a 10x stock solution (see Appendix, page 44) prepare enough 1x TAGZyme Buffer (20 mM sodium phosphate, pH 7.0; 150 mM NaCl) for desalting and the TAGZyme digestion. Approximately 50 ml 1x TAGZyme Buffer is sufficient for a small-scale experiment.

Note: Tags containing glutamate or aspartate residues can only be efficiently cleaved at pH 6.0–6.5. In this case, prepare the TAGZyme Buffer at lower pH (20 mM sodium phosphate, pH 6.0–6.5; 150 mM NaCl). Following cleavage, the pH of the reaction mixture must be increased to between pH 6.7 and 7.5 to enable efficient removal of His-tagged enzymes by IMAC. This can be accomplished by the addition of ~1/100 volume of 1M Tris·Cl.

Desalting

Desalt your His-tagged protein using gel filtration, ultrafiltration dialysis, hollow-fibre filtration, or the method best suited to your needs. It is recommended that proteins are exchanged into 1x TAGZyme Buffer.

Ni-NTA matrices show negligible leaching of nickel ions into the elution buffer (10). However, when using IMAC resins that bind metal ions more weakly, addition of 5–10 mM EDTA to the eluted protein fraction prior to the desalting procedure is recommended to remove nickel ions from the 6xHis tag.

Protocol 4. DAPase–Qcyclase digestion (small-scale pilot experiment)

The amount of DAPase and Qcyclase Enzyme and the length of incubation time required for complete digestion of the His tag depend on the target protein and its concentration. Generally, digestion is complete after treatment for 30 minutes at 37°C with 50 mU DAPase and 3 U Qcyclase Enzyme per mg protein but the optimal conditions for the processing of a tagged protein must be determined in pilot experiments. Table 9 gives guidelines for the conditions required for digestion of 50 µg target protein. Protein concentration should be at least 0.3 mg/ml.

Table 9. Recommendations for pilot DAPase–Qcyclase Enzyme digestion experiments

Enzyme/reagent	For processing of 50 µg His-tagged target protein in 30 minutes	
	Starting point	Titration range
DAPase (1 U/ml)*	2.5 mU (2.5 µl)	0.5–5 mU (0.5–5 µl)
Qcyclase (50 U/ml)	150 mU (3 µl)	30–300 mU (0.6–6 µl)
Cysteamine·HCl (2 mM)*	5 µl	1–10 µl

** Dilute an aliquot of the DAPase Enzyme and cysteamine stock solutions according to steps 1 and 2 of protocol 4.*

It is recommended that the progress of each digestion be analyzed by SDS-PAGE. Take 5 µl samples from each reaction every 10 minutes, add SDS sample buffer to inactivate the enzyme, and load samples onto an SDS gel including an undigested control. Alternatively, or in addition to varying the enzyme concentration, the incubation temperature can be varied.

Procedure

1. **Prepare a 1/10 dilution of DAPase Enzyme stock solution (10 U/ml) using 1x TAGZyme Buffer.**
2. **Prepare a 1/10 dilution of cysteamine·HCl stock solution (20 mM) using distilled water.**
3. **Mix 1 volume DAPase Enzyme solution (1 U/ml) with 2 volumes cysteamine·HCl (2 mM).**

   DAPase Enzyme is activated by cysteamine in this step.
4. **Incubate for 5 min at room temperature. The enzyme mixture must be used within 15 min of preparation.**

   During DAPase preparation, bring the protein solution to the desired incubation temperature (4–37°C).
5. **Add 1.2 volumes of Qcyclase (50 U/ml) to the prepared DAPase.**
6. **Start the digestion by adding the enzyme mix to the temperature-adjusted protein solution.**
7. **Incubate for a length of time that should deliver complete digestion (see Table 8, page 30). If desired, take samples during the reaction for SDS-PAGE analysis.**

   If removal of DAPase and Qcyclase Enzymes is required, please refer to the protocol for small-scale subtractive IMAC in the Appendix, page 51.

Protocol 5. Preparative DAPase–Qcyclase digestion

The TAGZyme Kit has been designed to enable processing and purification of at least 10 mg of desalted and IMAC-purified tagged protein. Suitable protein concentrations in digestion reactions are between 0.3 and 5 mg/ml. The amounts of DAPase and Qcyclase Enzyme to be used for digestion per mg of desalted protein are established in the pilot DAPase–Qcyclase digestion experiment. Table 10 lists the corresponding volumes to be used in a preparative digestion per mg of His-tagged protein.

Table 10. Recommendations for preparative DAPase–Qcyclase digestion

Enzyme/reagent	For processing of 1 mg His-tagged target protein	
	Starting point	Titration range
DAPase (10 U/ml)	50 mU (5 µl)	10–100 mU (1–10 µl)
Qcyclase (50 U/ml)	3 U (60 µl)	0.6–6 U (12–120 µl)
Cysteamine·HCl (20 mM)	5 µl	1–10 µl

In most cases a linear scaling up of the conditions found to give complete digestion in the pilot experiment will give satisfactory results in a preparative digestion. If desired, a time course can be carried out to verify the optimal digestion conditions determined in the pilot experiments.

Procedure

1. **Mix 1 volume DAPase solution (10 U/ml) with 1 volume cysteamine·HCl (20 mM).**

   DAPase is activated by cysteamine in this step.

2. **Incubate for 5 min at room temperature. The enzyme mixture must be used within 15 min of preparation.**

   During DAPase preparation, bring the protein solution and an aliquot of 1x TAGZyme Buffer to the desired incubation temperature (4–37°C).

3. **Add 12 volumes of Qcyclase (50 U/ml) to the prepared DAPase.**
4. **Start the digestion by adding the enzyme mix to the temperature-adjusted protein solution.**
5. **Incubate for a length of time that was found to be sufficient for complete digestion in the pilot experiment. If desired, take samples during the reaction for SDS-PAGE analysis.**

Protocol 6. Removal of DAPase and Qcyclase Enzymes by subtractive IMAC

This protocol applies to a cleavage reaction containing 1 mg target protein. Refer to Table 17, page 51 for Ni-NTA IMAC scale up recommendations.

1. **Resuspend the Ni-NTA Agarose suspension by shaking and pipet 1 ml of the 50% suspension (corresponding to 0.5 ml bed volume) into one of the disposable columns provided in the kit.**

   Alternatively, pack an LC or FPLC column with 0.5 ml bed volume of Ni-NTA Agarose*.

2. **Equilibrate the column with 5 ml (10 column volumes) 1x TAGZyme Buffer.**

   Steps 1 and 2 can be performed during enzyme incubation. When using a chromatography system, do not exceed a flow rate of 0.5 ml/min to ensure quantitative binding of the 6xHis-tagged DAPase and Qcyclase Enzymes.

3. **Pass the digestion reaction mixture through the column and collect the flow-through fraction.**

   Important! The flow-through fractions contain the processed pyroglutamyl-extended protein.

4. **Wash the column with 2 ml of 1x TAGZyme Buffer or until the flow-through no longer contains protein. Collect the fraction(s).**

   Important! The flow-through fractions contain the processed pyroglutamyl-extended protein.

5. **Add the flow-through fraction from step 3 to the protein-containing fractions from step 4. Discard the Ni-NTA resin used in this subtractive step.**

   The DAPase–Qcyclase Enzyme step is finished and the recovered detagged protein is ready for the removal of pyroglutamate by the action of pGAPase Enzyme (see page 35).

** An HR 5/2 or HR 5/5 FPLC column (Amersham Pharmacia Biotech) is suitable for such a volume of matrix. For processing of larger amounts of protein please refer to the bed-volume size and flow-rate recommendations on page 51. In general, column height should be ≥2x column diameter.*

Protocol 7. pGAPase digestion (small-scale pilot experiment)

This step is only necessary for proteins that have been incubated with Qcyclase Enzyme.

Table 11. Times generally required for pGAPase reaction at the given enzyme-to-target protein ratio

Target protein	pGAPase	Reaction temperature	Required reaction time
1 mg	1 U	4–8°C	Overnight (15 h)
50 µg	50 mU	20–25°C	3 h
		37°C	90 min

Generally, digestion with pGAPase Enzyme is complete after treatment for 90 minutes at 37°C with 1.0–1.25 U of pGAPase Enzyme per mg of protein (see Table 11). However, optimal conditions may vary from protein to protein. Therefore, it is recommended that a titration of the enzyme concentration (see Table 12) or a control reaction with DAPase Enzyme (see "Analysis during the TAGZyme Procedure", page 23) is carried out after completion of the TAGZyme procedure. Alternatively, vary time and/or temperature of incubation. Increase the incubation time when the incubation temperature is decreased.

Table 12. Recommendations for pilot pGAPase pyroglutamate removal experiments

Enzyme/reagent	For processing of 50 µg pyroglutamyl-extended target protein	
	Starting point	Titration range
pGAPase (25 U/ml)	50 mU (2 µl)	10–100 mU (0.4–4 µl)
Cysteamine·HCl (2 mM)*	2 µl	1–10 µl

** Dilute an aliquot of the cysteamine·HCl stock solution according to step 1 of protocol 7.*

1. **Prepare a 1/10 dilution of cysteamine·HCl stock solution (20 mM) using distilled water.**
2. **Mix 2 µl cysteamine·HCl (2 mM) with 2 µl of pGAPase Enzyme (25 U/ml) per 50 µg of pyroglutamyl-extended protein.**

   pGAPase Enzyme is activated by cysteamine in this step.
3. **Incubate for 10 min at 37°C. The mixture must be used within 30 min after preparation.**
4. **Add the activated pGAPase Enzyme to the pyroglutamyl-extended protein contained in the flow-through fraction from the first subtractive IMAC.**
5. **Incubate at the desired temperature. For time-period guidelines refer to Table 11.**

   If removal of pGAPase Enzyme is required, please refer to the protocol for small-scale subtractive IMAC in the Appendix, page 51.

Protocol 8. Preparative pyroglutamate removal using pGAPase Enzyme

This step is only necessary for proteins that have been incubated with Qcyclase Enzyme.

Generally, digestion with pGAPase Enzyme is complete after treatment for 90 minutes at 37°C with 1.0–1.25 U of pGAPase Enzyme per mg of protein (see Table 11). However, optimal conditions may vary from protein to protein. Therefore, it is recommended that a titration of the enzyme concentration or a control reaction with DAPase Enzyme (see "Analysis during the TAGZyme Procedure", page 23) is carried out after completion of the TAGZyme procedure. Alternatively, vary time and/or temperature of incubation. Increase the incubation time when the incubation temperature is decreased.

1. **Mix 4 µl cysteamine·HCl (20 mM) with 40 µl of pGAPase Enzyme (25 U/ml) per mg of pyroglutamyl-extended protein.**

   pGAPase Enzyme is activated by cysteamine in this step.
2. **Incubate for 10 min at 37°C. The mixture must be used within 30 min after preparation.**
3. **Add the activated pGAPase Enzyme to the pyroglutamyl-extended protein contained in the eluate from the first subtractive IMAC.**
4. **Incubate at the desired temperature. For incubation guidelines refer to Table 11.**

Protocol 9. Removal of pGAPase Enzyme by subtractive IMAC

This protocol applies to a cleavage reaction containing 1 mg target protein. Refer to Table 17, page 51 for Ni-NTA IMAC scale up recommendations.

1. **Resuspend the Ni-NTA Agarose suspension by shaking and pipet 1 ml of the 50% suspension (corresponding to 0.5 ml bed volume) into one of the disposable columns provided in the kit.**

   Alternatively, pack a LC or FPLC column with 0.5 ml bed volume of Ni-NTA Agarose*.

2. **Equilibrate the column with 5 ml (10 column volumes) 1x TAGZyme buffer.**

   Steps 1 and 2 can be performed during enzyme incubation. When using a chromatography system, do not exceed a flow rate of 0.5 ml/min to ensure quantitative binding of the 6xHis-tagged pGAPase Enzyme.

3. **Pass the digestion reaction mixture through the column and collect the flow-through fraction.**

   Important! The flow-through fractions contain the processed native protein.

4. **Wash the column with 2 ml of 1x TAGZyme buffer or until the flow-through no longer contains protein. Collect the fraction(s).**

   Important! The flow-through fractions contain the processed native protein.

5. **Add the flow-through fraction from step 3 to the protein-containing fractions from step 4. Discard the Ni-NTA resin used in this subtractive step.**

We recommend investigating the efficiency of the pGAPase Enzyme-catalysed removal of pyroglutamate residues by subjecting an aliquot of the fully processed native protein to a post-process DAPase Enzyme treatment and SDS analysis (see Analysis during the TAGZyme procedure, page 23).

The TAGZyme procedure is finished and the detagged native protein is ready for further applications.

** An HR 5/2 or HR 5/5 FPLC column (Amersham Pharmacia Biotech) is suitable for such a volume of matrix. For processing of larger amounts of protein please refer to the bed-volume size and flow-rate recommendations on page 51. In general, column height should be ≥2x column diameter.*

	Comments and suggestions
End product not sufficiently pure	
a) Poor quality of tagged protein preparation	Refer to *The QIA*expressionist for guidelines and limitations of Ni-NTA IMAC purification of His-tagged proteins.
b) Inefficient digestion	When working with proteins purified by IMAC using matrices other than Ni-NTA, metal ions may be leached from the matrix. These metal ions may have an inhibitory effect on digestion. Include EDTA in the desalting procedure prior to digestion. Check N-terminal amino acid sequence for inefficiently cleaved dipeptides (Table 2, page 17). Check for the presence of DAPase Enzyme stop points in odd-numbered positions in the protein sequence. Check whether pH of the reaction is correct. The pH must be lowered when Glu or Asp residues are in the tag sequence to be cleaved. Check the pH of the 1x TAGZyme Buffer preparation. pH optimum for the TAGZyme procedure is pH 6.7. to 7.5. Check for the presence of denaturants. Even low concentrations will inactivate DAPase Enzyme. Be sure that the length of the tag to be cleaved does not exceed 30 amino acids. The optimal DAPase Enzyme-to-protein ratio may vary from protein to protein. Determine optimum conditions as described in pilot experiments. In some cases, a certain percentage of the tagged protein is not cleaved, e.g., due to steric hindrance. This residual His-tagged protein will be efficiently removed by subtractive IMAC.
c) Too many amino acids removed (DAPase digestion into the body of the target protein)	DAPase stop point is not present at correct position. Check sequence for correct positioning of stop continues point (Table 1, page 9). When making use of glutamine (Q) stop point, be sure to include an excess of Qcyclase Enzyme in the incubation reaction with DAPase Enzyme. Refer to Table 8, page 30 for suitable ratio of DAPase Enzyme to Qcyclase Enzyme.

	Comments and suggestions
d) Continued digestion during pGAPase step	Inefficient first-round subtractive IMAC can lead to DAPase Enzyme being present during pGAPase incubation. Be sure to equilibrate the IMAC column as described in the protocol. Be sure not to exceed the recommended flow rate of 0.5 ml/min. Check pH of column load as binding of DAPase and Qcyclase Enzymes may be inefficient at a pH lower than 7.0. Inefficient subtractive IMAC – refer to *The QIA*expressionist for general guidelines for IMAC and its limitations.
e) Pyroglutamyl-extended protein remains in the final preparation	pGAPase activity was inhibited. Be sure that urea is not present in the pGAPase reaction. Titrate pGAPase concentration to find out optimal concentration for complete digestion. Check for complete digestion by performing a DAPase control reaction as described on page 23.
f) pGAPase Enzyme present after subtractive IMAC	Subtractive IMAC was inefficient. Refer to *The QIA*expressionist for general guidelines for IMAC and its limitations.

Appendix

Enzyme specifications

DAPase Enzyme

Recombinant dipeptidyl peptidase I, polyhistidine-tagged (DPPI/dipeptidyl aminopeptidase I/DAPI/Cathepsin C; EC 3.4.14.1).

Source	Recombinant baculovirus expression vector system expressing the cloned DPPI gene from rat liver
Supplied form	Solution in 3 mM sodium phosphate; 150 mM NaCl; 2 mM cysteamine·HCl; 50% glycerol, pH 6.7–7.0
Purity	>98% (determined by SDS-PAGE)
Concentration	10 units/ml
Storage	–20°C
Stability	9 months at –20°C; 1 week at 2–8°C
Assay conditions	DAPase Enzyme is assayed at 37°C in 20 mM citric acid, 150 mM NaCl, 1 mM EDTA, 5 mM cysteamine, pH 4.5, containing 4 mM Gly-Phe-*p*-nitroanilide as substrate.
Unit definition	One unit is defined as the amount of enzyme that converts 1 µmol of substrate per minute under the conditions used.
Specific activity	7–11 unit/mg protein (protein determined by Bradford assay using BSA as standard)

Qcyclase Enzyme

Recombinant glutamine cyclotransferase, polyhistidine-tagged (GCT; EC 2.3.2.5)

Source	Recombinant baculovirus expression vector system expressing the cloned glutamine cyclotransferase gene from papaya latex
Supplied form	Solution in 8 mM sodium phosphate; 150 mM NaCl; 50% glycerol, pH 7.0
Purity	>98% (determined by SDS-PAGE)
Concentration	50 units/ml
Storage	–20°C
Stability	1 year at –20°C; 1 week at 2–8°C
Assay conditions	Qcyclase Enzyme (0.5–2 units) is assayed at 37°C in 50 mM Tris·HCl, pH 8.0, containing 2.5 mM Gln-Trp-Glu as substrate. The product (pyroGlu-Trp-Glu) is separated from the substrate by eluting the column with a linear NaCl gradient in 10 mM Tris·HCl, pH 8.0. The portion of Gln-Trp-Glu converted to pyroGlu-Trp-Glu detected at 280 nm is calculated from the respective peak areas.
Unit definition	One unit is defined as the amount of enzyme, which converts 1 µmol of substrate per minute under the conditions used.
Specific activity	70–110 units/mg protein (protein determined by A_{280})

pGAPase Enzyme

Recombinant pyroglutamyl aminopeptidase, polyhistidine-tagged (PGAP; EC 3.4.19.3)

Source	*E. coli* expressing the cloned pyroglutamyl aminopeptidase gene from *Bacillus amyloliquefaciens*
Supplied form	Solution in 6 mM Tris·HCl; 40 mM NaCl; 2 mM EDTA; 2 mM cysteamine·HCl; 50% glycerol, pH 8.0
Purity	>98% (determined by SDS-PAGE)
Concentration	25 units/ml
Storage	–20°C
Stability	1 year at –20°C; 1 week at 2–8°C
Assay conditions	PGAP (0.01–0.025 Units) was assayed at 37°C in 20 mM Tris·HCl, 100 mM NaCl, 5 mM EDTA, 5 mM cysteamine, pH 8.0, containing 2 mM pyroglutamyl-*p*-nitroanilide as substrate.
Unit definition	One unit is defined as the amount of enzyme, which converts 1 µmol of substrate per minute under the conditions used.
Specific activity	4.5–7 units/mg protein (protein determined by A_{280})

Solutions required

10x TAGZyme Buffer	**for 500 ml**
200 mM NaH_2PO_4	13.80 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99 g/mol)
1.5 M NaCl	43.83 g NaCl (MW 58.44 g/mol)

Adjust to desired pH with NaOH. Filter (0.2 µm) into a sterile storage vessel and store at 2–8°C.

Note: Upon 1:10 dilution pH will shift from 6.3 to 7.0.

Use of blunt-end–cutting restriction enzymes to introduce various amino acids at the N-terminus of the mature protein

Blunt-ended restriction enzyme sites can be introduced at the 5' end of the protein-coding sequence by PCR, and cloned into the TAGZyme pQE-1 vector. Different restriction enzyme sites can be utilized to incorporate specific amino acids at the N-terminus of the mature protein (see Table 13). After expression of the construct, the His tag and glutamine residue are removed using the TAGZyme System.

For example, your (mature) protein of interest starts with the amino acid alanine (Ala). To introduce Ala into amino acid position 10 (immediately following the glutamine residue) in the TAGZyme pQE-1 vector, construct a primer containing the restriction enzyme sequence for *Fsp*I. A suitable sense PCR primer is shown below. GCA codes for the desired amino acid alanine, and the sequence TGC GCA forms the recognition sequence of *Fsp*I. *Fsp*I-restricted PCR products can be blunt-end ligated into *Pvu*II-restricted TAGZyme pQE-1 vector, to give a construct encoding for a glutamine residue in an odd-numbered position, followed by the desired N-terminal amino acid, in this case alanine.

PCR sense primer: *Fsp*I

N-terminal amino acid sequence: 5' N NNN TGC GCA NNN NNN NNN NNN NNN 3'

Ala[1] aa[2] aa[3] aa[4] aa[5] aa[6]

Table 13. Restriction enzyme recognition sequences for the incorporation of the indicated amino acid directly after the glutamine residue encoded by the TAGZyme pQE-1 vector or into the *PmlI* site in the MCS of the TAGZyme pQE-2 vector*.

Amino acid(s) to be introduced	Suitable restriction enzyme	Restriction enzyme recognition sequence
Any	*Mly*I	GAGTC (N)$_5$▼NNN CTCAG (N)$_5$▲NNN Xaa
Any-Ser	*Xmn*I	GAANN▼NNTTC N CTTNN▲NNAAG N Xaa-Ser
Any†-Cys	*Msl*I	CA Py NN▼NN Pu TG N GT Pu NN▲NN Py AC Any†-Cys
Any†-Trp		CA Py NN▼NN Pu TG N GT Pu NN▲NN Py AC N Any†-Trp
Ala	*Fsp*I	TGC▼GCA ACG▲CGT Ala
	*Sfo*I	GGC▼GCC CCG▲CGG Ala
	*Afe*I	AGC▼GCT TCG▲CGA Ala
Arg	*Bsr*BI	GAG▼CGG CTC▲GCC Arg
	*Nru*I	TCG▼CGA AGC▲GCT Arg

Table continued overleaf

Table 13. Continued

Asn	*Hpa*I	GTT▼AAC CAA▲TTG Asn
	*Hinc*II	GT Py▼Pu AC CA Pu▲Py TG Asn (AAC)
Gln-Pro‡	*Msc*I	TGG▼CCA ACC▲GGT ‡Gln-Pro (CCA)
	*Stu*I	AGG▼CCT TCC▲GGA ‡Gln-Pro (CCT)
Gly	*Nae*I	GCC▼GGC CGG▲CCG Gly
	*Sma*I	CCC▼GGG GGG▲CCC Gly
Ile	*Eco*RV	GAT▼ATC CTA▲TAG Ile
	*Ssp*I	AAT▼ATT TTA▲TAA Ile
Leu	*Pvu*II	CAG▼CTG GTC▲GAC Leu
Lys	*Dra*I	TTT▼AAA AAA▲TTT Lys
Lys-Leu, Lys-Pro, Lys-His, Lys-Gln, Lys-Arg	*Pme*I	GTTT▼AAAC CAAA▲TTTG Lys-Leu/Pro/His/Gln/Arg
Lys-Phe, Lys-Leu, Lys-Ile, Lys-Met,Lys-Val	*Swa*I	ATTT▼AAAT TAAA▲TTTA Lys-Phe/Leu/Ile/Met/Val

Table continued overleaf

Table 13. Continued

Thr	*Sca*I	AGT▼ACT TCA▲TGA Thr
Tyr	*Bst*Z17I	GTA▼TAC CAT▲ATG Tyr
Val	*Bsa*AI	Py AC▼GT Pu Py TG▲CA Py Val
	*Btr*I	CAC▼GTC GTG▲CAG Val
	*Pml*I	CAC▼GTG GTG▲CAC Val
	*Sna*BI	TAC▼GTA ATG▲CAT Val
Serine in position 2: Ala-Ser, Arg-Ser, Gln-Ser, Glu-Ser, Gly-Ser, Leu-Ser, Lys-Ser, Met-Ser,Pro-Ser, Ser-Ser, Thr-Ser, Trp-Ser, Val-Ser	*Psh*AI	GACNN▼NNGTC N CTGNN▲NNCAG N Xaa-Ser

* *This list may not be complete! Only 5- or 6-nucleotide cutters are listed.*

† *Any amino acid except Asp, Asn, His, Tyr, Cys, and Phe can be inserted (refer to the foottnote below‡ when Pro is incorporated).*

‡ *Use of this restriction site incorporates a proline residue in the position following the glutamine stop. Proline functions as a stop two positions ahead, thus generating a protein with the N-terminal amino acids Gln1-Pro2… Omit Qcyclase Enzyme from the DAPase reaction in this case, as Qcyclase Enzyme will convert the glutamine residue to pyroglutamate.*

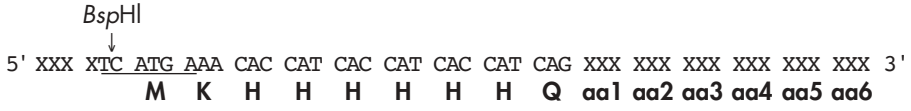
Recommended PCR cloning strategy for use of the vector pQE-TriSystem in combination with TAGZyme cleavage

The pQE-TriSystem vector allows parallel expression of a His-tagged recombinant protein in *E. coli*, baculovirus-infected insect cells, and mammalian cells using a single construct. The vector encodes a C-terminal His tag (which cannot be removed by the TAGZyme System). To make use of this extremely versatile expression vector, an N-terminal His tag must be introduced by PCR. A cloning strategy and primer design recommendations are given below.

- Clone into the most N-terminal restriction site, *Nco*I, to avoid the introduction of slowly cleaved dipeptides (see Table 2, page 17).
- Design a 5' (sense) oligonucleotide primer including the *Nco*I-compatible 5' restriction site *Bsp*HI and including sequences coding for a 6xHis tag (the use of the *Bsp*HI site allows the insertion of a lysine residue as the next amino acid following the start methionine).
- Include a C-terminal stop codon (UAA) after the last amino acid
- Clone into any one of the C-terminal restriction sites (e. g., *Hin*dIII)

Refer to *The QIA*expressionist (8) or visit us at www.qiagen.com for more details on the pQE-TriSystem vector.

Design of the 5' (sense) PCR primer with an introduced glutamine stop point*



* If the protein to be expressed contains a natural stop point within the amino acids aa1 – aa3 (see Table 1, page 9) this can be used as a DAPase Enzyme stop point instead of the glutamine residue.

Design of the 3' (antisense) PCR primer including a stop codon (TTA) for cloning into the *Hin*dIII site

Cloning into restriction sites located 3' of *Nco*I is not recommended, because this will result in the insertion of an alanine residue directly behind the initial methionine. Alanine does not efficiently prevent methionine processing (1) which may result in erroneously processed DAPase Enzyme digestion products. Furthermore, the second N-terminal dipeptide would be Ile-Ser which will be inefficiently cleaved (see Table 2, page 17).

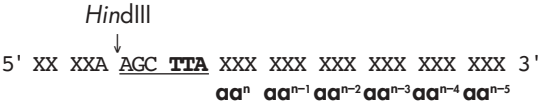


Table 14. The genetic code

First position (5' end)	Second position U	C	A	G	Third position (3' end)
U	Phe	Ser	Tyr	Cys	U
	Phe	Ser	Tyr	Cys	C
	Leu	Ser	Stop	Stop	A
	Leu	Ser	Stop	Trp	G
C	Leu	Pro	His	Arg	U
	Leu	Pro	His	Arg	C
	Leu	Pro	Gln	Arg	A
	Leu	Pro	Gln	Arg	G
A	Ile	Thr	Asn	Ser	U
	Ile	Thr	Asn	Ser	C
	Ile	Thr	Lys	Arg	A
	Met	Thr	Lys	Arg	G
G	Val	Ala	Asp	Gly	U
	Val	Ala	Asp	Gly	C
	Val	Ala	Glu	Gly	A
	Val	Ala	Glu	Gly	G

Table 15. *E. coli* codon usage table. For each codon, frequency of usage (%) and the corresponding amino acid are given

First position	Second position U		C		A		G		Third position
U	2.23	Phe	0.85	Ser	1.62	Tyr	0.52	Cys	U
	1.66	Phe	0.86	Ser	1.22	Tyr	0.65	Cys	C
	1.39	Leu	0.72	Ser	0.20	Stop	0.09	Stop	A
	1.36	Leu	0.89	Ser	0.02	Stop	1.52	Trp	G
C	1.10	Leu	0.70	Pro	1.29	His	2.09	Arg	U
	1.11	Leu	0.55	Pro	0.97	His	2.20	Arg	C
	0.39	Leu	0.84	Pro	1.53	Gln	0.36	Arg	A
	5.26	Leu	2.32	Pro	2.88	Gln	0.54	Arg	G
A	3.03	Ile	0.90	Thr	1.77	Asn	0.88	Ser	U
	2.51	Ile	2.34	Thr	2.17	Asn	1.61	Ser	C
	0.44	Ile	0.71	Thr	3.36	Lys	0.21	Arg	A
	2.53	Met	1.44	Thr	1.03	Lys	0.12	Arg	G
G	1.83	Val	1.53	Ala	3.21	Asp	2.47	Gly	U
	1.53	Val	2.55	Ala	1.91	Asp	2.96	Gly	C
	1.09	Val	2.01	Ala	3.94	Glu	0.80	Gly	A
	2.59	Val	3.36	Ala	1.78	Glu	1.11	Gly	G

His tags suitable for exoproteolytic cleavage by the TAGZyme System

The following His tags have been shown to deliver excellent expression rates in *E.coli* and be well-suited for convenient cleavage using the TAGZyme System. For a more detailed overview, see reference 12.

Table 16. Amino acid and encoding nucleotide sequences for recommended His tags

	1	2	3	4	5	6	7	8	9
Amino acid	M	K	H	H	H	H	H	H	(Q)
Codon	ATG	AAA	CAT	CAC	CAT	CAC	CAT	CAC	(GCT)
Amino acid	M	R	H	H	H	H	H	H	(Q)
Codon	ATG	CGT	CAT	CAC	CAT	CAC	CAT	CAC	(GCT)

Small-scale subtractive IMAC

- Pipet 50 µl Ni-NTA suspension (25 µl settled bed volume) into a microcentrifuge tube and equilibrate by spinning briefly (10 s), removing the supernatant, adding 500 µl TAGZyme buffer, spinning briefly (10 s), and removing the supernatant.
- Add the TAGZyme Enzyme reaction mixture and incubate at 4°C or room temperature for 10 min on a shaker platform or an end-over-end shaker. Ensure that the suspension is mixed well.
- To recover the target protein, spin briefly (10 s), and carefully remove the supernatant. Be sure not to disturb the Ni-NTA matrix, which contains the bound TAGZyme Enzyme.

Recommendations for subtractive IMAC scale-up

The use of a chromatography system with which the flow rate can be precisely controlled is recommended. When processing larger columns the flow rate is a critical factor for the efficient binding of His-tagged proteins to the Ni-NTA matrix. In addition, the use of Ni-NTA Superflow is recommended as it can withstand higher back pressures (up to 10 bar = 1 MPa). Table 17 gives guidelines for flow rates and column dimensions to be used when scaling up subtractive IMAC.

Table 17. Recommended column dimensions and flow rates for Ni-NTA IMAC scale up

Amount of protein to be processed	Ni-NTA bed volume	Column dimensions (internal diameter x length)	Flow rate (ml/min)		Linear flow rate (cm/h)	
			Load	Wash	Load	Wash
Up to 1 mg	0.5 ml	0.5 cm x 2.5 cm	0.5	1.0	150	300
50 mg	10 ml	1.6 cm x 5 cm	5	10	150	300
1 g	200 ml	5 cm x 10.2 cm	50	100	150	300

References

1. *Dalbøge, H., Bayne, S., and Pedersen, J. (1990) In vivo processing of N-terminal methionine in* E. coli*. FEBS Lett.* ***266****, 1.*
2. *Bujard, H., Gentz, R., Lanzer, M., Stüber, D. Müller, M., Ibrahimi, I. Häuptle, M.T., and Dobberstein, B. (1987) A T5 promotor based transcription-translation system for the analysis of proteins in vivo and in vitro. Methods Enzymol.* ***155****, 416.*
3. *Stüber, D., Matile, H., and Garotta, G. (1990) System for high-level production in* Escherichia coli *and rapid purification of recombinant proteins: application to epitope mapping, preparation of antibodies, and structure-function analysis. In: Lefkovits, I. and Pernis, B., eds., Immunological Methods, vol. IV, New York: Academic Press, p 121.*
4. *Schwarz, E., Scherrer, G., Hobom, G., and Kössel, H. (1978) Nucleotide sequence of cro, cII and part of the 0 gene in phage lambda DNA. Nature* ***272****, 410.*
5. *Sutcliffe, J.G. (1979) Complete nucleotide sequence of the* E. coli *plasmid pBR322. Cold Spring Harbor Symp. Quant. Biol.* ***43****, 77.*
6. *Farabaugh, P.J. (1978) Sequence of the lac I gene. Nature* ***274****, 765.*
7. *Gottesman, S., Halpern, E., and Trisler, P. (1981) Role of sulA and sulB in filamentation by Ion mutants of* Escherichia coli *K-12. J. Bacteriol.* ***148****, 265.*
8. The QIA*expressionist (03/2001). Handbook for high-level expression and purification of 6xHis-tagged proteins. QIAGEN, Hilden, Germany.*
9. *Sambrook, J. et al. (1989) Molecular Cloning A Laboratory Manual. Cold Spring Harbor Laboratory Press. New York.*
10. *Steinert, K., Artz, C., Fabis, R., and Ribbe, A. (1996) Comparison of chelating resins for purification of 6xHis-tagged proteins. QIAGEN News 1996 No. 5, 12.*
11. *Hirel, P.H.-H., Schmitter, J.-M., Dessen, P., Fayat, G., and Blanquet, S. (1989). Extent of N-terminal methionine excision from* Escherichia coli *proteins is governed by the side-chain length of the penultimate amino acid.*
    Proc. Natl. Acad. Sci. USA ***86****, 8247.*
12. *Pedersen, J., Lauritzen, C., Madsen, M.T., and Dahl, S.W. (1999) Removal of N-terminal polyhistidine tags from recombinant proteins using engineered aminopeptidases. Prot. Expr. Purif.* ***15****, 389.*

Trademarks

Patented or patent-pending technology and/or registered or registration-pending trademark of QIAGEN: QIAGEN®, QIA*express*®, QIA*expressionist*™, Ni-NTA.
Coomassie is a registered trademark of ICI Organics Inc.
FPLC is a registered trademark of Amersham Pharmacia Biotech.
TAGZyme, DAPase, pGAPase, and Qcyclase are trademarks of UNIZYME.
Triton is a registered trademark of Rohm & Haas Inc.
Tween is a registered trademark of ICI Americas Inc.

Registered names, trademarks, etc. used in this document, even when not specifically marked as such, are not to be considered unprotected by law.

Hoffmann-La Roche owns patents and patent applications pertaining to the application of Ni-NTA resin (Patent series: RAN 4100/63: USP 4.877.830, USP 5.047.513, EP 253 303 B1), and to 6xHis-coding vectors and His-labeled proteins (Patent series: USP 5.284.933, USP 5.130.663, EP 282 042 B1). All purification of recombinant proteins by Ni-NTA chromatography for commercial purposes, and the commercial use of proteins so purified, require a license from Hoffmann-La Roche.

TAGZyme technology is licensed under U.S. Patent No. 5,691,169, U.S. Patent No. 5,783,413 and E.U. Patent No. 00759931B1.

Ordering Information

Product	Contents	Cat. No.
TAGZyme Kit	0.5 Units DAPase Enzyme, 30 Units Qcyclase Enzyme, 10 Units pGAPase Enzyme, 20 mM Cysteamine·HCl (1 ml), Ni-NTA Agarose (10 ml), 20 Disposable Columns	34300
TAGZyme pQE Vector Set	TAGZyme pQE-1 and -2 Vector DNA, 25 µg each	32932
TAGZyme DAPase Enzyme (2.5 U)	2.5 Units DAPase Enzyme, 20 mM Cysteamine·HCl (1 ml)	34362
TAGZyme DAPase Enzyme (50 U)*	50 Units DAPase Enzyme, 20 mM Cysteamine·HCl (25 ml)	34366
TAGZyme Qcyclase/pGAPase Enzymes (150 U/50 U)	150 Units Qcyclase Enzyme, 50 Units pGAPase Enzyme	34342
TAGZyme Qcyclase/pGAPase Enzymes (3000 U/1000 U)*	3000 Units Qcyclase Enzyme, 1000 Units pGAPase Enzyme	34346
Related products		
Ni-NTA Agarose† (25 ml)	25 ml nickel-charged resin (max. pressure: 2.8 psi)	30210
Ni-NTA Superflow† (25 ml)	25 ml nickel-charged resin (max. pressure: 140 psi)	30410
Polypropylene Columns (1 ml)	50/pack, 1 ml capacity	34924

* *Bulk enzyme quantities are customized products; delivery may take up to 6 weeks. Enzymes are also available in GMP-grade. Please inquire.*

† *Available in bulk quantities; please inquire.*

QIAGEN Companies

Please see the inside front cover for contact information for your local QIAGEN office.

QIAGEN Distributors

Argentina
Tecnolab S.A.
Tel: (011) 4555 0010
Fax: (011) 4553 3331
E-mail: info@tecnolab.com.ar
Web site: www.tecnolab.com.ar

Austria
VWR International GmbH
Tel: (01) 576 00 0
Fax: (01) 576 00 600
E-mail: info@at.vwr.com
Web site: www.vwr.com

Belgium/Luxemburg
Westburg b.v.
Tel: 0800-1-9815
Fax: (31) 33-4951222
E-mail: info@westburg.nl
Web site: www.westburg.nl

Brazil
Uniscience do Brasil
Tel: 011 3622 2320
Fax: 011 3622 2323
E-mail: info@uniscience.com
Web site: www.uniscience.com

China
Gene Company Limited
Tel: (852)2896-6283
Fax: (852)2515-9371
E-mail:
Hong Kong:info@genehk.com
Beijing: gene@public2.bta.net.cn
Shanghai: gene@public.sta.net.cn
Chengdu: gene@public.cd.sc.cn
Guangzhou:
gzyitao@public.guangzhou.gd.cn

Croatia
INEL Medicinska Tehnika d.o.o.
Tel: (01) 2984-898
Fax: (01) 6520-966
E-mail:
inel-medicinska-tehnika@zg.tel.hr

Cyprus
Scientronics Ltd
Tel: 02-357 22 765416
Fax: 02-357 22 764614
E-mail: a.sarpetsas@biotronics.com.cy

Czech Republic
BIO-CONSULT spol. s.r.o.
Tel/Fax: (420) 2 417 29 792
E-mail: bio-cons@login.cz
Web site: www.bio-consult.cz

Denmark
VWR International A/S
Tel: 43 86 87 88
Fax: 43 86 87 90
E-mail: info@dk.vwr.com
Web site: www.vwr.com

Egypt
Clinilab
Tel: 52 57 212
Fax: 52 57 210
E-mail: Clinilab@link.net

Finland
VWR International Oy
Tel: (09) 804 551
Fax: (09) 8045 5200
E-mail: info@fi.vwr.com
Web site: www.vwr.com

Greece
BioAnalytica S.A.
Tel: (210)-640 03 18
Fax: (210)-646 27 48
E-mail: bioanalyt@hol.gr

Hungary
Kasztel-Med Co. Ltd.
Tel: (01) 385 3887
Fax: (01) 381 0695
E-mail: info@kasztel.hu
Web site: www.kasztel.hu

India
Genetix
Tel: (011)-542 1714
or (011)-515 9346
Fax: (011)-546 7637
E-mail: genetix@nda.vsnl.net.in

Israel
Westburg (Israel) Ltd.
Tel: 08-6650813/4
or 1-800 20 22 20 (toll free)
Fax: 08-6650934
E-mail: info@westburg.co.il
Web site: www.westburg.co.il

Korea
LRS Laboratories, Inc.
Tel: (02) 924-86 97
Fax: (02) 924-86 96
E-mail: webmaster@lrslab.co.kr
Web site: www.lrslab.co.kr

Malaysia
RESEARCH BIOLABS SDN. BHD.
Tel: (603)-8070 3101
Fax: (603)-8070 5101
E-mail: biolabs@tm.net.my
Web site: www.researchbiolabs.com

Mexico
Quimica Valaner S.A. de C.V.
Tel: (55) 55 25 57 25
Fax: (55) 55 25 56 25
E-mail: qvalaner@infosel.net.mx

The Netherlands
Westburg b.v.
Tel: (033)-4950094
Fax: (033)-4951222
E-mail: info@westburg.nl
Web site: www.westburg.nl

New Zealand
Biolab Ltd
Tel: (09) 980 6700
or 0800 933 966
Fax: (09) 980 6788
E-mail:
biosciences@nzl.biolabgroup.com
Web site: www.biolabgroup.com/nzl

Norway
VWR International AS
Tel: 22 90 00 00
Fax: 22 90 00 40
E-mail: info@no.vwr.com
Web site: www.vwr.com

Poland
Syngen Biotech Sp.z.o.o.
Tel: (071) 351 41 06
or 0601 70 60 07
Fax: (071) 351 04 88
E-mail: info@syngen.pl
Web site: www.syngen.pl

Portugal
IZASA PORTUGAL, LDA
Tel: (21) 424 7312
Fax: (21) 417 2674

Singapore
Research Biolabs Pte Ltd
Tel: 62731066
Fax: 62734914
E-mail: biolabs@singnet.com.sg

Slovak Republic
BIO-CONSULT Slovakia spol. s.r.o.
Tel/Fax: (02) 5022 1336
E-mail: bio-cons@post.sk
Web site: www.bio-consult.cz

Slovenija
MEDILINE d.o.o.
Tel: (01) 830-80-40
Fax: (01) 830-80-70
or (01) 830-80-63
E-mail: mediline@siol.net

South Africa
Southern Cross Biotechnology
(Pty) Ltd
Tel: (021) 671 5166
Fax: (021) 671 7734
E-mail: info@scb.co.za

Spain
IZASA, S.A.
Tel: (93) 902.20.30.90
Fax: (93) 902.22.33.66
E-mail: suministros@izasa.es

Sweden
VWR International AB
Tel: (08) 621 34 00
Fax: (08) 760 45 20
E-mail: info@se.vwr.com
Web site: www.vwr.com

Taiwan
TAIGEN Bioscience Corporation
Tel: (02) 2880 2913
Fax: (02) 2880 2916
E-mail: taigen@ms10.hinet.net

Thailand
Theera Trading Co. Ltd.
Tel: (02) 412-5672
Fax: (02) 412-3244
E-mail: theetrad@samart.co.th

Turkey
Medek Medikal Ürünler
ve Saglik Hizmetleri A. S.
Tel: (216) 302 15 80
Fax: (216) 302 15 88
E-mail: makialp@med-ek.com

All other countries
QIAGEN GmbH, Germany

1024037 03/2003