TECHNICAL BULLETIN

HisLink™ Protein Purification Resin

Instructions for use of Products
V8821 and V8823

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HisLink™ Protein Purification Resin

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1. Description

HisLink™ Protein Purification Resin[a,b] is a macroporous silica resin modified to contain a high level of tetradentate-chelated nickel (>20mmol Ni/ml settled resin) for efficient capture and purification of bacterially expressed polyhistidine- or HQ-tagged proteins. (For information on HQ-tagged vectors, see Section 7.) This resin also may be used for general applications that require an immobilized metal affinity chromatography (IMAC) matrix (1,2). HisLink™ Resin may be used in either column or batch purification formats. Gravity feed of a cleared lysate over a HisLink™ column is sufficient for complete capture and efficient elution of polyhistidine- or HQ-tagged proteins; however, the resin also may be used with vacuum filtration devices (e.g., the Vac-Man® Vacuum Manifold, Cat.# A7231) to allow simultaneous processing of multiple columns. HisLink™ Resin also is an excellent choice for affinity

purification using low- to medium-pressure liquid chromatography systems such as fast performance liquid chromatography (FPLC). In batch format, HisLink™ Resin may be easily separated from the lysate without filtration, allowing processing of larger quantities of lysate and purification of protein from lysate that has not been cleared of insoluble cellular debris.

Advantages

- HisLink™ Resin is provided precharged with nickel as a 50% slurry (v/v) in water and ready for use.
- Purify polyhistidine- or HQ-tagged proteins from cleared lysate by standard column chromatography or from crude lysate in batch. HisLink™ Resin is made using a chromatographic grade of silica and suitable for low- to medium-pressure applications such as FPLC.
- The amount of lysate that can be processed may be increased significantly without increasing the time needed for purification by using the batch purification method described in Section 3.E.
- The high level of nickel available on the particle surface means that larger quantities of polyhistidine- or HQ-tagged protein can be purified per volume of resin used. The HisLink™ Resin has a measured binding capacity of >15mg/ml (or >1,000nmol/ml) of settled resin as determined for an 8.5kDa polyhistidine-ubiquitin protein.

2. Product Components and Storage Conditions

Product	Size	Cat.#
HisLink™ Protein Purification Resin	50ml	V8821

Each bottle contains 50ml of resin.

- 50ml HisLink™ Protein Purification Resin, 50% (v/v) slurry

Product	Size	Cat.#
HisLink™ Protein Purification Resin	5ml	V8823

Each bottle contains 5ml of resin.

- 5ml HisLink™ Protein Purification Resin, 50% (v/v) slurry

Storage Conditions: Store at 4°C.

3. Protocol for Purifying Polyhistidine- or HQ-Tagged Proteins

Materials to be Supplied by the User

Required

- HEPES (pH 7.5)
- imidazole

Optional

- column [e.g., Fisher PrepSep Extraction Column (Cat.# P446) or Bio-Rad Poly-Prep® Chromatography Column (Cat.# 731-1550)]
- vacuum manifold [e.g., Promega Vac-Man® Jr. Laboratory Vacuum Manifold (Cat.# A7660) or Vac-Man® Laboratory Vacuum Manifold (Cat.# A7231) or Fisher vacuum manifold (Cat.# 11-131-31, 11-131-32)]

3.A. General Guidelines and Instructions

Recommended Buffers

Binding Buffer

100mM HEPES (pH 7.5)
10mM imidazole
500mM NaCl (for HQ-tagged proteins)

Wash Buffer

100mM HEPES (pH 7.5)
10–100mM imidazole (for polyhistidine-tagged proteins)
10–20mM imidazole (for HQ-tagged proteins)
500mM NaCl (for HQ-tagged proteins)

Elution Buffer

100mM HEPES (pH 7.5)
250–1,000mM imidazole (for polyhistidine-tagged proteins)
50–1,000mM imidazole (for HQ-tagged proteins)

The HEPES-based buffer system works well with the HisLink™ Resin for purifying polyhistidine- or HQ-tagged, expressed proteins from bacterial lysates. Unlike other resins, the HisLink™ Resin does not have a stringent requirement for salt in the buffer (usually NaCl) to reduce nonspecific protein interactions for polyhistidine-tagged proteins. In some cases, avoiding salt will increase the purity of the protein isolated. However, for HQ-tagged proteins, we recommend the addition of 500mM NaCl to the binding and wash buffers. While HEPES is an excellent buffer for HisLink™ Resin purification, the resin is compatible with other buffers commonly associated with polyhistidine- or HQ-tagged protein purification, including Tris or sodium phosphate buffers.

Adding imidazole to the binding and wash buffers reduces competitive binding and co-elution of nonspecific proteins under low stringency conditions. In cases where polyhistidine- or HQ-tagged protein interaction with the resin is particularly strong, increasing the amount of imidazole in the wash buffer may dramatically improve the purity of the recovered protein. We recommend that the concentration of imidazole remain lower than 100mM for polyhistidine-tagged proteins and 20mM for HQ-tagged proteins in the wash buffer. Carefully determine the tolerable wash stringency and imidazole concentration when working with novel proteins (see Section 5).

Eluting polyhistidine- or HQ-tagged proteins with imidazole is concentration-dependent. Some proteins can be effectively eluted with concentrations of imidazole at or below 50mM, whereas other proteins may require higher imidazole concentrations (≥500mM) for efficient release. HQ-tagged proteins may elute with a lower concentration of imidazole (50–100mM). When working with proteins that have not been previously characterized, we recommend using a step or linear gradient to determine the optimal concentrations of imidazole in the wash and elution buffers (see Section 5).

For polyhistidine-tagged proteins, sodium chloride (100–500mM) also may be added to reduce nonspecific protein binding. In general, NaCl is required when lysozyme is used to prepare a cell lysate. In this case, salt is required to prevent co-purification of lysozyme. Additionally, NaCl can increase the amount of protein purified for some tagged proteins and is recommended for HQ-tagged proteins.

3.B. Lysate Preparation

Cells may be lysed using any number of methods including sonication, French press, bead milling, treatment with lytic enzymes (e.g., lysozyme) or by using a commercially available cell lysis reagent. We recommend preparing the lysate as a 10- to 50-fold concentration of the cell culture, depending on the amount of expressed protein. Increasing the concentration allows the user to process more culture and recover more protein. However, at high expression levels, protein precipitation may limit the extent to which the culture may be concentrated.

Notes:

1. If you are using a column for purification, clear the lysate before loading the column by centrifuging at ≥10,000 × *g* for 30 minutes at 4°C and discarding the pellet. Further clearing the lysate by filtration through a 0.2 or 0.4µm filter will aid in column flow and is strongly recommended when using FPLC for purification.

2. Lysozyme will bind to and elute from the resin with the HEPES buffer described in Section 3.A. If lysozyme is used to prepare a lysate, add salt (>300mM NaCl) to the binding and wash buffers to prevent the lysozyme binding to the resin.

3. We recommend using protease inhibitors during cell lysis. Adding protease inhibitors such as 1mM PMSF to cell lysates does not inhibit the binding or elution of polyhistidine- or HQ-tagged proteins with the HisLink™ Resin. When preparing cell lysates from high-density cultures, adding DNase and RNase (concentrations up to 20µg/ml) will reduce the lysate viscosity and aid in purification.

3.C. Purification of Polyhistidine- or HQ-Tagged Proteins from Cleared Lysate by Gravity Flow Column Chromatography

1. Prepare the binding, wash and elution buffers described in Section 3.A.

   Note: If you do not know the appropriate concentrations of imidazole for the wash and elution buffers, see Section 5 for a protocol to define the optimal imidazole concentrations for purifying the protein of interest.

2. Determine the column volume required to purify the protein of interest. In most cases, 1ml of settled resin is sufficient to purify the amount of protein typically found in up to 1 liter of culture (cell density of $O.D._{.600}$<6.0). In cases of very high expression (e.g., 50mg/liter), up to 2ml of resin per liter of culture may be needed.

3. Once you have determined the volume of settled resin required, precalibrate this amount directly in the column by pipetting the equivalent volume of water into the column and marking the column to indicate the top of the water. This mark indicates the top of the settled resin bed. Remove the water before adding resin to the column.

4. Fill the column with resin to the line marked on the column by transferring the resin with a pipette. Allow the resin to settle and adjust the resin by adding or removing resin as necessary.

   Note: When pipetting the HisLink™ Resin, make sure that it is fully suspended, and pipet the resin immediately. The resin is heavy and settles quickly. If the resin cannot be pipetted within 10–15 seconds of mixing, significant settling will occur, and the resin will need to be resuspended. Alternatively, a magnetic stir bar may be used to keep the resin in suspension during transfer. To avoid fracturing the resin, do not leave the resin stirring any longer than the time required to pipet and transfer the resin.

5. Allow the column to drain, and equilibrate the resin with 5 column volumes of binding buffer, allowing the buffer to completely enter the resin bed.

6. Gently add the cleared lysate to the resin until the lysate has completely entered the column. The rate of flow through the column should not exceed 1–2ml/minute for every 1ml of column volume. Under normal gravity flow conditions the rate is typically about 1ml/minute. The actual flow rate will depend on the type of column used and the extent to which the lysate was cleared and filtered.

   Note: For HQ-tagged proteins, we recommend adding NaCl to a final concentration of 500mM in the binding and wash buffers. The presence of salt improves binding of the tagged protein to the resin and minimizes nonspecific background.

Do not let the resin dry out after you have applied the lysate to the column.

7. Wash unbound proteins from the resin using at least 10–20 column volumes of wash buffer. Divide the total wash buffer used into two or three aliquots, and allow each aliquot to completely enter the resin bed before adding the next aliquot.

8. Once the wash buffer has completely entered the resin bed, add elution buffer and begin collecting fractions (0.5–5ml fractions). Elution is protein-dependent, but polyhistidine- or HQ-tagged proteins will generally elute in the first 1ml. Elution is usually complete after 3–5ml of buffer have been collected per 1.0ml of settled resin, provided the imidazole concentration is high enough to efficiently elute the protein of interest. See Section 5 for information on optimizing elution conditions.

   Note: HQ-tagged proteins may elute with a lower concentration of imidazole (50–100mM) compared to polyhistidine-tagged proteins.

9. Analyze fractions by gel electrophoresis to determine protein amount and purity. Combine fractions as desired.

3.D. Purification of Polyhistidine- or HQ-Tagged Proteins from Cleared Lysate on a Vacuum Manifold

1. Determine the column volume required to purify the protein of interest. In most cases, 1ml of settled resin is sufficient to purify the amount of protein typically found in up to 1 liter of culture (grown to a density of $O.D._{600}$<6.0). In cases of very high expression (e.g., 50mg/liter), up to 2ml of resin per liter of culture may be desired.

2. Once you have determined the volume of settled resin required, precalibrate the amount directly in the column by pipetting the equivalent volume of water into the column and marking the column to indicate the top of the water. This mark indicates the top of the settled resin bed. Remove the water before adding resin to the column.

3. Fill the column with resin to the line marked on the column by transferring the resin with a pipette. Allow the resin to settle, and adjust its volume by using a pipette to add or remove resin.

   Note: When pipetting the HisLink™ Resin, make sure that it is fully suspended, and pipet the resin immediately. The resin is heavy and settles quickly. If the resin cannot be pipetted within 10–15 seconds of mixing, significant settling will occur, and the resin will need to be resuspended. Alternatively, a magnetic stir bar may be used to keep the resin in suspension during transfer. To avoid fracturing the resin, do not leave the resin stirring any longer than the time required to pipet and transfer the resin.

4. Place the column onto a vacuum manifold and apply just enough pressure to drain the water from the area above the resin. Equilibrate the resin with 5 column volumes of binding buffer, allowing the entire volume of buffer to enter the resin bed.

   Note: Be careful to not let the column dry out. If the column becomes dry at this point, reapply binding buffer and resuspend resin; after the resin has settled, reduce the level of buffer to the top of the resin.

5. Add lysate to the resin and apply a vacuum to the column at a pressure that maintains a flow rate of 1–2ml/minute (optimal flow rate is 1ml/minute for every 1ml of settled column resin). Care should be taken not to let the resin dry out during this step.

   Note: For HQ-tagged proteins, we recommend adding NaCl to a final concentration of 500mM in the binding and wash buffers. The presence of salt improves binding of the tagged protein to the resin and minimizes nonspecific background.

6. Wash the resin with at least 10–20 column volumes of wash buffer, applying vacuum to facilitate washing. Divide the total wash buffer used into two or three aliquots, and allow each aliquot to completely enter the column before adding the next aliquot. Care should be taken not to let the resin dry out during this step.

7. Once the final aliquot of wash buffer has completely entered the resin bed, add elution buffer and begin collecting fractions (0.5–5ml fractions). Elution may be performed under vacuum if the manifold used allows for the collection of the eluate. Alternatively, the elution may be done by gravity as described in Section 3.C, Step 8. Elution is protein-dependent, but polyhistidine- or HQ-tagged proteins will generally elute in the first 1ml. Elution is usually complete after 3–5ml of buffer per 1.0ml of settled resin, provided the imidazole concentration is high enough to efficiently elute the protein of interest. See Section 5 for information on optimizing elution conditions.

   Note: HQ-tagged proteins may elute with a lower concentration of imidazole (50–100mM) compared to polyhistidine-tagged proteins.

8. Analyze fractions by gel electrophoresis to determine protein amount and purity. Combine fractions as desired.

3.E. Batch Purification of Polyhistidine- or HQ-Tagged Proteins from Cleared or Crude Lysate

1. Batch purification may be performed on either cleared or crude lysate following the same general protocol. To purify in batch mode, first determine the amount of resin required for the amount of cleared or crude lysate. Generally, for expression levels on the order of 1–30mg/liter of culture, 2–4ml of 50% slurry should be sufficient to bind the polyhistidine- or HQ-tagged protein from 1 liter of culture.

   Add the resin to the cleared or crude lysate, and stir with a magnetic stir bar (or other device) for at least 30 minutes at 4°C, ensuring that the resin is well mixed throughout the lysate solution. Alternatively, the lysate and resin can be added to a conical tube and placed on an orbital shaker for 30 minutes.

   Note: For HQ-tagged proteins, we recommend adding NaCl to a final concentration of 500mM in the binding and wash buffers. The presence of salt improves binding of the tagged protein to the resin and minimizes nonspecific background.

   Note: Binding is efficient at 4–22°C. Depending on the stability of the particular protein being purified, you may choose to perform the binding at 22°C rather than 4°C.

2. Allow the resin to settle for approximately 5 minutes; then carefully decant the lysate. If necessary, use a pipette to completely remove the lysate, leaving the resin behind.

   Note: If purifying from a cleared lysate, the wash steps may be performed by transferring the bound lysate directly to a column and washing and eluting as described in Section 3.C.

3. To remove nonspecifically bound proteins, add wash buffer (10ml per 1ml of resin used) to the resin and fully resuspend. Allow the resin to settle for approximately 5 minutes; then carefully decant the wash solution. If necessary, use a pipette to remove as much of the wash volume as possible without disturbing the resin. Repeat wash step two times for a total of 3 washes.

4. After the third wash, thoroughly resuspend the resin in a volume of wash buffer sufficient to transfer the resin to a column. Allow the entire amount of buffer to enter the resin bed. Use as much wash buffer as necessary to transfer all of the resin.

5. Once the wash buffer has completely entered the resin bed, add elution buffer and begin collecting fractions (0.5–5ml fractions). Elution is protein-dependent, but polyhistidine- or HQ-tagged proteins will generally elute in the first 1ml. Elution is usually complete after 3–5ml of buffer per 1ml of settled resin, provided the imidazole concentration is high enough to efficiently elute the protein of interest. See Section 5 for information on optimizing imidazole concentration.

   Note: HQ-tagged proteins may elute with a lower concentration of imidazole (50–100mM) compared to polyhistidine-tagged proteins.

6. Analyze fractions by gel electrophoresis to determine protein amount and purity. Combine fractions as desired.

3.F. Purifying Polyhistidine- or HQ-Tagged Proteins from Cleared Lysate by FPLC

1. Determine the column volume required, calibrating the column if necessary. Pack and assemble the column according to the manufacturer's instructions. Do not introduce air bubbles into the resin. Place column inline on the instrument.

2. Equilibrate the column with at least 5 column volumes of binding buffer.

3. Apply lysate to the column at a flow rate of 1ml/minute for every 1ml of column volume.

   Note: For HQ-tagged proteins, we recommend adding NaCl to a final concentration of 500mM in the binding and wash buffers. The presence of salt improves binding of the tagged protein to the resin and minimizes nonspecific background.

4. Wash unbound proteins from the column with wash buffer, monitoring the absorbance until the A_{280} remains at or below baseline for at least 5–10 column volumes. If absorbance is not monitored, 20 column volumes of wash buffer should be adequate.

5. Elute bound protein with elution buffer, and collect fractions. Elution is protein-dependent, but the protein generally elutes in the first 1ml, and elution is usually complete after 3–5ml of buffer per 1ml of settled resin.

   Note: HQ-tagged proteins may elute with a lower concentration of imidazole (50–100mM) compared to polyhistidine-tagged proteins.

6. Analyze fractions by gel electrophoresis to determine protein amount and purity. Combine fractions as desired.

4. General Considerations

Elution

Imidazole (50–1,000mM): The amount of imidazole required to elute a polyhistidine- or HQ-tagged protein depends on the properties of the protein and its tag. You may need to empirically determine the optimal imidazole concentration for washing and elution of your protein of interest (see Section 5). We have observed elution from this resin using imidazole concentrations as low as 50mM. HQ-tagged proteins and polyhistidine-tagged proteins containing less than six histidines may require less imidazole for elution. Polyhistidine-tagged proteins containing more than six histidines may require a higher concentration of imidazole. The HQ tag contains three histidines and three glutamines (HQHQHQ). HQ-tagged proteins may elute with a lower concentration of imidazole (50–100mM) compared to polyhistidine-tagged proteins.

EDTA (100mM): Strong chelators such as EDTA will strip nickel from the resin and release bound protein. EDTA is generally not as efficient as imidazole for elution, and it has the added complication that Ni(EDTA) will be present in the eluate and must be removed.

pH 3–5: Polyhistidine binding to immobilized nickel is most efficient at a pH well above the pKa of polyhistidine (~ 6.0). Above pH 7, more than 90% of the imidazole moieties of histidine will be deprotonated and available to bind to nickel (3). Lowering the pH of a binding reaction below the pKa of histidine leads to protonation of the histidine tag and release from the resin. Buffers that can be used at this low pH for elution include citrate and acetate. Trifluoroacetic acid (i.e., 0.1% TFA) also can be used for elution, and because of its volatility, it is directly compatible with mass spectrometry analysis. As with EDTA elution, acidic elution is generally not as efficient as imidazole elution and is complicated by the leaching of nickel, which may become quite significant at pH values below 4. These considerations also apply to HQ-tagged proteins.

Denaturing Conditions

Proteins that are expressed as an inclusion body and have been solubilized with chaotrophic agents such as guanidine-HCl or urea can be purified by modifying the above protocols to include the appropriate amount of denaturant (up to 6M guanidine-HCl or up to 8M urea) in the binding, wash and elution buffers.

Reusing Resin

The HisLink™ Resin and packed column may be used to purify protein more than once (generally two or three times), but we strongly recommend that the resin only be used for the same protein. To reuse a packed column, re-equilibrate the column with 20 column volumes of binding buffer before re-applying lysate. We recommend that you store the columns for reuse in binding buffer or water, not in elution buffer. We do not recommend using a packed column or resin that has been stored in elution buffer for more than 4 hours.

Adjuncts for Lysis or Purification

We have found that the following materials may be used without adversely affecting the ability of HisLink™ Resin to bind and elute polyhistidine- or HQ-tagged proteins.

Additive	Concentration
HEPES, Tris or sodium phosphate buffers	≤100mM
NaCl	≤1M
Triton® X-100	≤2%
Tween® 20	≤2%
glycerol	≤20%
guanidine-HCl	≤6M
urea	≤8M
RQ1 RNase-Free DNase	≤5µl/1ml original culture

5. Determining Wash Stringency and Imidazole Concentration for Elution

1. This protocol can be used to determine the concentration of imidazole required for effective washing and elution of the protein of interest. Begin by preparing stock solutions of 1M HEPES (pH 7.5) and 2M imidazole (pH 7.5). Combine these solutions to obtain the correct compositions for the buffers defined in the table below.

   Table 1. Wash/Elution Buffer Recipes for Determining Optimal Imidazole Concentration.

Wash/Elution Buffer	Imidazole Concentration
100mM HEPES (pH 7.5)	25mM
	50mM
	75mM
	100mM
	250mM
	500mM
	1,000mM

2. Prepare column as described in Section 3.A, and equilibrate with 5 column volumes of binding buffer.

3. Add the lysate to the column until the entire volume has completely entered the resin bed.

4. Add 10 column volumes of binding buffer (preferably in two portions), and allow the binding buffer to fully enter the resin bed.

5. Add 5 column volumes of the first (25mM imidazole) test elution buffer, and collect the eluant in 1–2.5 column volume fractions.

6. After the first elution buffer with the lowest imidazole concentration has completely entered the resin bed, add the next buffer and collect fractions.

7. Continue with each buffer until all buffers have been used to elute the column or until you are sure that the target protein has been eluted.

8. Analyze fractions by gel electrophoresis to determine the amount of imidazole that may be used in the wash buffer without losing an undesirable amount of protein and the amount needed in the elution buffer to provide purity of the desired protein.

9. The number of fractions to be analyzed by gel electrophoresis can be reduced by screening the fractions for total protein content by Bradford assay. The fractions that contain the most protein are likely to contain the polyhistidine- or HQ-tagged protein of interest and can be further analyzed by gel electrophoresis.

6. References

1. Porath, J. *et al.* (1975) Metal chelate affinity chromatography, a new approach to protein fractionation. *Nature* **258**, 598–9.
2. Lönnerdal, B. and Keen, C.L. (1982) Metal chelate affinity chromatography of proteins. *J. Appl. Biochem.* **4**, 203–8.
3. Hochuli, E. (1990) Purification of recombinant proteins with metal chelate adsorbent. *Genet. Eng. (NY)* **12**, 87–98.

7. Related Products

Polyhistidine- or HQ-Tagged Protein Purification

Product	Size	Cat.#
MagneHis™ Protein Purification System	65 reactions	V8500
	325 reactions	V8550
MagneHis™ Ni-Particles	2ml	V8560
	10ml	V8565
MagZ™ Protein Purification System	30 purifications	V8830
HisLink™ 96 Protein Purification System	1 × 96 reactions	V3680
	5 × 96 reactions	V3681
HisLink™ Spin Protein Purification System	25 reactions	V1320

GST-Fusion Protein Purification

Product	Size	Cat.#
MagneGST™ Protein Purification System	40 reactions	V8600
	200 reactions	V8603
MagneGST™ Glutathione Particles	4ml	V8611
	20ml	V8612

Protein Analysis

Product	Size	Cat.#
RQ1 RNase-Free DNase*	1,000u	M6101
Broad Range Protein Molecular Weight Markers	100 lanes	V8491
Gel Drying Kit	1 kit	V7120
Gel Drying Film	100 sheets	V7131

*For Laboratory Use.

HQ-Tagged Flexi® Vectors

Product	Size	Cat.#
pFN6A (HQ) Flexi® Vector	20µg	C8511
pFN6K (HQ) Flexi® Vector	20µg	C8521
pFC7A (HQ) Flexi® Vector	20µg	C8531
pFC7K (HQ) Flexi® Vector	20µg	C8541

pFN6A and pFN6K (HQ) Flexi® Vectors: These vectors are designed for expressing N-terminal, HisGln (HQ) metal-binding peptide fusion proteins in bacteria and in vitro protein expression systems. The vectors are configured to append the peptide sequence MKHQHQHQAIA to the amino terminus of a protein. The vectors are designed for bacterial or in vitro protein expression via the T7 RNA polymerase promoter and are available with ampicillin (pFN6A (HQ) Flexi® Vector) or kanamycin (pFN6K (HQ) Flexi® Vector) resistance for selection in *E. coli*.

pFC7A and pFC7K (HQ) Flexi® Vectors: These vectors are designed for expressing C-terminal, HisGln (HQ) metal-binding peptide fusion proteins in bacteria and in vitro protein expression systems. The vectors are configured to append the peptide sequence VSHQHQHQ to the carboxy terminus of a protein. The vectors are designed for bacterial or in vitro protein expression via the T7 RNA polymerase promoter and are available with ampicillin (pFC7A (HQ) Flexi® Vector) or kanamycin (pFC7K (HQ) Flexi® Vector) resistance for selection in *E. coli*.

For further information regarding these HQ-tagged Flexi® Vectors and the Flexi® Vector Systems for cloning, refer to the *Flexi® Vector System Technical Manual* #TM254 or visit: **www.promega.com**

[a]U.S. Pat. Nos. 7,112,552 and 7,354,750.

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