Technical Manual

HaloLink™ Resin

INSTRUCTIONS FOR USE OF PRODUCTS G1911 AND G1912.

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HaloLink™ Resin

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I. Description

The HaloTag™ technology[a,b,c] comprises the HaloTag™ polypeptide, which can be fused to a protein of interest using the HaloTag™ Vectors[b,c,d], and a system of interchangeable synthetic ligands that covalently bind to the HaloTag™ polypeptide. Unique ligands enable customers to perform a variety of applications, such as immobilization, labeling and imaging. Construction of HaloTag™ fusion proteins is facilitated by a system of vectors. The pFC8A (HaloTag™) CMV Flexi® Vector[b,c,d] (Cat.# C3631), pFC8K (HaloTag™) CMV Flexi® Vector[b,c,d] (Cat.# C3641) and HaloTag™ pHT2 Vector[b,c] (Cat.# G8241) (see Section VII.C. Related Products), are designed for expressing HaloTag™ fusion proteins for in vitro protein expression systems or in mammalian cells. The pHT2 Vector allows C- and N-terminal fusions, while the Flexi® HaloTag™ Vectors are configured to append the HaloTag™ polypeptide to the carboxy

terminus of the fusion protein. The vectors contain the T7 RNA polymerase promoter for in vitro expression and the human cytomegalovirus (CMV) intermediate-early enhancer/promoter for expression in mammalian cells.

In this Technical Manual we describe the HaloLink™ Resin[a], which allows covalent and oriented surface immobilization of HaloTag™ fusion proteins. HaloLink™ Resin consists of Sepharose® beads with a HaloTag™-specific ligand on the surface. The resin shows high binding capacity and very low nonspecific binding. Furthermore, the binding affinity of HaloTag™ protein for HaloLink™ resin is very high.

- **Composition:** HaloTag™ binding ligand is covalently attached to the surface of Sepharose® beads via a carbamide linkage using an eleven-atom linker.
- **Particle Size:** 45–165 microns.
- **Concentration:** HaloLink™ Resin is supplied as 25% slurry in 25% ethanol.
- **Binding Capacity:** One milliliter of settled resin binds >7mg of HaloTag™ fusion protein. **Note:** One milliliter of settled resin corresponds to 4ml of the 25% slurry provided.

II. Product Components

Product	Size	Cat. #
HaloLink™ Resin	40 reactions (2ml)	G1911
	100 reactions (5ml)	G1912

Storage Conditions: Store the HaloLink™ Resin at 4°C. Do not freeze.

III. HaloLink™ Resin Applications

The HaloLink™ Resin can be used in a variety of applications including enzyme immobilization, detection of protein:protein interactions and purification of fusion proteins using protease cleavage. Due to the covalent linkage, HaloTag™ fusion proteins cannot be eluted from the resin, which allows extensive washing to remove nonspecifically bound proteins without the danger of eluting HaloTag™ fusion proteins. In addition, rapid binding, high binding capacity and low nonspecific binding contribute to reliable results.

III.A. Detection and Analysis of Protein:Protein Interactions

The covalent and oriented attachment of fusion proteins to HaloLink™ Resin provides an excellent choice for detection of protein interactions using the pull-down method. HaloLink™ Resin is predominantly intended for detection of protein interactions when both protein partners are expressed in the in vitro expression systems or for isolation of protein complexes from mammalian cells in vivo.

The typical pull-down assay comprises the protein of interest fused to a tag and a solid support that binds this protein. The fusion protein (also referred to as bait) bound to the resin is then introduced to a pool of proteins containing potential binding partners (prey). The bait-prey complex is isolated from a complex protein mixture by resin precipitation and extensive washing to remove nonspecifically-bound proteins. The specific binding partner is eluted and the identity of the prey is usually determined by SDS-polyacrylamide gel electrophoresis or mass spectrometry. Pull-down assays are prone to false positives due to nonspecific binding of proteins. Covalent binding of HaloTag™ fusion proteins to HaloLink™ Resin allows extensive washing without the fear of dissociation of HaloTag™ fusion proteins from the resin. Additionally, low nonspecific binding of HaloLink™ Resin reduces the incidence of false positives, resulting in increased specificity.

Due to the advantageous properties of HaloLink™ Resin (stable binding, high on-rate, high capacity and low nonspecific binding) protein:protein interactions have been successfully detected where both the bait and the prey are expressed using in vitro protein expression systems. Furthermore, we have circumvented the need to precharge resin with bait and have isolated bait-prey protein complexes that were preformed in vitro or in vivo.

III.B. Enzyme Immobilization

Immobilization of enzymes and study of their activities can be achieved with the HaloTag™ technology. Covalent attachment of proteins to the HaloLink™ Resin allows the assay of enzymatic activities over a long period of time in a variety of buffer conditions without protein dissociation from the resin. Other affinity purification resin tags are often used to attach enzymes to the surface; however, after incubation fusion proteins will dissociate from the resin. Because HaloTag™ fusion proteins are covalently bound to the HaloLink™ Resin, dissociation does not occur.

III.C. Purification of Fusion Proteins

The major application for HaloLink™ Resin is permanent attachment of proteins onto the resin, which prevents elution of HaloTag™ fusion proteins. However, the plasmids pFC8A (HaloTag™) CMV Flexi® Vector and pFC8K (HaloTag™) CMV Flexi® Vector contain a Factor Xa protease cleavage site situated in the linker sequence between the HaloTag™ peptide and the protein of interest. This allows release of the nontagged protein of interest from the HaloLink™ Resin by Factor Xa protease cleavage (1).

Section IV provides guidelines for immobilization of HaloTag™ fusion proteins expressed in vitro on HaloLink™ Resin. Immobilized proteins can then be evaluated for enzymatic activity, protein interactions or other applications.

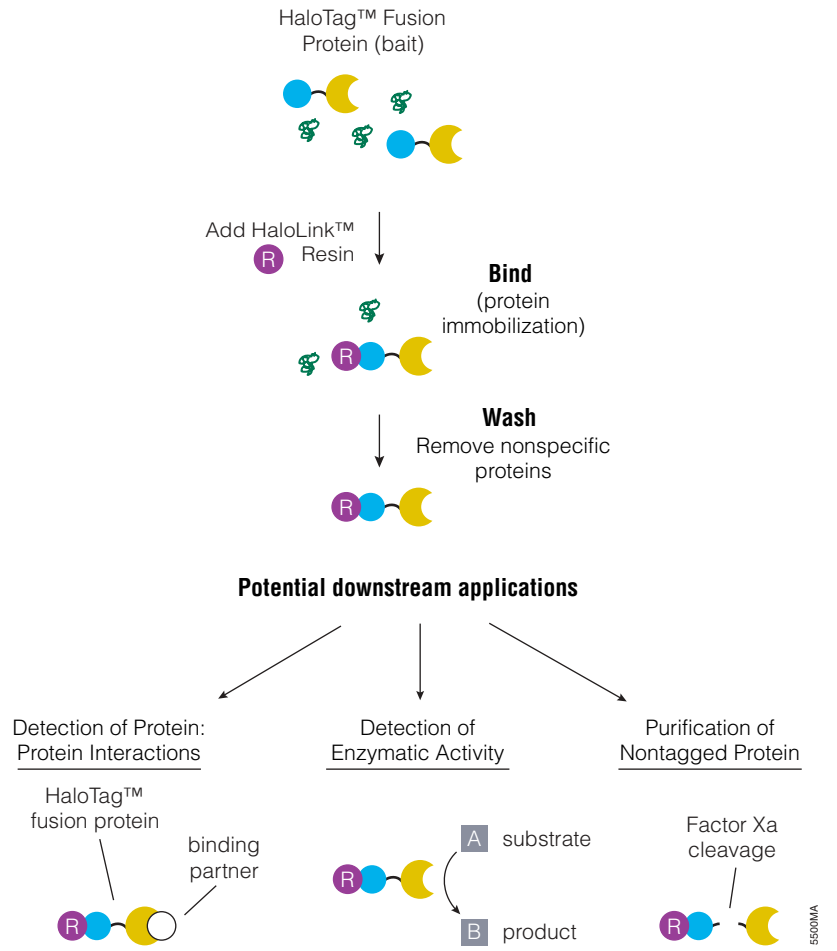

Figure 1. Overview of the HaloLink™ Resin immobilization protocol.

IV. Protocols

Materials to be Supplied by the User
(Buffer compositions provided in Section VII.A.)

- binding buffer
- wash buffer
- SDS-polyacrylamide gels
- rotating or shaking platform (i.e., tube rotator from Scientific Equipment Products, other mixing devices such as the IKA-SCHÜTTLER MTS2)
- BSA (Blot-Qualified BSA, Cat.# W3841)
- TNT® T7 Quick Coupled Transcription/Translation System (Cat.# L1170 or equivalent; see Section VII.C. Related Products)
- centrifuge **Note:** Speed in rpm can be calculated from the following formula: $RCF = (1.12)(r)(rpm/1000)^2$ where r = radius in millimeters measured from the center of spindle to bottom of rotor bucket. In a standard size microcentrifuge 800 x *g* corresponds to 3,000rpm (rpm = revolutions per minute).

IV.A. Immobilization of HaloTag™ Fusion Proteins on HaloLink™ Resin

The protocol below is optimized for binding of proteins synthesized using the in vitro expression systems (see Figure 1 for overview). We used the TNT® T7 Quick Coupled Transcription/Translation System (Cat.# L1170). Other in vitro expression systems can be used (see Section VII.C. Related Products and references 2 and 3). This protocol is a guideline and should be optimized for specific HaloTag™ fusion proteins. Volumes of protein samples and resin can be scaled up or down depending on the specific needs.

It is important that appropriate controls be included in the experiment to demonstrate that results are due to specific interactions. A negative control (resin only) without HaloTag™ fusion protein should be set up in parallel with the experimental sample to assay for nonspecific binding to the resin.

This protocol can also be used for immobilization of proteins expressed in vivo in mammalian cells. Immobilized HaloTag™ fusion proteins can be evaluated for enzymatic activity, protein:protein interactions, or can be cleaved from the resin with Factor Xa for use in other downstream applications.

Note: The HaloTag™ Vectors pFC8A (HaloTag™) CMV Flexi® Vector, pFC8K (HaloTag™) CMV Flexi® Vector and HaloTag™ pHT2 are optimized for expression in the in vitro coupled transcription/translation systems (see Section VII.C. Related Products) and in vivo in mammalian cells. **These vectors will not express proteins in bacterial cells.** See references 4 and 5 for more information.

Phase 1. Synthesis of the HaloTag™ Fusion Proteins

For in vitro synthesis refer to references 2 and 3 (Section VII.B.). For proteins expressed in mammalian cells we added 100µl of cytosolic fraction (from cells grown in a 10cm culture dish, resuspended in 1ml of 10mM HEPES [pH 7.0] and lysed) to 100µl of resuspended HaloLink™ Resin. General guidelines on the number of cells to use and cell lysis conditions can be found in reference 5. **Note:** For proteins expressed using Wheat Germ Extract Plus, 10mg/ml of BSA must be added to the binding buffer for efficient binding.

Phase 2. Resin Equilibration

Buffer compositions can be found in Section VII.A.

1. Mix HaloLink™ Resin by inverting to obtain a uniform suspension. Dispense 50µl of HaloLink™ Resin into a 1.5ml microcentrifuge tube and centrifuge for 1 minute at 800 x *g*. Carefully remove and discard the supernatant, leaving the resin at the bottom of the tube.
2. Add 400µl of binding buffer (Section VII.A.). Mix thoroughly by inverting the tube. Centrifuge for 2 minutes at 800 x *g*. Carefully remove and discard the supernatant, leaving the resin at the bottom of the tube. Repeat Steps 1–2 two additional times for a total of 3 washes.
3. After the final wash resuspend the resin in 50–100µl of binding buffer (Section VII.A.). **Note:** The volume used for resuspending the resin can be adjusted.

Phase 3. Binding of HaloTag™ Fusion Protein

1. Add 40–50µl of the in vitro transcription/translation reaction containing the HaloTag™ fusion protein to the equilibrated resin.
2. Incubate with mixing on a tube rotator (or equivalent device) for 30–60 minutes at room temperature (incubate at 4°C if proteins are unstable; at 4°C, longer incubation time may be required). Make certain that the resin does not settle to the bottom of the tube; settling will reduce the binding efficiency.
3. Centrifuge for 2 minutes at 800 x *g*. Save supernatant for analysis if desired.

Phase 4. Washing

1. Add 1ml of wash buffer (Section VII.A.) and mix thoroughly by inverting the tube several times. Centrifuge for 2 minutes at 800 x *g*. Discard the wash. Repeat two additional times.
2. Add 1ml of wash buffer and mix thoroughly by inverting the tube several times. Incubate at room temperature for 5 minutes with mixing. Centrifuge for 2 minutes at 800 x *g*. Discard the wash and repeat this step.
3. Resuspend the resin carrying covalently attached HaloTag™ fusion protein in the desired volume of a buffer compatible with downstream applications (see Figure 1).

IV.B. Detection of HaloTag™ Fusion Proteins

The HaloTag™ technology offers a quick and convenient way to test protein expression by fluorescent detection using the HaloTag™ TMR Ligand (Cat.# G8251). The fluorescent TMR-conjugated ligand can be added to a fraction of the in vitro transcription/translation reaction containing HaloTag™ fusion protein or to a fraction of cell lysate and analyzed by SDS gel electrophoresis (5). The same protocol can be used to monitor binding of HaloTag™ fusion proteins to the HaloLink™ Resin by analyzing the starting material and the supernatant recovered after protein binding (Phase 3, Step 3). This fraction contains unbound protein.

1. Prepare a 500-fold dilution of HaloTag™ TMR Ligand stock solution (5mM) in binding buffer (or TBS buffer) for a final concentration of 10µM.
2. Mix 2µl of in vitro transcription/translation reaction containing HaloTag™ fusion protein or an equivalent amount of unbound fraction (from Phase 3, Step 3) with 1µl of HaloTag™ TMR Ligand [10µM].
3. Add binding buffer or 1X TBS to a final volume of 10µl. Incubate at room temperature protected from light, for 30 minutes.
4. Remove 5µl of the reaction, add 5µl of 2X SDS gel loading buffer and heat to 70°C for 5 minutes. Separate on an SDS-polyacrylamide gel. Analyze on a fluorescent detection scanner (e.g., Typhoon®, Amersham Biosciences, Ltd.).

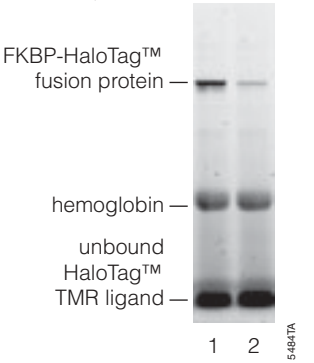

Figure 2. Detection of HaloTag™ fusion proteins using HaloTag™ TMR Ligand. Lane 1, TNT® T7 Quick Coupled Transcription/Translation System reaction expressing HaloTag™ FK506 binding protein (FKBP) fusion protein. Lane 2, FKBP-HaloTag™ fusion protein present in the unbound fraction. Five microliters of the HaloTag™ TMR Ligand labeling reaction was loaded onto the gel for both lanes. Hemoglobin is present in the rabbit reticulocyte lysate preparations; it autofluoresces and thus appears in both gel lanes.

V. General Considerations

V.A. Creating HaloTag™ Fusion Protein Constructs

Instructions for creating HaloTag™ Fusion Protein constructs can be found in the *Flexi® Vector Systems Technical Manual*, #TM254 (4) and the *HaloTag™ Interchangeable Labeling Technology Technical Manual*, #TM260 (5).

We suggest that the synthesis of proteins be verified. Section IV.B. provides guidelines for detection of HaloTag™ fusion proteins with a fluorescent TMR-conjugated ligand. If nontagged proteins are synthesized for detection of protein:protein interactions, we suggest using [^{35}S]met or FluoroTect™ Green in vitro Translation Labeling System (Cat.# L5001).

V.B. TNT® T7 Quick Coupled Transcription/Translation Reaction

We recommend using the TNT® T7 Quick Coupled Transcription/ Translation System for protein expression. Purified plasmid DNA used for the transcription/translation reaction should be free of residual ethanol and salts before it is added to the reaction. To isolate clean DNA we recommend using the Wizard® *Plus* Minipreps DNA Purification System, Wizard® *Plus* SV Minipreps DNA Purification System or the PureYield™ Plasmid Midiprep System (Cat.# A7100, A1330 and A2492, respectively). DNA prepared by the standard alkaline lysis method described by Sambrook, Fritsch and Maniatis (6) is also sufficiently clean for use in the TNT® T7 Quick Coupled Transcription/Translation System reaction.

For most plasmid constructs, optimal results are obtained when 1µg of plasmid DNA template is used. However, we have used 0.5–2.0µg of DNA template and obtained satisfactory levels of translation. The use of more than 1µg of plasmid does not necessarily increase the amount of protein produced. Linearized templates produced by restriction enzyme digestion of plasmids should be purified before use in an in vitro transcription/translation reaction. For more information refer to the *TNT® Quick Coupled Transcription/Translation System Technical Manual*, #TM045 and Hurst, R. *et al*. (2,7).

VI. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. E-mail: **techserv@promega.com**

Symptoms	Causes and Comments
Inefficient immobilization of HaloTag™ fusion protein onto resin.	Increase the volume of cell-free translation reaction added to HaloLink™ Resin.
	Increase the volume of reaction added to HaloLink™ Resin containing the bait, prey or bait-prey mix.
	Adjust the volume of HaloLink™ Resin used.

(continued)

VI. Troubleshooting (continued)

Symptoms	Causes and Comments
Inefficient immobilization of fusion protein to HaloTag™ resin	Increase time allowed for binding of HaloTag™ fusion protein onto HaloLink™ Resin.
(continued)	Adjust binding temperature.
	Proteolysis of translation product. Add protease inhibitor cocktail.
High background.	Volume of sample containing HaloTag™ protein was too large. Reduce the volume of reaction added to the HaloLink™ Resin.
	Nonspecific interactions can be diminished by increasing wash stringency. Increase the number and/or volume of washes and include salt or detergents in the wash buffer.
	Increase the amount of BSA in the buffers. Use Blot-Qualified BSA (Cat.# W3841) during washing.
	Add IGEPAL® CA-630 (NP 40 analog) at 0.05% final concentration, or 0.5% Triton®X-100 or 5% glycerol to reduce backgrounds.
	Too much HaloLink™ Resin results in high background. Reduce the amount of resin added to the reaction.
HaloTag™ fusion protein not detected with HaloTag™ TMR Ligand	Too much sample loaded onto SDS-polyacrylamide gel. Reduce the volume of the in vitro transcription/translation reaction loaded (e.g., 1–5µl). Too much sample causes hemoglobin precipitation and prevents protein migration into the gel due to aggregation.
	Fluorescent scanner does not have appropriate filters and/or sensitivity. Use fluorescent scanner with appropriate sensitivity (e.g., Typhoon®, Amersham Biosciences, Ltd.). For scanners with lower sensitivity, sample volume may need to be increased but use caution due to hemoglobin precipitation (see comment above).
	Samples may be overheated. Reduce denaturation temperature to 60°C for 5–10 minutes.

VII. Appendix

VII.A. Composition of Buffers

wash buffer

100mM Tris (pH 7.6)
150mM NaCl
1mg/ml BSA
0.05% IGEPAL® CA-630 (Sigma Cat# I 3021 or I 8896)

Note: If IGEPAL® CA-630 interferes with the activity of the protein of interest the concentration can be reduced to 0.001% or eliminated. However, this may result in higher nonspecific binding. If IGEPAL® CA-630 is reduced in concentration or eliminated, we recommend the use of either 0.5 % Triton® X-100 or 5% glycerol. BSA may also be eliminated if it interferes with protein activity, but higher nonspecific binding may result. Buffers other than Tris can be used in this protocol.

1X TBS

100mM Tris (pH 7.6)
150mM NaCl

binding buffer

100mM Tris (pH 7.6)
150mM NaCl
0.05% IGEPAL® CA-630 (Sigma Cat# I 3021 or I 8896)

Note: IGEPAL® CA-630 is added to prevent sticking of the resin to the sides of the tube. (IGEPAL® is an NP40 analog.) The effective range of IGEPAL® concentration is 0.001–0.05%. For ease of use we recommend preparing a 10% stock solution of IGEPAL® and making further dilutions from this solution.

Important: Buffers containing IGEPAL® should be stored at room temperature and used within one week of preparation.

4X SDS gel loading buffer

0.24M Tris-HCl (pH 6.8)
3mM bromophenol blue
50.4% glycerol
0.4M dithiothreitol
2% SDS

VII.B. References

1. *PinPoint™ Xa Protein Purification System Technical Manual*, #TM028, Promega Corporation.
2. *TNT® Quick Coupled Transcription/Translation Systems Technical Manual*, #TM045, Promega Corporation.
3. *TNT® Coupled Reticulocyte Lysate Systems Technical Bulletin,* #TB126, Promega Corporation.
4. *Flexi® Vector Systems Technical Manual,* #TM254, Promega Corporation.
5. *HaloTag™ Interchangeable Labeling Technology Technical Manual,* #TM260, Promega Corporation.
6. Sambrook, J. *et al.* (1989) *Molecular Cloning: A Laboratory Manual,* Second Edition. Cold Spring Harbor Press, Cold Spring Harbor, NY.
7. Hurst, R. *et al.* (1996) The TNT® T7 Coupled Transcription/Translation System. *Promega Notes* **58**, 8–11.

VII.C. Related Products

Vectors

Product	Size	Cat. #
HaloTag™ pHT2 Vector	20µg	G8241
pFC8A (HaloTag™) CMV Flexi® Vector	20µg	C3631
pFC8K (HaloTag™) CMV Flexi® Vector	20µg	C3641
Flexi® System, Entry/Transfer	5 entry and 20 transfer reactions	C8640
Flexi® System, Transfer	100 transfer reactions	C8820
Carboxy Flexi® System, Transfer	50 transfer reactions	C9320

Ligands

Product	Size	Cat. #
HaloTag™ TMR Ligand	30µl	G8251

Transcription/Translation Systems

Product	Size	Cat. #
TNT® T7 Quick Coupled Transcription/ Translation System	5 reactions	L1171
	40 reactions	L1170
TNT® T7 Coupled Reticulocyte Lysate System	40 reactions	L4610

For Laboratory Use.

Translation System

Product	Size	Cat. #
Wheat Germ Extract Plus	40 × 50µl	L3250
	10 × 50µl	L3251

Detection System

Product	Size	Cat. #
FluoroTect™ $Green_{Lys}$ in vitro Translation Labeling System	40 reactions	L5001

For Laboratory Use.

Reagents

Product	Size	Cat. #
Blot-Qualified BSA*	10g	W3841
Factor Xa Protease	50µg	V5581

*For Laboratory Use.

DNA Isolation System

Product	Size	Cat. #
PureYield™ Plasmid Midiprep System	25 preps	A2492
	100 preps	A2495

[a]Patent Pending. For research use only. Researchers may use this product in their own research. Research performed on behalf of another party, whether for monetary consideration or free of charge, is permitted. No reach-through payments shall be owed to Promega relating to an organization's commercialization of products that are the discoveries resulting from research use of this product or its derivatives, provided that such products of the organization do not fall within the scope of the valid claims of any issued patents assigned or licensed to Promega, or that such commercialization would not be a violation of the terms of this label license. No other use or transfer of this product is authorized without the express, written consent of Promega. With respect to manufacture or sale of research products, or any diagnostic, therapeutic or prophylactic uses, please contact Promega for supply and licensing information. If the purchaser is not willing to accept the conditions of this limited use statement, Promega is willing to accept return of the unopened product and provide purchaser with a full refund. However, in the event that the product is opened, then purchaser agrees to be bound by the conditions of this limited use statement.

[b]Patent Pending. For research use only. Researchers may use this product in their own research and they may transfer this product and its derivatives to others for research use provided that at the time of transfer a copy of this label license is given to the recipients and recipients agree to be bound by the terms of this label license. Research performed on behalf of another party, whether for monetary consideration or free of charge, is permitted. In addition, researchers must do one of the following: (1) use HaloTag™ ligands (including but not limited to a ligand linked to dyes, ligands that can be linked to other moieties, and ligands chemically linked onto surfaces) purchased exclusively from Promega or Promega's authorized distributors or (2) contact Promega to obtain a license. With respect to manufacture or sale of research products, or any diagnostic, therapeutic or prophylactic uses, please contact Promega for supply and licensing information. No reach-through payments shall be owed to Promega relating to an organization's commercialization of products that are the discoveries resulting from research use of this product or its derivatives, provided that such products of the organization do not fall within the scope of the valid claims of any issued patents assigned or licensed to Promega, or that such commercialization would not be a violation of the terms of this label license. No other use or transfer of this product or its derivatives is authorized without the express, written consent of Promega. If the purchaser is not willing to accept the conditions of this limited use statement, Promega is willing to accept return of the unopened product and provide purchaser with a full refund. However, in the event that the product is opened, then purchaser agrees to be bound by the conditions of this limited use statement.

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