INSTRUCTIONS

# GlycoLink™ IP Kit

## 88943

2312.2

| Number | Description |
|---|---|
| **88943** | **GlycoLink IP Kit,** contains sufficient reagents to perform 25 reactions using 20µL of immobilized antibody support |

**Kit Contents:**

**UltraLink® Hydrazide Gel,** 0.5mL of settled resin supplied as 50% slurry (e.g., 100µL of 50% slurry is equivalent to 50µL of settled resin)

**GlycoLink Coupling Buffer,** 50mL, 0.1M sodium acetate, 0.15M sodium chloride; pH 5.5

**Aniline,** 0.2mL

Molecular Weight: 93.13

**Wash Buffer,** 60mL, 1M sodium chloride, 0.05% sodium azide

**Sodium *meta*-Periodate,** 0.5g

Molecular Weight: 213.89

**Zeba™ Spin Desalting Columns, 7K MWCO,** 25 × 0.5mL

**IP Lysis/Wash Buffer,** 50mL, 0.025M Tris, 0.15M sodium chloride, 0.001M EDTA, 1% NP-40, 5% glycerol; pH 7.4

**BupH™ Phosphate Buffered Saline Pack,** 1 pack, yields 0.1M phosphate, 0.15M sodium chloride; pH 7.2 when reconstituted with 500mL of water

**Elution Buffer,** 50mL, pH 2.8, contains primary amine

**Lane Marker Sample Buffer, Non-reducing, (5X),** 5mL, 0.3M Tris•HCl, 5% SDS, 50% glycerol, lane marker tracking dye, pH 6.8

**Pierce® Spin Columns-Screw Cap,** 25 columns, includes accessories

**Microcentrifuge Collection Tubes,** 2mL, 100 each

**Storage:** Upon receipt store kit at 4°C. Product shipped at ambient temperature.

**Note: Aniline is highly toxic. Wear gloves and handle with extreme care.**

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## Introduction

The Thermo Scientific GlycoLink IP Kit contains all the components needed for immobilizing glycoproteins through oxidized sugar groups to produce oriented, covalent attachments for optimal immunoprecipitation. Polyclonal antibodies with abundant carbohydrates and monoclonal antibodies with adequate carbohydrates are ideal for GlycoLink Chemistry, resulting in immobilizations with unobstructed antigen-binding sites and optimal purification capability. The Thermo Scientific UltraLink Hydrazide Resin and a spin-column format enable efficient immobilizations and immunoprecipitations.

The immobilization chemistry uses sodium *meta*-periodate to oxidize glycoproteins and convert sugar *cis*-diol groups to reactive aldehyde groups. The aldehydes react with hydrazide groups on the GlycoLink Column to form stable hydrazone bonds (Figure 1) and aniline is added to catalyze the reaction, resulting in > 90% coupling in 4 hours or less. With the desired antibody immobilized, the resulting immunoprecipitation column can be used for studying a variety of protein-protein interactions.

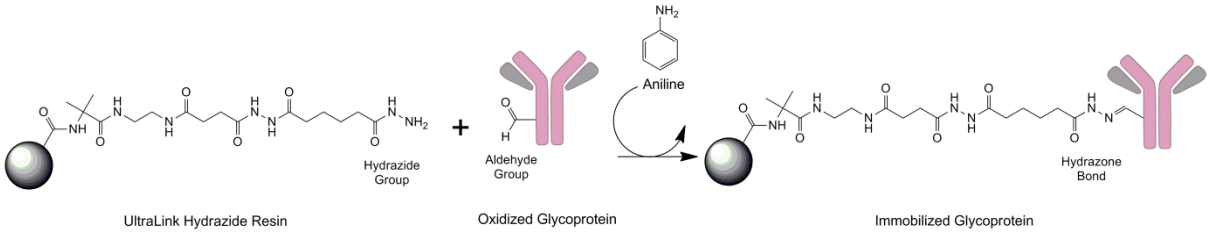


**Figure 1.** General structure and reaction scheme for the Thermo Scientific GlycoLink Column.

## Important Product Information

- Primary amines (e.g., Tris, glycine), glycerol and reducing agents in the antibody solution will interfere with coupling and oxidation. Remove these components before coupling using Thermo Scientific Zeba Spin Desalting Columns or Thermo Scientific Slide-A-Lyzer Dialysis Cassettes.
- Gelatin or carrier proteins in the antibody solution will compete for coupling sites on the resin. Remove gelatin and carrier proteins using the Thermo Scientific Pierce Antibody Clean-up Kit (Product No. 44600) or by performing Protein A/G purification (Product No. 20423) and dialysis.
- Perform antibody coupling at room temperature. Perform cell lysis and antigen IP at 4°C.
- Perform all resin centrifugation steps for 60 seconds at low speed (i.e., 1000 × *g*). Centrifuging at speeds greater than 5000 × *g* may cause the resin to clump and make resuspending difficult.
- When centrifuging spin columns with 2mL collection tubes, the flow-through volume should not exceed 600µL. Exceeding this volume may result in column back pressure and incomplete washing or elution.
- IP Lysis/Wash Buffer has been tested on representative cell types including, but not limited to, the following cell lines: HeLa, Jurkat, A431, A549, MOPC, NIH 3T3 and U2OS. Typically, $10^6$ HeLa cells yield ~10mg of cell pellet and ~3µg/µL (or 300µg) when lysed with 100µL of IP Lysis/Wash Buffer.
- For best results, add Thermo Scientific Halt Protease (Product No. 78429) and Phosphatase (Product No. 78420) Inhibitor Cocktails to lysis and wash buffers to minimize degradation and dephosphorylation of cell lysate proteins.
- The IP Lysis/Wash Buffer is compatible with the Thermo Scientific Pierce BCA Protein Assay (Product No. 23225).
- Proper controls are vital for identifying relevant interactions. Necessary negative controls include: antibody coupled to the UltraLink Hydrazide Gel without lysate added and resin without antibody coupled with lysate added.
- The Thermo Scientific Pierce Spin Columns package includes spin columns, screw caps, column plugs, Luer-Lok® Adapter Caps, large frits and a large frit tool. The large frit is not needed for the standard IP protocol. The Luer-Lok Caps have a flip top for use during wash steps. Use the screw caps for sealing the spin columns during storage (see the Additional Information Section).
- **Aniline is highly toxic. Wear gloves and handle with extreme care.**

## Procedure for the GlycoLink IP Kit

**Note:** The following protocol is for coupling 2-10µg of affinity-purified antibody in a solution free of primary amines (e.g., Tris, glycine), glycerol, BSA and gelatin (See the Important Product Information Section).

### A. Prepare Antibody for Coupling (oxidize carbohydrate groups)

1. Dilute or dissolve antibody in GlycoLink Coupling Buffer. Dilute samples at least three-fold in GlycoLink Coupling Buffer to a final volume of 100µL and pH < 6. For example, dilute 25µL of 0.4mg/mL rabbit polyclonal IgG in 75µL of GlycoLink Coupling Buffer. Alternatively, desalt to buffer exchange into GlycoLink Coupling Buffer to a final volume of 100µL.
2. Prepare 0.1M sodium *meta*-periodate by dissolving 5mg with 234µL of GlycoLink Coupling Buffer. Add 11µL of stock 0.1M periodate to 100µL of glycoprotein solution (10mM final periodate concentration). Cover tube with aluminum foil to protect from light and incubate for 30 minutes at room temperature. Stock periodate solution is stable for 1 week at 4°C when protected from light.

   **Note:** To prevent over-oxidation, do not exceed the 30 minute incubation.
3. Remove the top cap and the bottom tab of the 0.5mL desalting column and centrifuge at 1000 × *g* for 1 minute.
4. To equilibrate the desalting column, add 300µL of GlycoLink Coupling Buffer to the center of the column and centrifuge at 1000 × *g*. Repeat this step two times for a total of three equilibrations.
5. Slowly apply the oxidized glycoprotein solution to the center of the compact resin bed. Centrifuge at 1000 × *g* for 2 minutes and collect sample in a microcentrifuge tube. The collected solution contains the oxidized protein.

### B. Couple Oxidized Antibody to UltraLink Hydrazide Resin

1. Equilibrate the UltraLink Hydrazide Gel to room temperature and swirl to obtain an even suspension. Using a wide-bore tip or cut pipette tip, add 40µL of the resin slurry into a Pierce Spin Column. Place column into a microcentrifuge tube and centrifuge at 1000 × *g* for 1 minute. Discard the flow-through.
2. Add 100µL of GlycoLink Coupling Buffer and centrifuge to equilibrate resin. Repeat this step one time. Discard the flow-through, but save the collection tube. Remove excess liquid from the column by blotting the bottom on a paper towel and insert the bottom plug.
3. In a fume hood, prepare 0.2M GlycoLink Coupling Catalyst by adding 1.8µL of aniline to 100µL of GlycoLink Coupling Buffer. Vortex GlycoLink Coupling Catalyst for 10 seconds followed by brief high speed centrifugation. Add the entire volume to the oxidized antibody.

   **Note: Aniline is highly toxic. Wear gloves and handle with extreme care.**
4. Add oxidized glycoprotein sample to the column.

   Optional**:** Save 10µL of sample for evaluating binding efficiency using SDS-PAGE.
5. Replace the top cap and mix column by rocking or end-over-end mixing at room temperature for 2 hours.

   **Note:** Glycoproteins with > 3% glycosylation by weight may be incubated for < 2 hours.
6. Remove top and bottom column caps, place column into the same microcentrifuge tube from step B2 and centrifuge to collect non-bound protein. Discard flow-through.

   **Optional:** Decant flow-through from tube for evaluating binding efficiency using SDS-PAGE.
7. Wash the column with 200µL of GlycoLink Coupling Buffer and centrifuge. Repeat this step for a total of two washes.
8. Wash the column with 200µL of Wash Buffer and centrifuge. Repeat this step for a total of three washes.
9. Wash column with 200µL of degassed buffer at pH 7-8 (e.g., phosphate-buffered saline, PBS with optional 0.05% sodium azide) and centrifuge. Repeat this step for a total of three washes.

   Optional: Add 200µL of PBS and store column upright at 4°C for up to 1 week.

### C. Mammalian Cell Lysis

**Protocol I: Lysis of Cell Monolayer (Adherent) Cultures**

1. Carefully remove (decant) culture medium from cells.
2. Wash the cells once with ice cold PBS.
3. Add ice-cold IP Lysis/Wash Buffer (Table 1) to the cells. Incubate on ice for 5 minutes with periodic mixing.

**Table 1. Suggested volume of IP Lysis/Wash Buffer to use for different standard culture plates.**

| Plate Size/Surface Area | Volume of IP Lysis/Wash Buffer |
|---|---|
| 100 × 100mm | 500-1000µL |
| 100 × 60mm | 250-500µL |
| 6-well plate | 200-400µL per well |
| 24-well plate | 100-200µL per well |

4. Transfer the lysate to a microcentrifuge tube and centrifuge at ~13,000 × *g* for 10 minutes to pellet the cell debris.
5. Transfer supernatant to a new tube for protein concentration determination and further analysis.

**Protocol II: Lysis of Cell Suspension Cultures**

1. Centrifuge the cell suspension at 1000 × *g* for 5 minutes to pellet the cells. Discard the supernatant.
2. Wash cells once by suspending the cell pellet in 1X ice-cold PBS. Centrifuge at 1000 × *g* for 5 minutes to pellet cells.
3. Add ice-cold IP Lysis/Wash Buffer to the cell pellet. Use 500µL of IP Lysis/Wash Buffer per 50mg of wet cell pellet (i.e., 10:1 v/w). If using a large amount of cells, first add 10% of the final volume of IP Lysis/Wash Buffer to the cell pellet and pipette the mixture up and down to mix. Add the remaining volume of buffer to the cell suspension.
4. Incubate lysate on ice for 5 minutes with periodic mixing. Remove cell debris by centrifugation at ~13,000 × *g* for 10 minutes.
5. Transfer supernatant to a new tube to determine the concentration and further analysis.

### D. Antigen Immunoprecipitation General Protocol

**Note:** The amount of sample needed and the incubation time are dependent upon each specific antibody-antigen system and may require optimization for maximum yield.

1. Remove the bottom plug and loosen the screw cap of the spin column containing the antibody-coupled resin. Place the spin column in a collection tube and centrifuge to remove storage buffer. Discard the flow-through.
2. Remove the screw cap and place column into a collection tube. Wash resin two times with 200µL of ice-cold IP Lysis/Wash Buffer. Discard the flow-through after each wash.
3. Tap the bottom of the spin column on a paper towel to remove excess liquid. Replace the bottom plug.
4. Dilute the cell extract in IP Lysis/Wash Buffer. The recommended sample volume in the spin column is 300-500µL. The suggested amount of total protein per IP reaction is 250-500µg, as determined by the Pierce BCA Protein Assay.
5. Add the sample to the antibody-coupled resin in the spin column. Attach the screw cap and incubate column with gentle end-over-end mixing or shaking for 1 hour to overnight at 4°C.
6. Remove bottom plug, loosen the screw cap and place the column in a 2mL collection tube. Centrifuge column and save the flow-through. Do not discard flow-through until confirming that the IP was successful.
7. Remove the screw cap, place the column into a new 2mL collection tube, add 200µL of IP Lysis/Wash Buffer and centrifuge.

   **Note:** An alternative wash buffer (PBS Buffer) is supplied if a detergent-free wash is required.
8. Wash the sample three times with 200µL of IP Lysis/Wash Buffer and centrifuge after each wash.
9. Wash the sample once with 100µL of 1X PBS and centrifuge.

### E. Antigen Elution

1. Place the spin column into a new 2mL collection tube, add 25µL of Elution Buffer and centrifuge.

   **Optional:** To neutralize the low pH of the Elution Buffer (e.g., for downstream enzymatic or functional assays), add 5µL of 1M Tris, pH 9.5 to the collection tube, which will neutralize the pH upon centrifugation (Step E3). Alternatively, use a neutral pH elution buffer (i.e., Thermo Scientific Gentle Elution Buffer, Product No. 21027). For a more stringent elution, elute with 1X Lane Marker Sample Buffer, Non-reducing; however, this will result in loss of protein activity.

2. Keep the column in the tube and add 75µL of Elution Buffer. Incubate for 10 minutes at room temperature. The column does not need to be closed or mixed.

   **Note:** For a more concentrated eluate, less Elution Buffer may be used; however, overall yield may be reduced.

3. Centrifuge the tube and collect the flow-through. Analyze the eluate for presence of antigen. Perform additional elutions (i.e., Steps E1-E3) as needed. Analyze each eluate separately to ensure that the antigen has completely eluted.

### F. Sample Preparation for SDS-PAGE Analysis

1. Equilibrate the 5X Lane Marker Sample Buffer to room temperature. Gently mix the sample buffer by inverting 5-10 times. For a reducing gel, add 1M DTT to a final concentration of 100mM in the 5X Lane Marker Sample Buffer.
2. Add 5X Lane Marker Sample Buffer to sample to make a 1X final solution (i.e., add 5µL of 5X Lane Marker Sample Buffer to 20µL of sample).
3. Heat the sample at 95°C for 5 minutes. Allow the sample to cool to room temperature before applying to the gel.

## Troubleshooting

| Problem | Possible Cause | Solution |
|---|---|---|
| Antibody detected with the eluted antigen | Non-coupled antibody was not removed sufficiently with the Wash Buffer during the coupling procedure | Wash the antibody-coupled resin with Elution Buffer until no additional antibody elutes from the resin, as determined by protein assay or measuring the absorbance at 280nm |
| | Hydrazone linkage had been hydrolyzed during IP or elution procedure | Repeat conjugation immediately using the antibody-resin complex |
| | | Use gentle elution conditions |
| | Antibody light chain was present in elution | Avoid reducing agents |
| | | Use the Thermo Scientific Pierce Crosslink IP Kit (Product No. 26147) |
| Antigen did not immunoprecipitate | Sample did not contain enough antigen to detect | Verify protein expression and/or lysis efficiency of the lysate by SDS-PAGE or Western blot |
| | Antibody did not couple to the resin | Ensure the antibody solution does not contain amines, glycerol, or carrier proteins |
| | | Verify the antibody coupling by monitoring the flow-through and wash fractions (i.e., measure the absorbance at 280 nm or analyze by SDS-PAGE) |
| | Component in the IP Lysis/Wash Buffer interfered with antibody-antigen binding | Perform the IP and washes using Phosphate-Buffered Saline |

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|---|---|---|
| Antigen did not elute | The antibody-antigen interaction was not disrupted by the Elution Buffer | Optimize the elution conditions (see Additional Information Section) |
| | | Add 100µL of 1X non-reducing SDS sample buffer to the column and incubate at room temperature for 5-10 minutes; keep the spin column in the tube while incubating and do not plug or cap the column* |
| Antigen is non-functional in the downstream application | Antigen was sensitive to low pH and had become inactive during the elution process | Repeat the IP and use a high-salt, neutral pH elution buffer (e.g., Gentle Elution Buffer, Product No. 21027) |

*After eluting the antibody-coupled resin with SDS sample buffer, the resin cannot be reused and must be discarded.

## Additional Information

**A. Please visit the website for additional information including the following:**

- Tech Tip #27: Optimize elution conditions for immunoaffinity purification
- Tech Tip #40: Convert between times gravity (× *g*) and centrifuge rotor speed (RPM)
- Tech Tip #43: Protein stability and storage

**B. Pierce Spin Columns**

The Pierce Spin Columns can hold up to 900µL. Columns can be placed in 1.5mL or 2mL microcentrifuge tubes or used with a Luer-Lok Adapter (see Figure 1) for processing samples with a syringe. When using a syringe, sample size and wash volumes are limited only by the volume capacity of the syringe. For small volumes of resin, use columns with only the small, pre-inserted frit (Figure 2A). For applications requiring more than 100µL of resin, the large frit may be used at either the top or bottom (Figure 2B and 2C). Resins may be used repeatedly when the resin is in-between the small and large frit (Figure 2B).

- To remove a frit from a column, use an unfolded paper clip and insert the wire through the column tip to push the frit up.
- To insert a frit, place the frit inside the column and use the frit tool to push the frit into position.
- To remove the top frit from an already-packed column with a top and bottom frit, use an unfolded paper clip to tip the top frit up. The top frit can be then removed with tweezers.

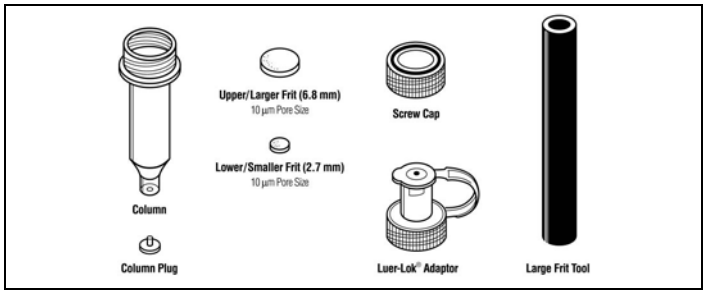


**Figure 1.** Schematic of the Thermo Scientific Pierce Spin Column contents.

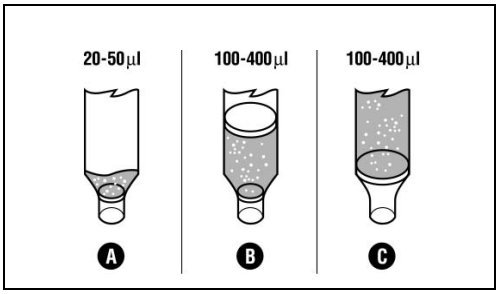


**Figure 2.** Three formats for frit placement.

## Related Thermo Scientific Products

| | |
|---|---|
| **78428** | **Halt™ Phosphatase Inhibitor Single-Use Cocktail (100X),** 100µL × 24 microtubes |
| **78440** | **Halt Protease and Phosphatase Inhibitor Cocktail (100X),** 1mL |
| **78430** | **Halt Protease Inhibitor Single-Use Cocktail (100X),** 24 × 100µL |
| **69705** | **Pierce Spin Columns-Screw Cap,** 25/pkg |
| **69720** | **Pierce Microcentrifuge Tubes, 2mL,** 72/pkg |
| **21027** | **Gentle Ag/Ab Elution Buffer, pH 6.6,** 500mL |
| **88941** | **GlycoLink Immobilization Kit** |
| **88942** | **GlycoLink Micro Immobilization Kit** |
| **88944** | **GlycoLink Coupling Catalyst, 100mL** |

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