

Instruction Manual
for
Catch and Release[®]
Phosphotyrosine, clone 4G10[®]
Immunoprecipitation Kit
Catalog # 17-502

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DO NOT USE IN HUMANS.

I. Introduction and Principle

Immunoprecipitation (IP) is a frequently used method to purify specific proteins from complex samples such as cell lysates or extracts. Traditional IP protocols use Protein A or Protein G coupled to an insoluble resin, such as agarose beads, to capture an antigen/antibody complex in solution. The complex is then “precipitated” by centrifugation. Limitations of traditional IP include sample handling and processing difficulties, the inability to release native antigen from the beads for functional assays and poor reproducibility and recovery due to multiple wash steps.

The Catch and Release[®] Phosphotyrosine, clone 4G10[®] Immunoprecipitation Kit overcomes many of the limitations associated with traditional IP of phosphorylated tyrosine side chain residues within proteins. Its unique Spin-Column format was designed to make IP faster, simpler and more reproducible. The Catch and Release[®] Phosphotyrosine, clone 4G10[®] Immunoprecipitation Kit enables the elution of the tyrosine phosphorylated antigen/antibody complex without denaturation, while ensuring minimal contamination by non-specific proteins in the eluate. In addition, the convenient Spin-Column format of Catch and Release[®] Phosphotyrosine, clone 4G10[®] Immunoprecipitation Kit improves performance and makes higher throughput processing of samples possible.

Catch and Release[®] Phosphotyrosine, clone 4G10[®] Immunoprecipitation Kit contains a proprietary resin in a microfuge-compatible Spin Column secured by a screw cap top and a breakaway closure on the bottom. Most lysate proteins and antibodies will exhibit little or no binding to the resin. It is this feature that enables the fast and simple elution of the tyrosine phosphorylated antigen: Phosphotyrosine, clone 4G10[®] antibody complex from the resin in either a denatured or native form.

Kit Description

Quantity: 50 Immunoprecipitations per kit.

Storage and Stability: All components to be stored at 4°C except the Catch and Release[®] Capture Tubes which are stored at room temperature. Components are stable for 6 months from date of shipment.

Use: This kit allows for quick and reproducible immunoprecipitation (IP) of phosphorylated tyrosine residues on proteins by using a spin column system. The system is more reproducible than regular IP's, which are problematic with regards to washing the protein A/G agarose without disrupting the agarose bed. The binding of the antibody/antigen complex in Catch and Release[®] is reversible, and elution of the immune complex can occur with native or denaturing buffers. This kit is only suitable for use with Anti-Phosphotyrosine, recombinant clone 4G10[®], Catalog # 05-777. **This kit cannot be used with any other general or phosphotyrosine* antibody other than the one listed above.** Please read the attached protocol before use.

*****Native 4G10[®] antibody, Catalog # 05-321, CANNOT be used with this kit.******

Other Recommended Kits and Control Reagents:

- For Immunoblot Analysis:
 - Visualizer™ Western Blot Detection Kit, mouse (Upstate Catalog # 64-201)
 - Visualizer™ Western Blot Detection Kit, rabbit (Upstate Catalog # 64-202)
 - ChemiBlot™ Molecular Weight Markers, (Chemicon Catalog # 2230)
 - Blot -FastStain™ (Chemicon Catalog # 2076)
 - ChemiBLOCKER™ (Chemicon Catalog # 2170)
 - ReBlot™ Western Blot Recycling Kit (Chemicon Catalog # 2060)
- For negative (IP) species controls:
 - Normal Mouse IgG (Upstate Catalog # 12-371)
 - Mouse Serum (Chemicon Catalog # S25-10ML)
 - Normal Rabbit IgG (Upstate Catalog #12-370)
 - Rabbit Serum (Chemicon Catalog # S20-100m)

II. KIT COMPONENTS

A. Provided Kit Components (Note Storage Temperatures)

Note: This kit **CANNOT** be used with any other general or phosphotyrosine* antibody other than the one included with this kit, Catalog # 05-777 or 05-777B.

*Native 4G10® antibody, Catalog # 05-321, **CANNOT** be used with this kit.

Anti-Phosphotyrosine, recombinant clone 4G10®, Catalog # 05-777B. One vial containing 250µg of protein G purified, recombinant clone 4G10® mouse IgG_{2bκ} in 267µl of PBS, pH 7.5. Liquid at 4°C. Store at 4°C.

Catch and Release® Wash Buffer, 10X, Catalog # 20-210. One vial containing 15ml of 10X buffer, pH 7.4 containing the following detergents: 10% NP-40, 2.5% deoxycholic acid and 150mM imidazole. Store at 4°C. **Note:** If crystallization occurs when buffer is stored at 4°C, warm to room temperature and vortex briefly before use.

Catch and Release® Non-denaturing Elution Buffer, 4X, Catalog # 20-209. One vial containing 10ml of 4X PBS-based IP Elution Buffer. Store at 4°C.

Catch and Release® Denaturing Elution Buffer, 1X, Catalog # 20-284. One vial containing 4ml of 1X Tris-based IP Elution Buffer. Add β-mercaptoethanol (bME) to a final concentration of 5% v/v immediately before use. Store at 4°C.

Catch and Release® v2.0 Spin Columns, 50 columns containing 0.5ml of prepacked IP capture resin. Store at 4°C

Catch and Release® Capture Tubes, 100, 2ml reservoir tubes.

B. Required Materials Not Provided

- Cell lysates
- Specific primary antibodies
- Milli-Q® water
- Variable volume (5-200µl) pipet + tips
- Sterile microcentrifuge tubes
- Microcentrifuge
- Rotator or rocker
- Forceps
- **If performing Immunoblot Analysis on immunoprecipitated material, material required might include:**
- SDS-PAGE reagents and apparatus
- Immunoblotting reagents and apparatus
- PVDF or other membrane
- Saran Wrap®
- Kimwipes®
- Specific primary antibodies
- Wash buffer
- Blocking buffer
- X-ray film and dark room or digital imaging system
- Ponceau stain (optional)
- Stripping buffers (optional)
- Membrane incubation containers
- Timer

III. Catch and Release® Phosphotyrosine, clone 4G10® IP Procedure

A. General Notes

- Unless otherwise noted, all dilutions of stock reagents provided in the kit are to be done with high-quality water, such as Milli-Q® water.
- If gel electrophoresis is to be performed, it should be done according to the specifications set by the manufacturers of the gel and the apparatus, taking into consideration the specific protein(s) that need to be resolved.
- When transferring the resolved proteins to a membrane, follow the recommendations set by the manufacturer of the transfer apparatus.
- IP with Catch and Release® Phosphotyrosine, clone 4G10® Immunoprecipitation Kit and Western blot detection with Visualizer™ is compatible with either nitrocellulose or PVDF membranes.

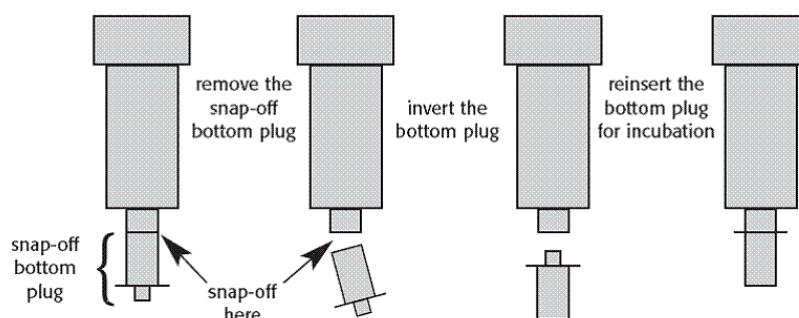
B. Optimizing Incubation Time and Temperature

The Catch and Release® Phosphotyrosine, clone 4G10® Immunoprecipitation Kit procedure as outlined below uses incubation times and temperatures that have been demonstrated by Upstate Biotech, Inc. to work well with the anti-Phosphotyrosine, recombinant clone 4G10® monoclonal antibody included in this kit. These conditions may differ from the conditions your laboratory uses in a traditional IP procedure. The IP method may be modified by adjusting incubation times: Your protein might exhibit optimal binding to the anti-Phosphotyrosine, recombinant clone 4G10® monoclonal antibody in as little as 10-15 minutes, or may require an overnight incubation. Some incubations will work at room temperature, while others are best performed at 4°C. These two key parameters should be empirically determined by the researcher for every lysate and target phosphorylated tyrosines on the protein of interest.

C. Catch and Release® Protocol

1. Dilute enough 10X Catch and Release® Wash Buffer to the 1X working concentration with Milli-Q® water for incubation and all washes. You will need approximately 2.5ml for washes and some additional volume possibly for the antibody incubation (Step 4).
2. Label the Spin Columns, Capture Tubes and microcentrifuge tubes to be used. Remove the snap-off bottom plug (save for later use; see figure 1 on the next page), and insert the Spin Column into a Capture Tube. Remove the screw-on cap and centrifuge at 5000 rpm (2000 x g) for 15-30 seconds to remove the resin slurry buffer. Wash the resin twice with 400µl 1X Wash Buffer. Empty the Capture Tube, and plug the bottom end of the Column with the snap-off bottom plug.

Figure 1



3. Determine the volume of combined reagents:
 - a. 500µg of cell lysate.

Notes: 1. This is a recommended starting amount, but the optimal amount may need to be empirically determined for each individual antibody and antigen.

2. High concentrations (>1mM) of reducing agents, such as dithiothreitol (DTT) or β-mercaptoethanol (bME), may denature antigens or antibodies and prevent capture.
 - b. 1-4µg of a specific primary antibody and negative control antibody or 5-10µl of whole antiserum or ascites fluid.

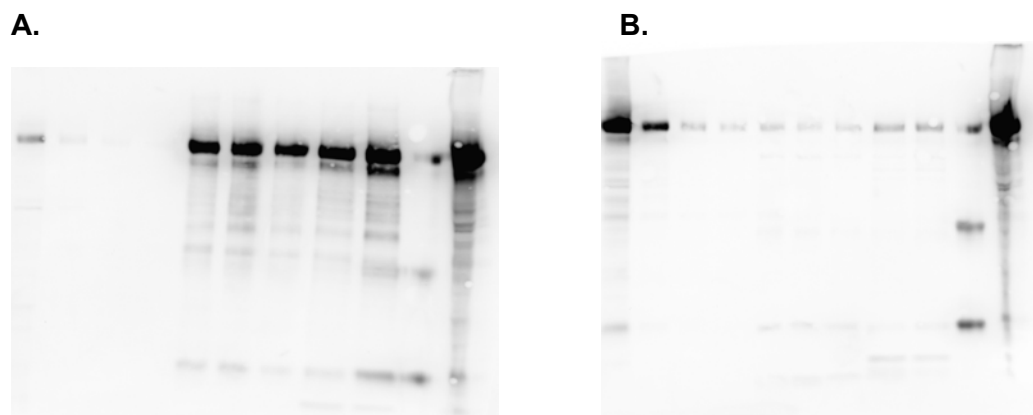
Note: These are recommended starting amounts but the optimal amounts may need to be empirically determined for each individual antibody and sample containing antigen. Upstate highly recommends performing corresponding negative IP controls (for any immunoprecipitation procedure) as side-by-side comparison. Please see page 3 for a listing of recommended negative IP control antibodies.
 - c. Sufficient 1X Wash Buffer to provide a final total volume of 500µl.

<u>Component</u>	<u>Amount</u>
Cell Lysate	_____ µl
Antibody	_____ µl
1X Wash Buffer	_____ µl
Total	500µl

4. With the bottom end of the Spin Column plugged, add the reagents in the following order to the column:
 - a. 1X Wash Buffer
 - b. Cell lysate
 - c. Specific primary antibody or negative control antibody
5. Cap the top of the Column (using the screw-on cap), and incubate on a rotator or mixer at room temperature for 30 minutes (see page 4 for more information on incubation times and temperatures), ensuring that the slurry remains suspended during incubation.
6. Remove the snap-off bottom plug and discard. Place the Column in the Capture tube. Remove the screw-on cap and centrifuge at 5000 rpm (2000 x g) for 15-30 seconds to collect flow-through. Transfer the flow-through to a microcentrifuge tube and save for Western blot analysis, if desired (it may be useful for trouble-shooting, if necessary).
7. Wash the Column 3 times with 400µl of 1X Wash Buffer, spinning at 5000 rpm (2000 x g) 15-30 seconds for each wash. Washes may be saved (if desired) for Immunoblot analysis and trouble-shooting.
8. Place the Column into a fresh Capture Tube.
9. Proteins may be eluted from the Column in either a denatured form (i.e. for SDS-PAGE and Immunoblotting), or a native form, following 9A or 9B.
 - a. For elution of protein in its denatured, reduced form:
Add 70µl of 1X Denaturing Elution Buffer containing β-mercaptoethanol to the Spin Column. Centrifuge and save eluate for Immunoblot analysis.
Note: This can be done as an additional step after Step 9B.
 - b. For elution of protein in its native form:
Dilute 4X Non-Denaturing Elution Buffer to 1X, and add 70µl to the Spin Column. Centrifuge the Spin Column at 5000 rpm (2000 x g), and save the eluate for Immunoblot analysis or other assays.

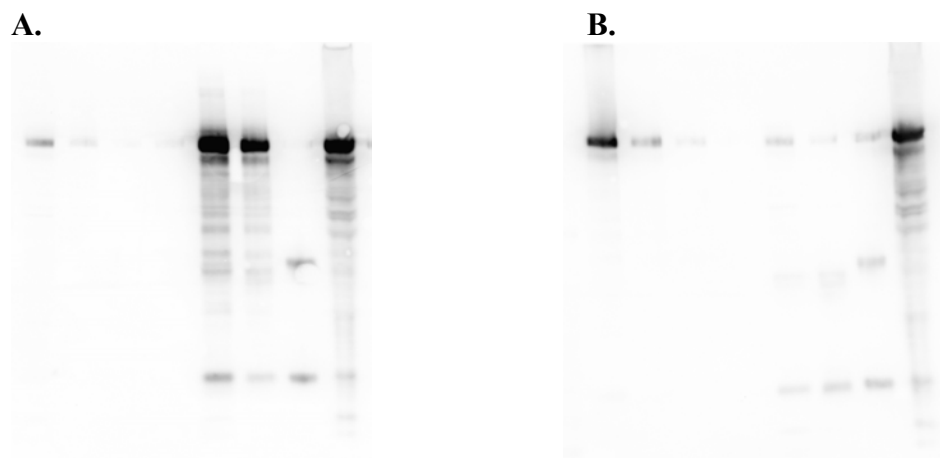
IV. Catch and Release® vs. Traditional Immunoprecipitation Data

Figure 1. Catch and Release® Phosphotyrosine, clone 4G10® Immunoprecipitation Kit



Catch and Release Spin columns and protocol were used with the Non-denaturing Elution Buffer to immunoprecipitate tyrosine phosphorylated proteins. EGF-stimulated A431 cell lysate (Catalog # 12-302) was mixed with A. Anti-Phosphotyrosine, recombinant clone 4G10® (Catalog #05-777) or B. Normal, Mouse IgG (Catalog # 12-371) as a negative control for 1 hour at room temperature. Samples from each fraction were run on an SDS-PAGE gel and immunoblotted with Anti-Phosphotyrosine, recombinant 4G10® (Catalog #05-777). Bands indicate tyrosine phosphorylated proteins Lane 1: Flow through, Lane 2: Wash 1, Lane 3: Wash 2, Lane 4: Wash 3, Lane 5: 1X Non-denaturing Elution Buffer, Lane 6: 2X Non-denaturing Elution Buffer, Lane 7: 4X Non-denaturing Elution Buffer, Lane 8: 1X Denaturing Elution Buffer, Lane 9: Boiled beads (to detect complex remaining following elutions), Lane 10: 1µg anti-Phosphotyrosine, recombinant clone 4G10®, (Panel A) or 1µg Normal, Mouse IgG (Panel B), Lane 11: 30µg EGF-stimulated A431 lysate.

Figure 2. Traditional IP with denaturing elution



Traditional immunoprecipitation was performed for tyrosine phosphorylated proteins using Protein A agarose and the denaturing elution buffer. EGF-stimulated A431 cell lysate (Catalog # 12-302) was mixed with A. Anti-Phosphotyrosine, clone 4G10® (Catalog # 05-777) or B. Normal, Mouse IgG (Catalog # 12-371) as a negative control for 1 hour at room temperature. Samples from each fraction were run on an SDS-PAGE gel and immunoblotted with anti-Phosphotyrosine, recombinant clone 4G10® (Catalog #05-777). Bands indicate tyrosine phosphorylated proteins. Lane 1: Flow through, Lane 2: Wash 1, Lane 3: Wash 2, Lane 4: Wash 3, Lane 5: Denaturing elution, Lane 6: Boiled beads (to detect complex remaining following elution), Lane 7: 1µg anti-Phosphotyrosine, clone 4G10®, (Panel A) or 1µg Normal mouse IgG (Panel B), Lane 8: 30µg EGF-stimulated A431 cell lysate (Catalog # 12-302).

V. Technical Support and Troubleshooting

A. Catch and Release® Frequently Asked Questions

Q: What is the blue eluate sometimes seen after initial washes?

A: This has been seen when spinning the columns at high speeds during the initial Spin Column resin washes. However, this should not affect column binding efficiency. Reducing centrifuge speed to 5000 rpm (2000 x g) should eliminate the blue color in the eluate.

Q: What should the user do if a viscous precipitate forms in the column during the initial incubation step?

A: Genomic DNA is present in the lysate. Try clarifying the lysate by spinning out genomic DNA at 15,000 rpm for 5 minutes at 4°C. Some of the protein of interest might also be removed in the process. Re-check protein concentration of the clarified sample, and increase volume of lysate to use if necessary.

Q. Why did everything (i.e., my primary antibody and target protein) come through in the flow-thru and column wash fractions?

A: No antibody added. Repeat incubation adding antibody[tp1].

Q: Why are bands faint or not present in the elution fractions and flow-thru?

- A:**
- Insufficient exposure time during Immunoblot detection procedure. Increase exposure time on x-ray film or digital imaging system.
 - Antibody concentration not sufficient or not optimized for either IP or Immunoblot detection. Repeat procedures with increased primary antibody concentration.
 - Secondary antibody concentration is not optimal. Optimize for Immunoblot detection, and re-probe blot after stripping.
 - Immunoblot detection reagents old or expired. Retry using fresh reagents.
 - Cell lysate contained low levels of antigen.
 - Increase incubation time for IP[tp2].

B. Immunoblot Analysis Troubleshooting:

Smear Pattern or Distorted Bands

- Uneven contact between gel and membrane: Cassettes used should allow a tight fit, leading to even pressure over the entire surface of the gel and membrane.
- Gel not equilibrated in buffer prior to transfer: The gel should be soaked in transfer buffer containing methanol for 15 to 30 minutes before assembling the transfer sandwich.

"Bald Spots"

- Bubbles between gel and membrane: Bubbles create areas of low transfer efficiency. Bubbles should be completely removed when putting together the transfer sandwich.

Incomplete Transfer

- Incomplete protein transfer: This often occurs with high molecular weight proteins, especially when using a methanol-based transfer buffer. One way to prevent this is by using a nylon membrane, which does not require methanol in the transfer buffer. Adding SDS to the transfer buffer and using higher field strengths also improve protein transfer.
- Proteins transferred through membrane: This may occur when working with proteins of very low molecular weight. Optimizing/shortening transfer times and using a double layer of membrane usually enhances retention of small proteins.
- Inappropriate transfer buffer used: The most stable and commonly used buffers are Tris-Glycine based.
- Impurities in the transfer buffer: This will lead to a pattern on the membrane that mirrors the holes in the transfer cassette. Fresh buffer should be prepared for each transfer.

High Background

- Cross-reactivity between blocking agent and primary antibody: this will result in overall membrane background. Usually, the addition of detergent (Tween®-20) to the Washing Buffer will eliminate the problem. If background persists, changing the blocking agent is recommended.
- Concentration of antibody too high or incubation time too long: the higher the antibody concentration and the longer the incubation time, the greater the non-specific binding. Raising the incubation temperature (e.g. to 37°C) is recommended over lengthening the incubation time. Also, many short washing steps are better than a few long ones.
- Membrane drying during incubation process: care should be taken to keep membrane from drying out during incubation.

Little or No Signal

- Antigen is not recognized by primary antibody: this can occur especially with monoclonal antibodies that were raised against a native protein. In some cases, a non-reducing gel system may need to be used.
- Inhibition of secondary antibody conjugate: HRP-labeled antibodies should not be used in conjunction with sodium azide or hemoglobin.
- Detergent is too harsh: SDS, Nonidet P-40, and Triton X-100 disrupt binding between proteins. Tween®-20 is the most commonly used and recommended detergent for washing and incubation solutions.

VII. References

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