Instruction Manual
for
Catch and Release®
Catch & Release® v2.0 High Throughput (HT) Immunoprecipitation Kit – 96 well

Catalog # 17-501

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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® High Throughput (HT) Immunoprecipitation Kit overcomes many of the limitations associated with traditional IP methods. Catch and Release® enables the elution of the antigen: antibody complex while ensuring minimal contamination by non-specific proteins in the eluate. The 96 well format of Catch and Release® improves performance and makes higher throughput processing of samples possible and reduces the required repeated washings to efficiently remove unbound material. The labor of this approach is compounded by the number of IP reactions being performed.

The High Throughput Immunoprecipitation protocol (HTIP) developed by Upstate Biotech, Inc. offers a significant savings in time and reagents. The protocol is performed in a filter-bottom 96-well plate, allowing for decreased washing time of the solid matrix by using vacuum in place of centrifugation. Using vacuum filtration to remove unbound materials in this way has improved the efficiency of the wash steps and increased the number of concurrent immunoprecipitations the user can perform (up to 96 wells). Our quality control data shows that absorption of antibody to the Catch and Release® resin followed by the capturing of antigen from cell lysates is as efficient as the traditional microcentrifuge tube methods. The results also show that the amount of reagents needed for each IP reaction are generally much less than required by the tube methods.

Kit Description

Quantity: 96 Immunoprecipitations per kit.

Storage and Stability: All components to be stored at 4°C except the Catch and Release® plates 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) by using a 96 well filter plate. 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. The system has been tested successfully with rabbit, mouse, sheep and goat antibodies. IP using human IgG_{1-4} should be suitable. IP using chicken antibodies or human IgA, IgD, IgE or IgM is not recommended with this kit. Please read the enclosed product manual before use.

Table 1: **Select Proteins Tested with Catch and Release® v2.0 High Throughput (HT) IP Kit**

Protein	Mol. Weight (kDa)	Upstate Antibody Catalog #	Host
Akt1/PKBα	60	07-416	Rabbit
cdk2	33	06-505	Rabbit

Proteins have also been tested with antibodies to the following epitope tags:

- Flag
- GFP
- HA
- Myc

Note:

- Catch and Release® is not suitable to immunoprecipitate proteins expressing 6X Histidine epitope tagged fusion proteins (i.e., His-tagged fusion proteins) due to non-specific interactions with the resin.

Table 2: **Negative Control Recommendations:**

- Normal Mouse IgG (Upstate Catalog # 12-371)
- Mouse Serum (Chemicon Catalog # S25-10ml)
- Normal Rabbit IgG (Upstate Catalog # 12-370)
- Rabbit Serum (Chemicon Catalog # S20-100ml)

II. KIT COMPONENTS

A. Provided Kit Components (Note Storage Temperatures)

Antibody Capture Affinity Ligand, Catalog # 20-216. One vial containing **60µg** Antibody Capture Affinity Ligand in **600µl** PBS containing 2mM PMSF and 10% glycerol. Store at 4°C.

Catch and Release® v2.0 HT Affinity resin, Catalog # 16-250. One vial containing **1.2ml** (20% w/v) of IP capture resin in suspension. 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. 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® 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® 96 well filter plate, 1 plate. Store at room temperature.

Catch and Release® Capture 96 well plate, 1 plate. Store at room temperature.

10X PBS, Catalog # 20-281. One bottle containing **24ml** 10X PBS. Dilute to 1X with water prior to use. Store at 4°C.

Required Materials Not Provided

- Cell lysates
- Specific primary antibodies
- Milli-Q® water
- Variable volume (5-200µl) pipet + tips
- 96 well plate Microcentrifuge
- Rotator or rocker
- Millipore Multiscreen vacuum manifold (Millipore Catalog # MAVM0960R)
- **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® v2.0 (HT) Immunoprecipitation 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® and Western blot detection with Visualizer™ is compatible with either nitrocellulose or PVDF membranes.

B. Optimizing Incubation Time and Temperature

The Catch and Release® procedure as outlined below uses incubation times and temperatures that have been demonstrated to work well with antibodies for many proteins* (see Table 1, page 2). If these conditions differ from the conditions used in your traditional IP procedure, you should follow your traditional method in your first use of the kit. For example, if you normally incubate a sample with your primary antibody for 1 hour at 4ºC, then you should do the same in Step 7 (see page 4). Once you have verified that Catch and Release® works with your antibody and protein, you may choose to optimize the procedure by adjusting incubation times. Some antibodies will exhibit optimal antigen binding in as little as 10-15 minutes, others may require an overnight incubation; some incubations will work at room temperature, while others are best performed at 4ºC. These two key parameters should be empirically determined by the researcher for every antibody, lysate and protein of interest.

C. Catch and Release® v2.0 HT Immunoprecipitation Protocol

1. Pre-wet the 96 well filter plate with 50µl 1X PBS.
2. Add 5µl of 20% w/v slurry resin and 2µl of Affinity Ligand with 50µl of 1X PBS to each well.
3. Gently shake the reaction mixture on a microtiter plate shaker at room temperature for 30 minutes.
4. Wash 3 times with 100µl 1X PBS by aspirating the filter plate on the Millipore Multiscreen vacuum manifold (Millipore Catalog # MAVM0960R).
5. Add 2-4µg of antibody to the reaction mixture with 50µl of 1X PBS in 96 well filter plate.
6. Gently shake the reaction mixture on a microtiter plate shaker at room temperature for 30 minutes.
7. Wash three times with 100µl 1X PBS by aspirating the filter plate on Millipore Multiscreen vacuum manifold.
8. Dilute the cell lysate to ~1µg/µl total cell protein with 1X PBS.
9. Add 100µg/100µl of the cell lysate to the reaction mixture in each well of the 96 well filter plate.
10. Gently shake the reaction mixture on a microtiter plate shaker at room temperature for 1 hour.
11. Wash three times with 100µl 1X Wash Buffer by aspirating the filter plate on the Millipore Multiscreen vacuum manifold.
12. Add 100µl of 1X Wash Buffer to the well and incubate for five minutes with gentle shaking.
13. Wash two times with 100µl 1X wash buffer by aspirating the filter plate on the Millipore Multiscreen vacuum manifold.
14. Store the beads frozen at -80°C for future analysis or resuspend with 30µl of 2X RSB to each well, and place the filter plate on a heat block at 90°C for 5 minutes.
15. Collect samples by placing a 96-well plate underneath the 96-well filter plate and centrifuge @ 1500rpm for one minute.
16. SDS-PAGE and immunoblot analysis on a sample of the supernatant fraction.

IV. Catch & Release v2.0 HT Immunoprecipitation Worksheet

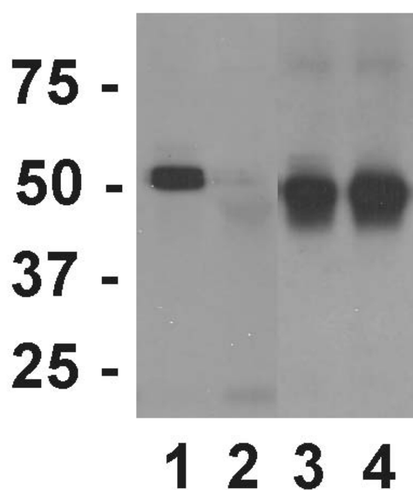
Amount of Capture resin: _____µl   Amount of lysate: _____µg

Amount of Affinity Ligand _____µl   Amount of 1X PBS: _____ µl

Amount of 1X wash buffer: _____µl   Amount of 2X RSB: _____µl

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

V. Catch and Release® v2.0 HT Immunoprecipitation Data



Catch and Release® v2.0 High Throughput (HT) Immunoprecipitation Assay Kit and protocol were used with the Denaturing Elution Buffer to immunoprecipitate Akt1. 100µg HEK 293 cell lysate was mixed with 2µg anti-Akt1/PKBα (Catalog # 07-416) (Lane 1) or 2µg of normal, rabbit IgG (Catalog # 12-370) (Lane 2) as a negative control for 1 hour at room temperature. Samples from each IP fraction were run on an SDS-PAGE gel and immunoblotted with anti-Akt/PKB (Catalog # 05-591). Lanes 3 and 4 were only probed with anti-rabbit IgG, HRP conjugate (Catalog # 12-348) to show equal amounts of the IP fractions were loaded per lane.

VI. Technical Support and Troubleshooting

A. Catch and Release® Frequently Asked Questions

Q: What should the user do if a viscous precipitate forms in the 96 well filter plate during the initial incubation step?

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 4ºC. 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.

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

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

B. Immunoblot Analysis Troubleshooting:

<u>Smeared Pattern or Distorted Bands</u>

- 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.

<u>"Bald Spots"</u>

- 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 37ºC) 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

1. Morgan, K, Protein Labeling and Immunoprecipitation in Current Protocols in Cell Biology, (J.S. Bonifacino, M. Dasso, J.B. Harford, J. Lippincott-Schwartz, and K.M. Yamada, eds.) Volume 1, Chapter 7, pp. 7.2.1-7.2.14., John Wiley & Sons, 2003.

2. Springer, T.A., Immunoprecipitation in Current Protocols in Immunology (J.E. Coligan, A.M. Kruisbeck, D.H. Margulies, E.M. Shevach, and W. Strober, eds.) pp.8.3.1-8.3.11. John Wiley & Sons, New York, 1996.

3. Irving, R.A., *et al.*, Construction, Screening and Expression of Recombinant Antibodies in Monoclonal Antibodies: Principals and Practice (J.W. Goding, ed.) Academic Press, London. 1996.

4. Harlow, E. and Lane, D., Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1988.

5. Nisonoff, A., Introduction to Molecular Immunology. Sinauer Associates, Sunderland Mass., 1984.

6. Gersten, D.M. and Marchalonis, J.J., J. Immunol. Meth. **24**: 305-309, 1978.

7. Rapley, R., Mol. Biotech. **3**: 139-154, 1995.

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