

Warning:

Wearing gloves is highly recommended when handling the kit contents.

All kit buffers are filtered, autoclaved and are bacterial free.

IPeX

Immunoprecipitation of Antigen Free from Antibody

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Applications

- Small-scale purifications of protein.
- Small-scale purifications of recombinant protein (e.g. with His or GST tags)
- Immunoprecipitations.
- Co-immunoprecipitations.
- Studying of protein-protein interaction.
- Eliminating interference from antibody heavy and light chain bands on SDS-PAGE or Western blots.

IPeX Kit Contents

<i>IPeX</i> kit (Cat # IP-10)	10 units
<i>IPeX</i> spin columns	10
Collection tubes	10
<i>IPeX</i> beads	150 μ l
5X Coupling Buffer (A)	10 ml
Stabilization Powder (B)	0.5 g
Blocking Buffer (C)	3.5 ml
10X Binding Buffer (D)	2 ml
Washing Buffer (E)	35 ml
Elution Buffer (F)	2 ml
Gentle Elution Buffer	2 ml
Information and protocols manual	1

IMPORTANT: *IPeX* kit contains sufficient reagent to immobilize 10 antibodies and to perform a total 10 immunoprecipitations (IP's) when using 10 µl of *IPeX* beads per precipitation (90 µg of antibody). Once an antibody has been immobilized, the affinity support may reuse up to 10 times for total of 100 IP's. **However, extra reagents need to be purchased separately to perform 100 reusable immobilized IP's (see Ordering Information Cat # IPR-100).**

Storage Conditions

IPeX kit must be stored in a dry place at room temperature (15-25°C) except *IPeX* beads that must be stored at 4°C. Under these conditions, *IPeX* kit can be stored for up to 12 months without any deterioration in performance and quality.

Product Use Limitations

IPeX kit is developed, designed and sold for research purposes only. It is not to be used for human diagnostic purposes or drug production nor for producing any substance intended to be administered to humans unless expressly cleared for that purpose by the Food and Drug Administration in the USA or the appropriate regulatory authorities in the country of use. All due care and attention should be exercised in the handling of materials described in this text.

Quality Control

The performance of *IPeX* kit is regularly monitored. *IPeX* kit is tested by, using it for immunoprecipitation of proteins and checking the recovery yields in Western Blot.

IPeX Technology

The *IPeX* kit is a novel tool for immobilizing of purified antibody directly onto an agarose beads to create a covalently and permanent affinity support, based on antibody. This technology eliminates the need for Protein A or Protein G agarose beads. An immune complex is forming when crude sample is incubated with the beads covalently bound antibody. After several washings of the immune complex the non-bound material is removed. The elution step dissociates the bound antigen from the complex without dissociation of the antibody from the agarose beads. The immobilizing antibody can also be used for small-scale antigen purification for protein assays, ELISAs, binding studies, Western blotting, etc. Binding and elution buffer conditions may have to be optimized to preserve the activity of the antigen if the kit buffer conditions are not suitable. Once the antibody-coupled beads have been prepared, subsequent IP's can be easily performed.

Two options to use the kit once the affinity purified antibody has been immobilized:

1. To store the affinity support beads for long-term storage (see protocol, step 23, IMPORTANT, step 23 IMOPRTENT, page 12).
2. To use immediately for antigen immunoprecipitation (see protocol, part **B**, page 12).

Kit Advantages

Advantages in using an *IPeX* kit rather than classical IP methods:

- Immobilizing the antibody provides faster and easier IP's.
- Purified antigen free from antibody contamination.
- Coupling of all primary amine-containing molecules.
- Coupling of all antibody species and subclasses.
- Antibody is coupled directly to the beads without cross-linker.
- The affinity support may be reused up to 10 times.
- Detecting at Western blots assay proteins close or of similar size as the heavy and light chains.

Specifications

<i>IPex</i> Beads: binding capacity	9 µg/µl
Maximum <i>IPeX</i> spin column volume capacity	700 µl
Numbers of reuse of the affinity support after immobilization	10 times

Important Information Before Starting:

- The antibody solution must not contain amines (e.g., Tris or glycine), as they will compete for coupling sites. Remove amines before coupling by dialysis using *GeBAflex-tube* (see Ordering Information page 21).
- The antibody solution must not contain gelatin or carrier proteins, as they will compete for coupling sites. Remove gelatin or carrier

proteins by performing a Protein A or Protein G purification and subsequent dialysis using PBS. Alternatively, inquire with the antibody vendor if a carrier/gelatin-free antibody is available.

- Perform all steps at room temperature unless otherwise indicated. The steps may be performed at 4°C but will take longer time for completion.
- Stabilization Buffer needs to be prepared 1 min before use. Pre-preparing of the buffer long before using will cause to inactive buffer. Stabilization powder (**B**) contains toxic material. Wear gloves and use caution when handling.
- For optimal results, first-time users should follow the suggested monitoring protocol, step by step to track the coupling and the coupling and immunoprecipitation progress.

Table 1: *IPeX* beads capacity coupling antibody

Amount of <i>IPeX</i> beads (6% beads slurry)	Antibody binding capacity	Total volume for the coupling reaction
10 µl	90 µg	500 µl
20 µl	180 µg	500 µl

IPeX Procedure

A. Coupling of affinity-purified antibody to *IPeX* beads

1. Re suspends *IPeX* beads by mixing the tube vigorously to obtain an even suspension. Transfer the desired amount of beads from the *IPeX* beads stock to a fresh 1.5 ml microcentrifuge tube. Take a minimum of 10 µl of *IPeX* beads for one *IPeX* Procedure.

For example, take 10 µl of *IPeX* beads for 2 µg or up to 90 µg of antibody sample.

2. Equilibrate *IPeX* beads by adding 700 µl of 1X Coupling Buffer; mix the tube vigorously by vortex for 5 seconds.

To prepare 1X Coupling Buffer, take 400 µl of 5X Coupling Buffer (A) and dilute with 1600 µl of water.

3. Pellet the beads by centrifuge the tube at +4°C for 2 min at 14,000 RPM. Remove the solution without disturbing the *IPeX* beads pellet.

IMPOTRTANT: Leaving 20 µl of the supernatant at the bottom of the tube will ensure the remaining of *IPeX* beads in the tube.

4. Repeat this steps 2-3 once more time.
5. To Couple: add to the left over from step 4, 100 µl of 5X Coupling Buffer (A); add the desired amount of affinity-purified antibody (see Table 2 for guidelines); complement the reaction volume to 500 µl with dH₂O.

Table 2: Example of coupling reaction

Left over from step 4	dH ₂ O	5X Coupling Buffer (A)	Affinity-purified Antibody	Reaction Volume
20 µl	370 µl	100 µl	10 µl	500 µl

IMPORTANT: Using these guidelines results in a minimum coupling efficiency of 85% after 4 hours. Do this step at 4°C if over night incubation is preferred.

The antibody solution must not contain amines (e.g., Tris or glycine), carrier proteins or gelatin (see Important Product Information).

6. Incubate the sample for at least 4 hours with gentle end-over-end mixing at room temperature.

Important: Coupling at 4°C will slow the reaction and will need longer time such as overnight.

- 7. In a fume hood, add 250 μ l of Stabilization Buffer to the tube containing 500 μ l coupling reaction (see blow preparation protocol for the Stabilization Buffer) and incubate for 30 min with gentle end-over-end mixing at room temperature.**

Preparing of Stabilization Buffer: In a fume hood, weight 0.02 g of the Stabilization powder (B) and mix it with 5 ml of dH₂O. **Do step 7 immediately.**

Stabilization powder (B) contains toxic material. Wear gloves and use caution when handling.

- 8. Centrifuge the sample from Step 7 at +4°C for 2 min at 14,000 RPM.**
- 9. Carefully pipette out the supernatant without disturbing the pellet of *IPeX* beads. Always, leave some supernatant in the bottom of the tube (~20 μ l).**

IMPOTRTANT: Leaving 20 μ l of the supernatant at the bottom of the tube will ensure the remaining of *IPeX* beads in the tube.

Coupling Monitoring: Measuring the absorbance of the antibody solution at 280 nm before and after coupling can approximate coupling efficiency. Alternatively, keep supernatant for a Western blotting analysis to determine whether any unbound affinity purified antibody remained in the supernatant. Use as secondary antibody, antibody against the same antibody species and subclasses used for the coupled.

- 10. To wash, add 700 μ l of 1X Coupling Buffer to the tube and mix by inverting the tube.**

To prepare 1X Coupling Buffer takes 400 μ l of 5X Coupling Buffer (A) for 1600 μ l of water.

- 11. Centrifuge the sample from Step 10 at +4°C for 2 min at 14,000 RPM.**
- 12. Carefully pipette out the supernatant without disturbing the pellet of *IPeX* beads. Always, leave about 20 μ l supernatant in the bottom of the tube.**
- 13. Repeat steps 10-12 once more time.**

14. To Block: add 100 μl of 5X Coupling Buffer (A) and 333 μl of Blocking Buffer (C) (see Table 3 for guidelines); complement the reaction volume to 500 μl with dH_2O .

Table 3: Example for blocking reaction

Left over form step 13	dH_2O	5X Coupling Buffer (A)	Blocking Buffer (C)	Reaction Volume
20 μl	47 μl	100 μl	333 μl	500 μl

15. Incubate the sample for 30 min with gentle end-over-end mixing at room temperature.

16. In a fume hood, add 250 μl of Stabilization Buffer (see blow preparation protocol for the Stabilization Buffer) to the tube containing the 500 μl blocking reaction. Incubate for 30 min with gentle end-over-end mixing at room temperature.

Preparing the Stabilization Buffer: In a fume hood, weight 0.02 g of the Stabilization powder (B) and mix it with 5 ml of dH_2O . **Do step 16 immediately.**

Stabilization powder (B) contains toxic material. Wear gloves and use caution when handling.

17. Centrifuge the sample from step 16 at $+4^\circ\text{C}$ for 2 min at 14,000 RPM.

18. Carefully pipette out the supernatant without disturbing the *IPeX* beads pellet. Always, leave some supernatant in the bottom of the tube ($\sim 20 \mu\text{l}$).

Important: Leaving 20 μl of the supernatant at the bottom of the tube will ensure the remaining of *IPeX* beads in the tube.

Blocking Monitoring: Measuring the absorbance of the supernatant at 280 nm before and after coupling can approximate coupling efficiency. Alternatively, keep supernatant for a Western blotting analysis to determine whether any unbound affinity purified antibody remained in the supernatant. Use as secondary antibody, antibody against the same antibody species and subclasses used for the coupled.

19. To wash, add 700 µl of 1X Binding Buffer to the tube left over from step 18 and mix by inverting the tube.

To prepare 1X Binding Buffer takes 100 µl of 10X Binding Buffer (D) and 900 µl of water.

20. Centrifuge the sample from Step 19 at +4°C for 2 min at 14,000 RPM.

21. Carefully pipette out the supernatant without disturbing the pellet of *IPeX* beads. Always, leave some supernatant in the bottom of the tube.

22. Repeat steps 19-21 one more time.

23. For immediately use go to step 24

IMPORTANT: TO STORE, immerse the antibody-coupled *IPeX* beads in 500 µl 1X PBS buffer and store at 4°C. For long-term storage, add sodium azide to a final concentration of 0.02%.

B. Immunoprecipitation of the Antigen

IMPORTANT: The amount of antigen needed and the incubation time are dependent upon the antibody-antigen system used and will have to be optimized for the specific system.

If using stored antibody coupled *IPeX* beads centrifuge the sample at 4°C for 2 min and proceed from step 24

24. To antibody-coupled *IPeX* beads from step 23, add 50 µl of 10X Binding Buffer (D); add the sample containing the desired antigen, complement the reaction volume to 500 µl with dH₂O (see Table 4 for binding reaction example).

IMPORTANT: Dilute the sample containing the desired antigen sample at least 1:1.

Table 4: Example for binding reaction

Left over from step 23	10X Binding Buffer (D)	Sample containing the desired antigen	dH₂O	Reaction Volume
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20 µl	50 µl	100 µl	330 µl	500 µl
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25. Incubate for at least 4 hours with gentle end-over-end mixing at +4°C.

IMPORTANT: Typical incubation times range from 4 hours to overnight.

To keep the antigen activity for downstream protocols, it is recommended to perform all steps of the antigen immunoprecipitation protocol at +4°C.

26. Place the *IPeX* spin column into a collection tube.

27. Transfer the binding reaction to the *IPeX* spin column.

28. Centrifuge the sample from step 27 at +4°C for 2 min at 6,000 RPM.

Antigen Binding Monitoring: Keep supernatant for a western blotting analysis to determine whether any unbound antigen remained in the supernatant. Use as secondary antibody, antibody against the same antibody species and subclasses used for the antigen.

29. To wash, add 700 µl Washing Buffer (E) to the *IPeX* spin column and mix by inverting it for several times.

30. Centrifuge the sample from step 29 at +4°C for 2 min at 6,000 RPM after placing the tube in to the collection tube.

31. Repeat Steps 29-30 four more times.

Washing Monitoring: Keep flow-through for a Western blotting analysis to determine whether any unspecific binding occur to the beads. Use as secondary antibody, antibody against the same antibody species and subclasses used for the antigen detection.

32. To Elute add the appropriate volume of Elution Buffer (F) (for 10 µl of *IPeX* beads add 50 µl) to *IPeX* beads in the *IPeX* spin column. Incubate for 10 minutes.

33. Centrifuge the sample from step 32 at +4°C for 2 min at 6,000 RPM.

34. Repeat steps 32-34 two more times.

Elution Options:

1. Before using the purified material in functional application, neutralize the Elution Buffer after the antigen is eluted. The elution buffer has a pH of 2.8 and can be neutralized by adding **18 µl** of 1M Tris, pH 9.1 per **50 µl** of Elution Buffer. Alternatively, if the protein or antibody is sensitive to the low pH system use **Gentle Elution Buffer** or use *GeBAflex-tube* for the dialysis of the antigen to a desired buffer (see Ordering Information page 21).
2. If performing SDS-PAGE analysis, it is not necessary to neutralize the eluted samples. However, the dye in the sample buffer may change color by the low pH, but this color change will disappear after the sample enters into the gel.

Elution Monitoring: Keep flow-through for a Western blotting analysis to determine whether the entire antigen has been eluted from the beads. Use as secondary antibody, antibody against the same antibody species and subclasses used for the antigen detection.

Troubleshooting Guide

Problem	Cause	Comments and Suggestion
Antibody is detected along with eluted protein complex	Uncoupled antibody was not removed sufficiently by diluted Coupling Buffer during the washing (steps 10-13) Stabilization of the coupling reaction was not sufficient (step 7)	1. Wash the antibody-coupled beads with diluted Coupling Buffer until no additional antibody elutes from the beads (Coupling Monitoring, page 10). 2. Better Stabilize the antibody by preparing fresh Stabilization Buffer or do a longer stabilization reaction.

<p>Protein are detected in the control experiment (beads without antibody)</p>	<ol style="list-style-type: none"> 1. Protein bind nonspecifically to the coupling <i>IPeX</i> beads 2. Insufficient Blocking or Stabilization of the blocking. 	<ol style="list-style-type: none"> 1. Increase the number of washes before the elution step or add Triton X-100 to the Washing Buffer to decrease nonspecific binding (steps 29-31). 2. Increase incubation time at the blocking reaction (steps 14-15). 3. Prepare fresh Stabilization Buffer or do a longer stabilization reaction (step 16).
<p>Target protein is not captured from sample</p>	<p>Antibody is not coupled to the beads or an insufficient amount of antibody is coupled to the beads causing low protein binding</p>	<ol style="list-style-type: none"> 1. Check supernatant and washing fraction to verify that antibody is coupled to the beads (Coupling and Blocking Monitoring, pages 10-11). 2. Increase antibody amount to ensure that sufficient antibody is coupled to the beads. 3. Use a more sensitive detection method.
	<p>Antibody is sensitive to low pH and has become</p>	<p>Prepare more antibody-coupled beads and used a</p>

	inactive during the elution steps (rare)	gentle elution buffer, neutral pH Elution Buffer (Cat # GEB-2).
	Antibody does not recognize native form of target protein (common with antibodies made against peptides)	Verify by other methods that the antibody recognize the native form before performing the coupling.
	Antibody is sensitive to amine coupling (occasionally a problem with monoclonal antibodies)	Increase antibody amount, reduce coupling time or try a different antibody.
	The target protein does not elute from the antibody using acidic conditions	Use lithium bromide, guanidine-HCl ^{^^} , urea ^{^^} , potassium thiocyanate ^{^^} or nonionic detergents to elute antigen (^{^^} using denaturants disrupts antibody structure and, therefore, the antibody-coupled beads cannot be reused).
Co- <i>IpeX</i> : Target protein is captured but no interacting with its protein in his complex	Protein:protein interaction are weak and cannot withstand the washing protocol	Another method must be used to capture weak or transient interactions.
	Antibody binds only to non-complexed form of the protein because of shared binding sites or conformational changes	Try Co- <i>IpeX</i> using antibodies that recognize different epitopes on the target protein.
	Co- <i>IpeX</i> buffer conditions do not promote protein:protein interaction	Specific ions, cofactors, etc. may need to be added to the Co- <i>IpeX</i> buffer to

		promote the interaction.

Immunoprecipitation of Actin Free from Antibody: case study

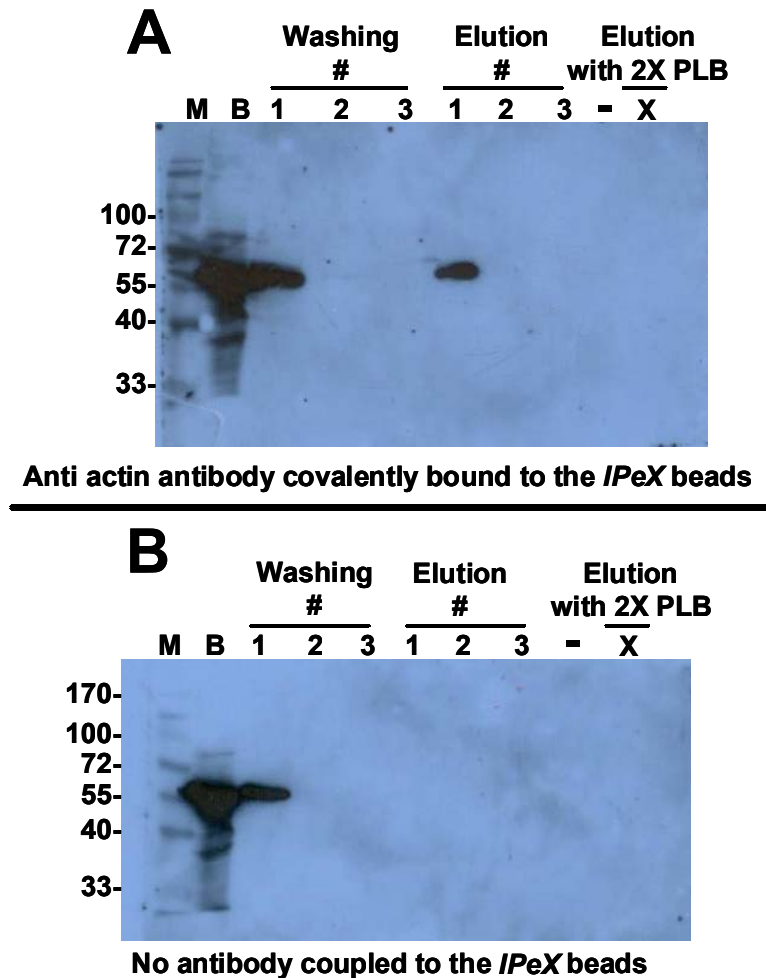


Figure 1: Immunoprecipitation of actin from whole HeLa cell extract with *IPeX* kit.

(A) Immunoprecipitation of actin with monoclonal mouse anti-actin (2 μ g) (ICN) covalently coupled to 15 μ l of *IPeX* beads for 4 hr at room temperature and stabilized for 30 min in room temperature. 100% of the antibody was coupled (for detailed protocol see Gene Bio-Application Ltd. *IPeX* handbook) (also, see schematic short protocol). (B) Immunoprecipitation of actin using *IPeX* beads was performed identically without the antibody as a control for antibody specific binding.

The immunoprecipitation of actin was performed with a whole HeLa cell extract (100 μ l) (total protein 4 μ g/ μ l), mixed with mouse anti-actin-coupled to *IPeX* beads (15 μ l of antibody-coupled *IPeX* beads) for 4 hr at 4oC (for detailed protocol see Gene Bio-Application Ltd. *IPeX* handbook) (also, see schematic short protocol ppt file).

Immunoprecipitation and coupling of the antibody to the *IPeX* beads were monitoring by sampling different steps of the reactions (in this experiment: Washing and Elution monitoring was performed), separating the samples on 12% SDS-PAGE, transfer the protein(s) to nitrocellulose membrane by a semi dry blotter. Western blot of actin is performing by using monoclonal mouse anti-actin as first antibody (1:5000 dilution) and mouse anti-IgG conjugated to HRP as second antibody (1:6000 dilution) (Jackson ImmunoResearch).

Gels marking

(M): Protein molecular weight marker (Fermentas).

(B): 5% of the flow through from the whole HeLa cell extract after biding reaction.

(Washing # 1,2 and 3): 3% of washing number 1,3 and 5 (five washing were performed).

(Elution # 1,2 and 3): 50 % from the elution volume number 1,2 and 3.

(-): Space in loading of the gel.

(Elution with 2X PLB, X): 100% from the elution volume with 2X Protein Loading Buffer.

Ordering Information Related Products

Product	Contents	Cat. No.
<i>IPeX</i> kit (for 10 preparations)	10 <i>IPeX</i> spin columns, 10 collection tubes, beads, buffers and handbook	IP-10
Gentle Elution Buffer	2 ml of Gentle Elution Buffer	GEB-2
Gentle Elution Buffer	20 ml of Gentle Elution Buffer	GEB-20
<i>IPeX</i> refill kit (for 100 preparations)	20 ml of Binding buffer, 350 ml of Washing buffer and 20 ml Elution Buffer	IPR-100
Related Products		
<i>GeBAflex-tube</i> kits for dialysis only		

Maxi <i>GeBAflex-tube</i> (15)	15 <i>GeBAflex-tube</i> of 3500 cut-off, floating rack	D035
Maxi <i>GeBAflex-tube</i> (15)	15 <i>GeBAflex-tube</i> of 6000-8000 cut-off, floating rack	D045
Maxi <i>GeBAflex-tube</i> (15)	15 <i>GeBAflex-tube</i> of 12000-14000 cut-off, floating rack	D055
<i>Midi GeBAflex-tube</i> (30)	30 <i>GeBAflex-tube</i> of 3500 cut-off and floating rack	D012
<i>Midi GeBAflex-tube</i> (30)	30 <i>GeBAflex-tube</i> of 6000-8000 cut-off and floating rack	D022
<i>Mini GeBAflex-tube</i> (30)	30 <i>GeBAflex-tube</i> of 6000-8000 cut-off and floating rack	D070-6-30
<i>Mini GeBAflex-tube</i> (30)	30 <i>GeBAflex-tube</i> of 12000-14000 cut-off and floating rack	D070-12-30
Related Proteomic Products		
<i>Stripping Buffer</i>	500 ml solution and handbook	ST010
<i>SDS Removing Buffer (WBD2)</i>	30 ml of SDS removing buffer	PDS010
<i>SeeBand</i> protein staining solution	500 ml solution and handbook	SB010
<i>SeeBand Forte</i> protein staining solution	500 ml solution and handbook	SB020