Insect RoboPop™ Ni-NTA His•Bind® Purification Kit

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Insect RoboPop™ Ni-NTA His•Bind® Purification Kit

Description

Insect RoboPop Ni-NTA His•Bind Purification Kit 71257-3

The Insect RoboPop Purification Kit is designed for high throughput (HT) purification of His•Tag® fusion proteins directly from transfected or infected Sf9 cultures without cell harvest, mechanical disruption or extract clarification. The kits feature Insect PopCulture® Reagent, Benzonase® Nuclease, Ni-NTA His•Bind Resin and buffers for efficient protein extraction and affinity purification. The Insect RoboPop Purification Kit is designed to purify fusion proteins from 10 ml cultures using the 2 ml 96-Well Filter Plate, which is compatible with standard filter manifolds for manual and robotic processing. A Collection Plate and Sealer is provided for storage of the purified proteins. The RoboPop Ni-NTA His•Bind Purification Kit will purify up to 400 µg His•Tag fusion proteins per well, based on the binding capacity of the resin. Using 10 ml cultures, protein yields ranging from 60–140 µg/culture were achieved with several mouse and human protein kinases expressed in pIEx™ transient expression vectors (1).

Components

RoboPop Ni-NTA His•Bind Purification Kit

• 50 ml	Insect PopCulture Reagent
• 1	2 ml 96-Well Filter Plate
• 1	Collection Plate with Sealer
• 10 ml	Ni-NTA His•Bind Resin, 10 ml settled volume (20 ml of a 50% v/v suspension)
• 125 ml	4X Ni-NTA Bind Buffer (4X = 200 mM Na phosphate pH 8.0, 40 mM imidazole, 1.2 M NaCl)
• 2 × 125 ml	4X Ni-NTA Wash Buffer (4X = 200 mM Na phosphate pH 8.0, 80 mM imidazole, 1.2 M NaCl)
• 50 ml	4X Ni-NTA Elute Buffer (4X = 200 mM Na Phosphate pH 8.0, 1.0 M imidazole, 1.2 M NaCl)
• 10 KU	Benzonase® Nuclease, Purity > 90%

Storage

Store Insect PopCulture Reagent, Collection Plate with Sealer, and 2 ml 96-Well Filter Plate at room temperature. Store Ni-NTA His•Bind Resin, 4X Ni-NTA Bind Buffer, 4X Ni-NTA Wash Buffer, and 4X Ni-NTA Elute Buffer at 4°C. Store Benzonase Nuclease at –20°C.

Notes: *Storage of Insect PopCulture Reagent at or below 4°C may cause precipitation. Incubate Insect PopCulture in a room temperature water bath with gentle swirling or inversion to redissolve.*

DO NOT FREEZE the Ni-NTA His•Bind Resin. Freezing results in complete loss of activity.

DO NOT store Benzonase Nuclease at –70°C. Freezing results in loss of activity.

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Protocol

The general procedure is described below, followed by detailed protocols in the subsequent sections. If manual processing is desired, an 8- or 12-tip multichannel pipetting device can be used instead of programming a robotic liquid handling system. An optimized robotic program for the Packard-brand MultiPROBE® II HT EX using WinPrep® software can be downloaded from http://www.novagen.com/robopopsoftware. Note that the WinPrep software requires the user to establish Gripper™ positions for each instrument. For Tecan Genesis Freedom™ workstation software, please contact your Tecan Sales Representative.

Quick protocol: cell culture, extraction and purification

1. Transfect or infect Sf9 cells with desired construct.
2. Harvest culture at the time of maximal expression of target protein by adding 0.05X culture volume of Insect PopCulture® Reagent. Add 0.4 µl (10U) Benzonase® Nuclease per 1 ml of the original culture volume. For a 10 ml culture use 500 µl Insect PopCulture and 4 µl Benzonase.

Note: *Benzonase Nuclease can be pre-mixed with Insect PopCulture Reagent prior to treating the cultures. Pre-mixed Insect PopCulture + Benzonase should be used on the same day and stored at 4°C until use.*

3. Mix by inverting gently several times and incubate 15 min at room temperature.
4. **Optional:** Take a sample from each well for screening expression levels using a compatible assay, e.g. FRETWorks™ S•Tag Assay, SDS-PAGE, Western blot or other method.
5. Add 200 µl Ni-NTA His•Bind Resin equilibrated in 1X Ni-NTA Bind Buffer (50% slurry). Incubate with mixing 30 min at 4°C.
6. Transfer the mixture to the 2 ml 96-Well Filter Plate and collect the resin with the vacuum manifold.
7. Wash the resin 2 times by resuspending in 2–3 ml (20–30 settled bed volumes) 1X Ni-NTA Wash Buffer. More extensive washing may be required if serum was present in the medium. Remove the supernatant for each wash by applying a gentle vacuum.
8. Position the Collection Plate on the robotic platform to capture the filtrate.
9. Elute the target protein by resuspending the resin in 200–300 µl (2–3 settled bed volumes) 1X Ni-NTA Elute Buffer. Collect the filtrate containing the target protein into the Collection Plate using the vacuum manifold.

Infection/transfection

Baculovirus infection

1. Seed a shake flask with an appropriate volume of serum-free medium (e.g. 10 ml in a 125 ml flask, 50 ml in a 250 ml flask) containing cells in log phase at 5×10^5 cells/ml. Incubate at 28°C with shaking (150 rpm) until the cell density reaches 2×10^6 cells/ml (not higher). If using monolayers for expression, seed a T-75 flask with 2×10^7 cells in 10 ml serum-free medium and allow to grow until almost confluent.
2. Infect the culture with a recombinant baculovirus at an MOI of at least 5 pfu per cell (> 10 if possible) by adding the appropriate volume of a freshly titered stock of virus.
3. Incubate at 28°C (with shaking at 150 rpm for suspension culture) until time of maximum expression (usually 72 hours).

Transient transfection using Insect GeneJuice® Transfection Reagent

1. Seed 1×10^7 Sf9 cells in 8 ml serum-free medium in a 125 ml shake flask.
2. In a sterile tube, dilute 20 µg plasmid DNA with 1 ml of serum-free medium. Also, dilute 100 µl Insect GeneJuice Transfection Reagent with 1 ml of serum-free medium.
3. Add the DNA *dropwise* to the Insect GeneJuice Transfection Reagent and mix immediately by gentle vortexing to avoid precipitation.
4. Incubate at room temperature 15 min.
5. Add the transfection mixture to the cells.
6. Incubate at 28°C with shaking at 150 rpm for 48 hrs.

Insect PopCulture® cell extract preparation

1. Extract the culture at the time of maximal expression of target protein by adding 0.05 culture volume of Insect PopCulture Reagent. Add 0.4 µl (10U) Benzonase® Nuclease per 1 ml of the original culture volume.

Note: *Benzonase Nuclease can be pre-mixed with Insect PopCulture Reagent prior to treating the culture. Pre-mixed Insect PopCulture + Benzonase should be used on the same day and stored at 4°C until use.*

2. Mix by gentle inversion several times and incubate 15 min at room temperature.

Note: *Protease Inhibitors may be added with the Insect PopCulture Reagent if desired. Protease inhibitor cocktails that produce a final concentration of > 1mM EDTA are not compatible with Ni-NTA His•Bind Resin or Benzonase treatment.*

3. **Optional:** Remove a sample from each well for screening expression levels using compatible assays. The FRETWorks™ S•Tag™ Assay (see Technical Bulletin 251), SDS-PAGE and Western blotting (His•Tag® Monoclonal Antibody, Cat. No. 70796-3) are compatible with Insect PopCulture extraction. The sample can be stored at 4°C until purification is complete.
4. The prepared extract is combined directly with equilibrated Ni-NTA His•Bind Resin for protein purification as described in the following sections.

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Insect RoboPop Ni-NTA His•Bind purification

Note: In these protocols, one volume is defined as the settled resin volume (e.g. 200 µl slurry yields 100 µl settled resin). The resin volume used per 10 ml culture can be adjusted according to expression levels. However, generally 200 µl slurry or 100 µl settled resin is recommended per 10 ml culture.

Buffer preparation

The buffer volumes required will vary depending on the pipetting capabilities of the liquid handling instrument employed. To eliminate the possibility for insufficient buffer/reagent addition, a 20% reservoir overfill is recommended. Excess buffers are provided in the kit for this purpose.

1. Dilute an appropriate amount of the supplied stock of 4X Ni-NTA Bind Buffer to 1X with sterile deionized water. Approximately 100 ml (overfill, 120 ml) 1X Ni-NTA Bind Buffer is required for Ni-NTA His•Bind Resin preparation per 96-well plate.
2. Dilute an appropriate amount of the supplied stock of 4X Ni-NTA Wash Buffer to 1X with sterile deionized water. Approximately 576 ml (overfill, 690 ml) 1X Ni-NTA Wash Buffer is required per 96-well plate.
3. Dilute the 4X Ni-NTA Elute Buffer to 1X with sterile deionized water. Approximately 29 ml (overfill, 35 ml) 1X Ni-NTA Elute Buffer is required per 96-well plate.

Ni-NTA His•Bind Resin preparation

1. Gently mix the bottle of Ni-NTA His•Bind Resin by inversion until completely suspended. Transfer the desired amount of suspended slurry to a sterile conical tube.
2. Centrifuge at 500 × g for 3 min to collect the resin.
3. Carefully remove the supernatant and wash the resin 3 times with 3 volumes 1X Ni-NTA Bind Buffer. For each wash, completely resuspend the resin by inversion, centrifuge at 500 × g for 3 min, and remove the supernatant carefully by pipetting.
4. After the final wash, resuspend the resin in 1 volume 1X Ni-NTA Bind Buffer.

Ni-NTA His•Bind purification

Tip: Disposable pipet tips should be used where appropriate to prevent cross contamination between sample wells.

1. Add 200 µl equilibrated Ni-NTA His•Bind Resin slurry to each prepared cell culture extract (10 ml cultures).
2. Incubate at 4°C with mixing for 30 minutes.
3. Transfer the mixture to a 24-well deep well plate (available from Whatman, Cat. No. 7701-5102). Split each 10 ml sample into 2 × 5 ml wells of the 24-well deep well plate.
4. Mix thoroughly on a shaking platform for 5–10 min. Continue to mix the suspensions during transfer to the Filter Plate in step 5.
5. Place the 2 ml 96-Well Filter Plate on the vacuum manifold and transfer the suspension to the Filter Plate while applying a gentle vacuum. Transfer the contents of the split 10 ml sample (2 deep wells) to a single well of the filter plate. Multiple deep wells can be transferred to a single filter plate well.

   Caution: Do not pull excess air through the resin bed after the filtrate has been removed. Drying of the resin bed can reduce yield and quality of the purified protein. Vacuum parameters should be adjusted to optimize the filtrate removal without resin bed drying.
6. Wash the resin in each well 2 times with 20–30 volumes (2–3 ml) 1X Ni-NTA Wash Buffer. For each wash:
   a) Rapidly dispense the Ni-NTA Wash Buffer to resuspend the resin. Disposable pipet tips are not necessary during the wash procedure.
   b) Separate the resin from the wash buffer with vacuum application. Separation time may vary depending of the type of manifold and vacuum parameters.

   Caution: Do not pull excess air through the resin bed after the filtrate has been removed. Drying of the resin bed can reduce yield and quality of the purified protein. After the final

wash the resin should be a moist cake with no excess liquid. Vacuum parameters should be adjusted to optimize the filtrate removal without resin bed drying.

7. Position the Collection Plate on the robotic platform to capture the filtrate.
8. Elute the bound protein with 2–3 volumes (200–300 µl) 1X Ni-NTA Elute Buffer. For each elution:
   a) Rapidly dispense the Ni-NTA Elute Buffer to resuspend the resin. Disposable pipet tips are not necessary during the elution procedure.
   b) Separate the Ni-NTA His•Bind Resin from the purified proteins in the Elute Buffer with vacuum application and collect into the Collection Plate.
9. Analyze the elution fractions from step 8. The eluate is compatible with Western blotting (His•Tag® Monoclonal Antibody, Cat. No. 70796-3), SDS-PAGE analysis, and other assays (e.g. FRETWorks™ S•Tag™ Assay, Technical Bulletin 251).
10. Store the eluted samples at 4°C. Storage conditions to retain activity are protein-dependent. Apply the Collection Plate Sealer to prevent evaporation.

Instructions for the Collection Plate Sealer

The two-layer Collection Plate Sealer has positioning tabs at both ends. After the Sealer is fully secured on the culture plate, you can remove positioning tabs or layers on the tabs. Do not remove positioning tabs or layers from the tabs until the sealer is fully secured on the Collection Plate. The bottom layer of the Sealer is brown and when removed exposes the adhesive. The top layer is foil and covers the purified protein samples in the Collection Plate.

1. Remove the brown bottom layer to expose the adhesive. Use the positioning tabs for handling.
2. Position the Sealer on the Collection Plate.
3. Secure the Sealer by rubbing across the foil to ensure the adhesive is attached between each well.
4. To remove the Sealer from the plate, lift from a corner and gently pull.
5. To remove a sample from a well, cut the foil above the well with a razor blade to allow the pipet tip to reach the well. Sealers are single-use and after removal cannot re-seal the wells.

Processing the Sample after Elution

The purified protein samples can be concentrated or the buffer changed by one of several methods. The storage buffer for your purified protein is often determined through an empirical process. Inappropriate storage buffer may lead to precipitation of the protein.

1. Dialyze into the buffer of choice. A 3-fold concentration can be achieved by dialysis into storage buffer containing 50% glycerol. Glycerol often stabilizes proteins for long-term storage. 96-well dialysis plates are available from Harvard Apparatus to facilitate processing.
2. Use 96-well microconcentrator units (e.g., Microcon-96 Filtrate Assemblies, Millipore) or 96-well solid phase extraction plates (SPE Bioplates, Whatman) as directed by the manufacturer to concentrate the sample or perform additional rounds of purification.

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Additional Guidelines

Medium

TriEx™ Insect Cell Medium (Cat. No. 71022-3) and BacVector® Insect Cell Medium (Cat. No. 70590-3) have been used successfully with Insect PopCulture® extraction. Other serum-free media are likely to be compatible. Serum many interfere with downstream application such as protein assays and purification.

Cell lines

Novagen's TriEx Sf9 Cells and Sf9 Insect Cells have been used successfully with Insect PopCulture Reagent. Other insect lines should be compatible, though it is possible that line-dependent differences could occur.

TriEx Sf9 cells (Cat. No. 71023-3) are derived from a high-yielding clone of Sf9 cells and are pre-adapted for growth in TriEx Insect Cell Medium. This matched cell/medium combination has been selected based on rapid, vigorous cell growth and high protein expression levels when using recombinant baculovirus for protein production.

Sf9 Insect Cells (Cat. No. 71104-3) plus BacVector Insect Cell Medium are recommended for co-transfection of transfer plasmid with BacVector Triple Cut Virus DNA and transfer plasmids to construct recombinant baculoviruses, for transfection of transient expression vectors (e.g. pIEx™ and pBiEx™ Vectors) and plaque assays. The cells can be used for protein production from recombinant baculovirus, although, the TriEx Sf9 cells + TriEx Insect Cells Medium (see above) have been found to yield higher protein expression levels in this application.

Volume of Insect PopCulture Reagent

Although the recommended amount of Insect PopCulture Reagent to use is 0.05 culture volume, there are no adverse effects to using higher ratios of Insect PopCulture Reagent per ml of culture.

Temperature of extraction

Insect PopCulture extraction along with Benzonase Nuclease treatment can be performed at room temperature or at 4°C. However, incubation times may need to be increased because Benzonase Nuclease activity is decreased at lower temperatures.

pH of extraction

Acidic pH (< 5.0) can degrade components of Insect PopCulture and will inhibit binding to Ni-NTA His•Bind® Resin.

Protein assays

Because proteins generally retain their activities and conformation, protein-specific activity and immunoassays are likely to be compatible with Insect PopCulture extraction. Insect PopCulture extracts are compatible with the S•Tag™ Rapid Assay, FRETWorks™ S•Tag Assay, and BCA protein assays. Direct analysis of the extract by SDS-PAGE with Coomassie blue staining generally requires loading the maximum well volume of crude extract containing a highly expressed target protein, combined with loading buffer.

Proteins purified with Ni-NTA His•Bind Resin are compatible with GST•Tag™, S•Tag, and BCA Assays. The BCA assay requires approximately a 1:10 dilution of the eluate.

Protease inhibitors

Protease inhibitors may be added with the Insect PopCulture Reagent to the culture medium. Note that protease inhibitor cocktails that produce a final concentration of > 1mM EDTA are not compatible with Ni-NTA His•Bind Resin or Benzonase® Nuclease treatment.

Benzonase Nuclease

Benzonase® Nuclease treatment is not generally recommended for purification of proteins that must be nuclease free. However, depending on the processing methods, Benzonase Nuclease may be removed during purification by anion exchange chromatography. Residual nuclease activity can be checked by incubation of the purified protein with RNA or DNA markers followed by gel analysis. For further details on Benzonase Nuclease see Technical Bulletin 261.

References

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