QIAGEN Supplementary Protocol:

Manual purification of 6xHis-tagged proteins from *E. coli* using the Ni-NTA Superflow 96 BioRobot® Kit

The two protocols given below are for the use of the Ni-NTA Superflow 96 BioRobot® Kit in manual procedures. The kit has been specially designed and optimized for automated 6xHis-tagged protein purification on QIAGEN® BioRobot Systems. For more details of the advantages of BioRobot Systems see the *Ni-NTA Superflow 96 BioRobot Kit Handbook* supplied with the kit or contact one of the QIAGEN Technical Service Departments or local distributors listed on the last page of the handbook.

The following protocols have been designed for use on the QIAvac 96 vacuum manifold or on the QIAGEN Centrifuge 4K15C with the Plate Rotor 2 x 96 that is suitable for centrifuging 96-well plates. Approximately 150 µg 6xHis-tagged protein can be purified from 3–5 ml cultures grown in 24-well blocks.

For the vacuum method, we strongly recommend using a vacuum source that can be conveniently regulated to provide vacuum pressures of between –100 and –800 mbar. Excessive vacuum pressures can cause samples to foam and splash, while insufficient vacuum pressures lead to increased protocol times. The vacuum setup should include a waste trap between the vacuum manifold and the pump.

Please read the *Ni-NTA Superflow 96 BioRobot Kit Handbook* carefully before beginning any of these procedures. **All the buffers used in these protocols are described in the handbook.**

Protocol 1. Purification of 6xHis-tagged proteins from *E. coli* under native conditions

Cell lysis

1. **Grow cell cultures, induce protein expression, culture for a previously optimized time period, harvest the cells by centrifugation, and store the pellets at –20°C or at –70°C for at least 1 h.**

   For details, see the protocol "*Cultivation of* E. coli *M15[pREP4] Harboring pQE Expression Constructs*" in the *Ni-NTA Superflow 96 BioRobot Kit Handbook*.

2. **Add 0.2–1.0 ml of Buffer NPI-10 containing 0.2 mg/ml lysozyme to each pellet.**

   When the OD_{600} value of the cultures before harvesting is 6, it is recommended the bacterial pellet be resuspended at a 5:1 ratio of original cell culture volume to buffer NPI-10. Specifically, for every 1 ml of cell culture used to obtain the bacterial pellet, add 0.2 ml of buffer NPI-10.

3. **Shake the block or tubes at 750 rpm for 30 min at room temperature to resuspend and lyse the pelleted bacteria.**

   The length of time needed to resuspend and lyse the bacteria may vary depending on the density of the culture and the host strain. The time allowed for resuspension may require optimization.

Protein purification on the QIAvac 96 vacuum manifold under native conditions

Reagents and equipment to be supplied by user

QIAvac 96 (cat. no. 19504)

Channeling Block (cat. no. 9232720)

96-well elution vessel (Square-Well Block [cat. no. 19573] or Collection Microtube rack [cat. no. 19560])

1. **During cell lysis, prepare the QIAvac 96 vacuum manifold.**
2. **Resuspend the Ni-NTA Superflow resin thoroughly. Pipet 200 µl Ni-NTA Superflow suspension into each well of the QIAfilter™ 96 Plate.**

   200 µl Ni-NTA Superflow suspension corresponds to a bed-volume of 100 µl.
3. **Place the Ni-NTA QIAfilter 96 Plate into the QIAvac 96 top plate, making sure that the underside of the plate contacts the gasket. Seal any unused wells of the Ni-NTA QIAfilter 96 Plate with tape.**
4. **Place the QIAvac top plate squarely over the QIAvac base. Attach the QIAvac to a vacuum source.**
5. **Equilibrate the Ni-NTA Superflow resin by adding 600 µl Buffer NPI-10 to each well and apply a vacuum for approximately 2 min or until the buffer has been completely drawn through the Ni-NTA QIAfilter 96 plate.**

   Apply a vacuum of approximately –500 mbar.
6. **Place the Ni-NTA QIAfilter 96 Plate into the QIAvac base. Place the TurboFilter® 96 Plate into the QIAvac top plate, and fit the QIAvac top plate squarely over the base.**
7. **Transfer the cell lysates into the wells of the TurboFilter 96 Plate.**

   Transfer a maximum of 0.8 ml.
8. **Carefully overlay the samples with 200 µl absolute ethanol (analytical grade) per well and apply a vacuum until the samples have been completely drawn through the TurboFilter 96 Plate.**

   Ethanol reduces foaming and thereby minimizes the possibility of cross-contamination.

   Apply a vacuum of approximately –500 mbar.
9. **Remove the TurboFilter module and place the Ni-NTA QIAfilter 96 Plate in the QIAvac top plate. Place the Channeling Block into the QIAvac base. Apply a weak vacuum of approximately –100 mbar for approximately 5 min or until all of the samples have been drawn completely through the Ni-NTA QIAfilter 96 Plate.**

   Application of a weak vacuum extends the time available for binding of the 6xHis-tagged proteins to the Ni-NTA matrix. This allows more efficient binding, which leads to higher yields. Although in some wells buffer may be drawn through earlier than in others, this will not affect elution or yield.

10. **Perform the first wash step by pipetting 1 ml Buffer NPI-20 into each well and applying a vacuum for approximately 5 min or until the buffer has been drawn completely through the Ni-NTA QIAfilter 96 Plate.**

    Apply a vacuum of approximately –500 mbar. Although in some wells buffer may be drawn through earlier than in others, this will not affect elution or yield.

11. **Perform a second wash step by repeating step 10.**

    If the lysates were highly concentrated or were from cells with low expression levels, it may be necessary to perform a third wash step to achieve higher purity.

    Very rarely, imidazole concentrations of 20 mM can interfere with binding of 6xHis-tagged proteins to the resin. If binding is inefficient, reduce the imidazole concentration in Buffer NPI-20 (e.g., to 10 mM).

12. **Remove the Channeling Block from the QIAvac base and place the chosen 96-well elution vessel inside the QIAvac base.**

13. **To elute the 6xHis-tagged proteins, pipet 450 µl Buffer NPI-250 into each well. Apply a weak vacuum of approximately –200 mbar for approximately 5 min or until the samples have been completely drawn through the Ni-NTA QIAfilter 96 Plate.**

    An elution volume of between 350 and 600 µl is recommended. The standard elution volume is 450 µl. Reduced recovery may result from reducing the elution volume.

    Approximately 70% of the bound 6xHis-tagged protein is eluted within the first fraction. If desired, a second elution step can be performed to increase recovery, by repeating step 13. The second elution can be collected into the same 96-well vessel (check the volume capacity of single wells) or into a second 96-well elution vessel.

Protein purification under native conditions using centrifugation

Reagents and equipment to be supplied by user

QIAGEN Centrifuge 4K15C

Plate Rotor 2 x 96 (cat. no. 81031)

96-well elution vessel (Square-Well Block [cat. no. 19573] or Collection Microtube rack [cat. no. 19560])

1. **During cell lysis, prepare the Ni-NTA QIAfilter 96 Plate.**
2. **After removing the seal, place the Ni-NTA QIAfilter 96 Plate onto a Square-Well Block.**
3. **Resuspend the Ni-NTA Superflow resin thoroughly. Pipet 200 µl Ni-NTA Superflow suspension into each well of the QIAfilter 96 Plate.**

   200 µl Ni-NTA Superflow suspension corresponds to a bed-volume of 100 µl.
4. **Place both modules into a plate holder in the centrifuge rotor.**
5. **Balance the centrifuge rotor.**
6. **Equilibrate the Ni-NTA Superflow resin by adding 600 µl Buffer NPI-10 to each well and centrifuging at 1500 x *g* for 2 min or until the buffer has completely passed through the plate.**

   Drain and rinse the Square-Well Block for reuse.
7. **Transfer up to 1 ml of the cell lysates into the wells of the TurboFilter 96 Plate. Place the TurboFilter 96 Plate onto a Square-Well Block and centrifuge at 500 x *g* for 5 min or until the lysates have completely passed through the TurboFilter 96 Plate.**

   The time necessary for centrifugation depends on the volume and viscosity of the cell lysates.
8. **Transfer the cleared cell lysates from the Square-Well Block into the wells of the equilibrated Ni-NTA QIAfilter 96 Plate. Place the Ni-NTA QIAfilter 96 Plate onto a clean Square-Well Block and centrifuge at 100 x *g* for 15 min or until samples have completely passed through the Ni-NTA QIAfilter 96 Plate.**

   Do not centrifuge at speeds significantly higher than 100 x *g*. Centrifugation at a lower speed extends the time available for binding of the 6xHis-tagged proteins to the Ni-NTA matrix. This allows more efficient binding, which leads to higher yields. Although in some wells buffer may pass through earlier than in others, this will not affect elution or yield.

   Drain and rinse the Square-Well Block for reuse.
9. **To perform the first wash step, place the Ni-NTA QIAfilter 96 Plate onto a clean Square-Well Block, pipet 1 ml Buffer NPI-20 into each well of the plate, and centrifuge at 1000 x *g* for 1 min or until the buffer has completely passed through the Ni-NTA QIAfilter 96 Plate.**

   Drain and rinse the Square-Well Block for reuse.

10. **To perform the second wash step, repeat step 9.**

    If the lysates were highly concentrated or were from cells with low expression levels, it may be necessary to perform a third wash step to achieve higher purity.

    Very rarely, imidazole concentrations of 20 mM can interfere with binding of 6xHis-tagged proteins to the resin. If binding is inefficient, reduce the imidazole concentration in Buffer NPI-20, (e.g., to 10 mM).

11. **Replace the Square-Well Block from step 9 with the 96-well elution vessel. To elute the 6xHis-tagged proteins, transfer 450 µl Buffer NPI-250 into each well. Centrifuge for 2 min at 5000 x *g* or until the samples have completely passed through the Ni-NTA QIAfilter 96 Plate.**

    An elution volume of between 350 and 600 µl is recommended. The standard elution volume is 450 µl. Reduced recovery may result from reducing the elution volume.

    Approximately 70% of the bound 6xHis-tagged protein is eluted within the first fraction. If desired, a second elution step can be performed to increase recovery by repeating step 11. The second elution can be collected into the same 96-well vessel (check volume capacity of single wells) or into a second 96-well elution vessel.

Protocol 2. Purification of 6xHis-tagged proteins from *E. coli* under denaturing conditions

Cell lysis

1. **Grow cell cultures, induce protein expression, culture for a previously optimized time period, harvest the cells by centrifugation, and store the pellets at –20°C or at –70°C for at least 1 h.**

   For details on the growth a bacterial expression cultures, refer to the *Ni-NTA Superflow 96 BioRobot Kit Handbook "Cultivation of* E. coli *M15[pREP4] Harboring pQE Expression Constructs."*

2. **Add 0.5 ml Buffer B–8 M urea to each pellet.**

3. **Shake the block or tubes at 750 rpm for 30 min at room temperature to resuspend and lyse the pelleted bacteria.**

   The length of time needed to resuspend and lyse the bacteria may vary depending on the density of the culture and the host strain. The time allowed for resuspension may require optimization.

Protein purification on the QIAvac 96 vacuum manifold under denaturing conditions

Reagents and equipment to be supplied by user

QIAvac 96 (cat. no. 19504)

Channeling Block (cat. no. 9232720)

96-well elution vessel (Square-Well Block [cat. no. 19573] or Collection Microtube rack [cat. no. 19560])

1. **During cell lysis, prepare the QIAvac 96 vacuum manifold.**

2. **Resuspend the Ni-NTA Superflow resin thoroughly. Pipet 200 µl Ni-NTA Superflow suspension into each well of the QIAfilter 96 Plate.**

   200 µl Ni-NTA Superflow suspension corresponds to a bed-volume of 100 µl.

3. **Place the Ni-NTA QIAfilter 96 Plate into the QIAvac 96 top plate, making sure that the underside of the plate contacts the gasket. Seal any unused wells of the Ni-NTA QIAfilter 96 Plate with tape.**

4. **Place the QIAvac top plate squarely over the base. Attach the QIAvac to a vacuum source.**

5. **Wash the Ni-NTA Superflow resin by adding 600 µl of distilled water to each well and applying a vacuum for approximately 2 min or until the water has been completely drawn through the plate.**

   Apply a vacuum of approximately –300 mbar.

6. **Place the Ni-NTA QIAfilter 96 Plate into the QIAvac base. Place the TurboFilter 96 Plate into the QIAvac top plate, and fit the QIAvac top plate squarely over the base.**

7. **Transfer the cell lysates into the wells of the TurboFilter 96 Plate.**

   Transfer a maximum of 0.5 ml.

8. **Carefully overlay the samples with 100 µl absolute ethanol (analytical grade) per well and apply a vacuum until the samples have been completely drawn through the filter.**

   Ethanol reduces foaming and thereby minimizes the possibility of cross-contamination.

   Apply a vacuum of approximately –500 mbar.

9. **Remove the TurboFilter module and place the Ni-NTA QIAfilter 96 Plate in the QIAvac top plate. Place the Channeling Block into the QIAvac base. Apply a weak vacuum of approximately –100 mbar for approximately 5 min or until all of the samples have been drawn completely through the Ni-NTA QIAfilter 96 Plate.**

   Application of a weak vacuum extends the time available for binding of the 6xHis-tagged proteins to the Ni-NTA matrix. This allows more efficient binding, which leads to higher yields. Although in some wells buffer may be drawn through earlier than in others, this will not affect elution or yield.

10. **Perform the first wash step by pipetting 1 ml Buffer B–4 M urea into each well, allowing to stand for 5 min, and applying a vacuum for approximately 2 min or until the buffer has been drawn completely through the Ni-NTA QIAfilter 96 Plate.**

    Apply a vacuum of approximately –500 mbar. Although in some wells buffer may be drawn through earlier than in others, this will not affect elution or yield.

11. **Perform a second wash step by repeating step 10, but using Buffer C–4 M urea.**

12. **Perform a third wash step by repeating step 11.**

13. **Remove the Channeling Block from the QIAvac base and place the chosen 96-well elution vessel inside the QIAvac base.**

14. **To elute the 6xHis-tagged proteins, transfer 450 µl of Buffer E–8 M urea into each well. Apply a weak vacuum of approximately –100 mbar for approximately 2 min or until the samples have been completely drawn through the Ni-NTA QIAfilter 96 Plate.**

    An elution volume of between 350 and 600 µl is recommended. The standard elution volume is 450 µl. Reduced recovery may result from reducing the elution volume.

    Approximately 70% of the bound 6xHis-tagged protein is eluted within the first fraction. If desired, a second elution step can be performed to increase recovery by repeating step 14. The second elution can be collected into the same 96-well vessel (check volume capacity of single wells) or into a second 96-well elution vessel.

Protein purification under denaturing conditions using centrifugation

Reagents and equipment to be supplied by user

QIAGEN Centrifuge 4K15C

Plate Rotor 2 x 96 (cat. no. 81031)

96-well elution vessel (Square-Well Block [cat. no. 19573] or Collection Microtube rack [cat. no. 19560])

1. **During cell lysis, prepare the QIAfilter 96 Plate.**
2. **Remove the seal from the QIAfilter 96 Plate and place it onto a Square-Well Block.**
3. **Resuspend the Ni-NTA Superflow resin thoroughly. Pipet 200 µl Ni-NTA Superflow suspension into each well of the QIAfilter 96 Plate.**

   200 µl Ni-NTA Superflow suspension corresponds to a bed-volume of 100 µl.
4. **Place both modules into a plate holder in the centrifuge rotor.**
5. **Balance the centrifuge rotor.**
6. **Wash the Ni-NTA Superflow resin by adding 600 µl distilled water to each well and centrifuging at 1500 x *g* for 2 min or until the water has completely passed through the QIAfilter 96 Plate.**

   Drain the Square-Well Block for reuse.
7. **Transfer the cell lysates from the Square-Well Block to the wells of the TurboFilter 96 Plate. Place the TurboFilter 96 Plate onto a Square-Well Block and centrifuge at 500 x *g* for 5 min or until the lysates have completely passed through the TurboFilter 96 Plate.**

   The time necessary for centrifugation depends on the volume and viscosity of the cell lysates.
8. **Transfer the cleared cell lysates from the Square-Well Block into the wells of the washed Ni-NTA QIAfilter 96 Plate. Place the Ni-NTA QIAfilter 96 Plate onto a fresh Square-Well Block and centrifuge at 100 x *g* for 15 min or until samples have completely passed through the Ni-NTA QIAfilter 96 Plate.**

   Do not centrifuge at speeds higher than 100 x *g*. Centrifugation at a low speed extends the time available for binding of the 6xHis-tagged proteins to the Ni-NTA matrix. This allows more efficient binding, which leads to higher yields. Although in some wells buffer may pass through earlier than in others, this will not affect elution or yield.

   Drain and rinse the Square-Well Block for reuse.
9. **To perform the first wash step, place the Ni-NTA QIAfilter 96 Plate onto a fresh Square-Well Block, pipet 1 ml Buffer B–4 M urea into each well of the plate, and centrifuge at 3000 x *g* for 1 min or until the buffer has completely passed through the plate Ni-NTA QIAfilter 96 Plate.**

   Drain the Square-Well Block for reuse.

10. **Perform a second wash step by repeating step 9, but using Buffer C–4 M urea.**
    Drain the Square-Well Block for reuse.

11. **Perform a third wash step by repeating step 10.**

12. **Replace the Square-Well Block from step 11 with the chosen 96-well elution vessel.**

13. **To elute the 6xHis-tagged proteins, transfer 450 µl of Buffer E–8 M urea into each well. Centrifuge at 5000 x *g* for 2 min or until the samples have been completely drawn through the Ni-NTA QIAfilter 96 Plate.**

    An elution volume of between 350 and 600 µl is recommended. The standard elution volume is 450 µl. Reduced recovery may result from reducing the elution volume.

    Approximately 70% of the bound 6xHis-tagged protein is eluted within the first fraction. If desired, a second elution step can be performed to increase recovery by repeating step 13. The second elution can be collected into the same 96-well vessel (check volume capacity of single wells) or into a second 96-well elution vessel.