DATA SHEET

STABIL-P.A.C

Incorporating NVoy Technology

Introduction

Protein processing and production is often hampered by the formation of aggregates that restrict and complicate the handling of proteins, antibodies and enzymes. NVoy is designed to minimise the sequential losses in consecutive protein processing steps which would otherwise dramatically reduce the overall protein yield.

Utilising NVoy technology is an alternative to the use of detergents, fusion proteins, arginine, chaperones and a range of other common additives employed to increase protein solubility and enable the handling of proteins in solution. This Stabil-P.A.C contains proprietary carbohydrate polymers, NV polymers, designed to increase the solubility and stability of proteins whilst preventing aggregation and reducing non-specific binding.

NV polymers are linear, uncharged carbohydrate polymers of around 5kD, derivatised to make them highly amphipathic.. They associate at multiple points with surface exposed hydrophobic patches of proteins in a dynamic fashion to form multipoint reversible complexes. Multiple-binding points allow NV polymers to be used at low concentrations relative to alternative reagents and their size prevents them from entering the protein core and inhibiting normal structural bonding or blocking catalytic/binding sites. Based on simple carbohydrate polymers, NV polymers are easily separated from the protein when they are no longer required in solution.

Impact Areas of NVoy Technology

The impact of NVoy technology can be seen in many areas of protein research including stabilisation, purification, analysis and crystallisation.

- Maintain activity over several freeze/thaw cycles
- Prolonged storage at 4ºC for unstable proteins
- Maintain solubility of fusion proteins after “tag” is removed
- Stable formulation of protein / antibody for immobilisation + conjugation
- Maintain soluble proteins that usually require ligand to be present
- Increase concentration of proteins that would otherwise aggregate when concentrated

- Endotoxin removal
- Minimise protein losses
- Improve purification strategy
- Cleaner protein preparations

- Allow full structural characterisation (MS, crystallisation, NMR, CD)
- Use in HTS assays to keep proteins soluble and reduce non-specific binding
- Replacing detergents which are more difficult to remove downstream

- Controllable crystal growth when rapid formation produces poor crystals

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• Concentrate and maintain high protein concentration without the need for any other additives
• Solubilise and stabilise proteins for longer period of time

• Simple, effective, generic technique

Kit Contents

1) *NV10 polymer (10 mg, x6)*
2) *Strong Release Agent (4 ml, x1)*
3) *Dimethylsulfoxide (DMSO, 4 ml, x1)*

Storage

Upon receipt store at + 4°C. Discard any reagents that show discoloration or evidence of microbial contamination. NV10 is stable as supplied. Reconstituted solutions of NV10 can be stored refrigerated for short term (1-7 days) and for longer term when frozen (6 – 12 months). For further information on storage please refer to question 3 in the FAQ on page 14.

General Notes on Usage

1) Each Tube contains 10 mg NV10. Generally a 1 mg/ml protein solution can be protected by 1 mg/ml to 5 mg/ml NV10. This corresponds to a 5-fold excess of NV10 to protein by mass.
2) If protein aggregation occurs, increase the NV10 concentration or decrease the final protein concentration. If no aggregation is observed it may be possible to decrease the NV10 or increase the protein concentration.
3) Two release agents have been provided to allow you to control the interaction between the NV10 molecules and your protein.

• Dimethylsulfoxide (DMSO) is a weak release agent and will facilitate a slow, gentle release.

• The Strong Release Agent and will facilitate rapid release.

For a solution containing 1 mg/ml NV10 (0.1% w/w), up to 2.5% (v/v) strong release agent or up to 15 % of DMSO may be added. Alternatively increased temperature (37 °C) can be used to facilitate a gentle release. Refrigeration will induce a stronger interaction between protein and NV10.

Recommended Protocol for Protein Stabilisation

Stability is very protein specific, but a general protocol is given below.

1) Determine the protein concentration (for example with Expedeon's Bradford*ULTRA* assay, BCA assay or absorbance at 280nm).
2) Typically a fivefold excess, by mass, of NV10 will protect and stabilise the target protein. For example, use 200 µg/ml NV10 for 40 µg/ml protein.
3) The protein solution can be added directly to dry NV10 to get the desired concentration. Alternatively a concentrated stock solution of up to 50 mg/ml NV10 can be made in an aqueous buffer of choice, which can then be added to the protein solution.
4) Stock solutions containing 2.5 mg/ml NV10 can be stored for 1 week at 4 °C or for longer term at -20 °C.

EXAMPLE : Use of NV10 to Improve Enzyme Stability

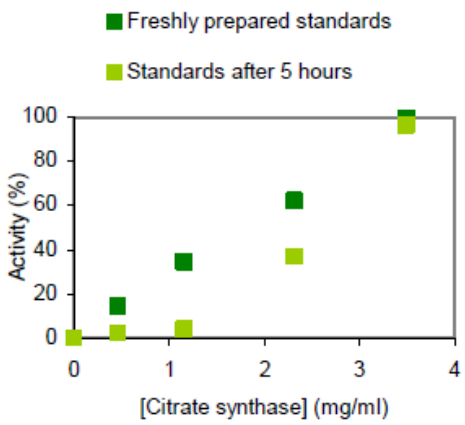


Figure 1: Within 5 hours of preparing a set of citrate synthase standards the standard curve has lost linearity, and the activities of the standards at 0.4 µg/ml and 1.1 µg/ml have virtually disappeared.

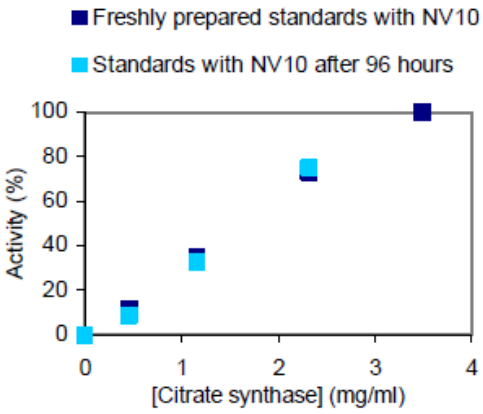


Figure 2: The citrate synthase standard curve produced from samples containing NV10 remains linear even after 96 hours.

Recommended Protocol for Protein Concentration

NVoy technology can be used to minimise protein losses due to aggregation and non-specific binding during concentration.

1) Determine the protein concentration.
2) Typically a fivefold excess, by mass, of NV10 will protect and stabilise the target protein e.g. 100 µg/ml NV10 for 20 µg/ml protein.
3) Concentrate the protein / NV10 solution to the desired protein concentration.
4) NV10 will co-concentrate with the protein in solution to give continuing protection.

EXAMPLE: Concentration of Bovine Serum Albumin

Duplicate samples containing 10 µg/ml BSA in PBS and supplemented with varying concentrations of NV10 were concentrated tenfold in Vivaspin 2 spin concentrators (5,000 mwco, Hydrosart low protein-binding membrane) according to the manufacturer's instructions.

Starting solution (1 ml)	Recovered yield (%)
10 µg/ml BSA	46 %
10 µg/ml BSA + 10 µg/ml NV10	60 %
10 µg/ml BSA + 40 µg/ml NV10	85 %
10 µg/ml BSA + 100 µg/ml NV10	90 %

Recommended Protocol for Protein Refolding

1) Solubilise the target protein at 5 mg/ml using chaotropes such as urea and guanidine hydrochloride. Refold the protein by 20-fold dilution into refolding buffer containing NV10. Incubate overnight at room temperature, add the protection release solution and perform a further overnight incubation.
2) The refolding buffer should contain 0.3 mg/ml – 2 mg/ml concentration of NV10 as a starting point
3) If protein aggregation occurs increase the NV10 concentration or decrease the final protein concentration in the refold.
4) The NV10 protection agent will suppress protein aggregation and may allow refolding at final protein concentrations greater than 0.25 mg/ml. However, the NV10 may also slow down the rate of refolding especially at 4°C.
5) The addition of the protection removal solution is not always required and extended incubation of the refolding target protein at room temperature may increase yield and allow complete refolding without NV10 release.
6) A 5% v/v addition of the protection removal solution will achieve rapid protection release. If the protein is not completely refolded aggregation may be caused by sudden protein release. If this occurs add the protection removal solution stepwise with small initial additions or consider performing analysis in the presence of NV10 without release.
7) NV10 can be added into the denaturant buffer to assist protein solubilization and then carried over into the refolding buffer without affecting functionality (e.g. add 20 mg/ml concentration of NV10 to the denaturant buffer to achieve 1 mg/ml final NV10 concentration in the refolding buffer). Please note that NV10 alone can not solubilize aggregated protein or inclusion bodies. It is recommended to use chaotropes such as urea or guanidine hydrochloride in combination with reducing agents.
8) Proteins vary widely in properties and requirements. To obtain correct refolding, the inclusion of co-factors, metal ions and a redox couple in the refolding buffer may be required. When using redox reagents please note that DTT and other strong reducing agents (potentially carried over from the denaturant solution) will reduce the oxidized component of the redox couple. We recommend the oxidized/reduced glutathione redox couple at a final ratio of 2mM reduced to 1 mM oxidized as a good starting point.

Recommended Protocol for Endotoxin Removal

1) Bind the target protein to an affinity chromatography or ion exchange media under conditions appropriate to the target protein.
2) Wash the medium with 10 column volumes (CV) of wash buffercontaining 2% NV10.
3) Wash the medium with 10 CV wash buffer containing 0.2% NV10.
4) Wash the medium with 5 CV wash buffer (at 4 °C - optional).
5) Elute the target protein in elution buffer.

EXAMPLE : Comparison of Endotoxin removal methods.

Target protein was bound to an affinity resin and processed according the NVoy procedure, the Detoxi Gel procedure (Pierce) and the method by Reichelt et al.[1] using Triton X114.

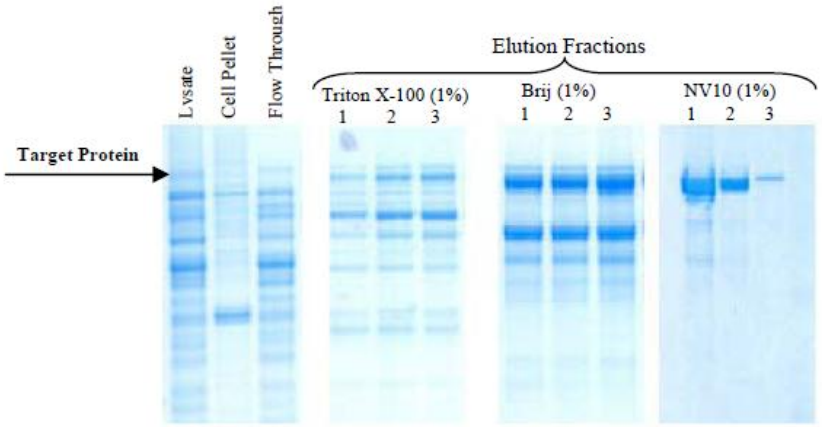
Method	Protein Yields (% Control)	Endotoxin EU	Activity (% Control)
Control	100	120,000	100
Detoxi Gel	32	3000	55
Triton X114	45	80	32
NVoy	78	None Detected	72

NVoy assisted endotoxin removal was the only method that resulted in a final endotoxin concentration below the detection limit. Moreover the Novexin method resulted in significantly higher protein recovery and protein activity.

Protocol for Protein Processing & Purification

1) Harvest the Cultured cells and lyse with a buffer containing co-factors and up to 1.0% NV10 (w/v). Do not use detergents in the lysate buffer.
2) If the protein expressed is soluble continue to step 3. If inclusion bodies have formed please refer to the protein refolding protocol on page 8.
3) Clarify the lysate by centrifugation
4) Bind the protein on a chromatography support of choice and wash the medium with 10 column volumes of wash buffer containing up to 1% NV10 (w/v).
5) Elute in a buffer of choice which may contain a five fold mass excess NV10 to the expected protein concentration (e.g. 5mg/ml NV10 for a 1mg/ml protein solution) to enhance protein stability and storage.

EXAMPLE : Comparision of protein processing using NV10 vs. detergents.



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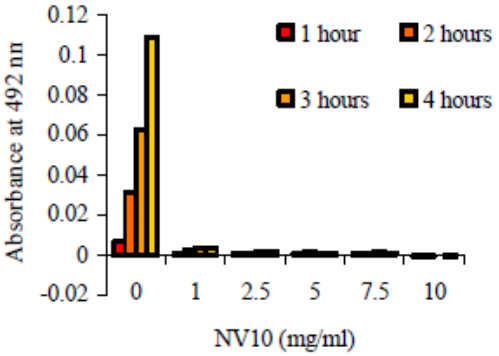
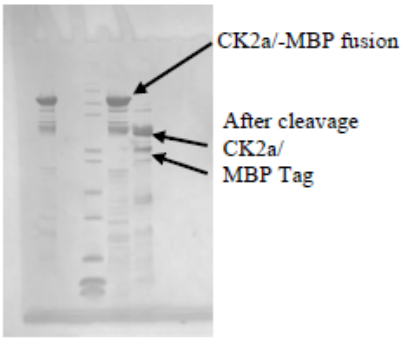
Protocol for Fusion Protein 'tag-removal'

1) Determine the starting protein concentration (for example with Expedeon's Bradford*ULTRA* assay, BCA assay or absorbance at 280nm).
2) Typically a fivefold excess, by mass, of NV10 will protect the target fusion protein. For example, use 5 mg/ml NV10 for 1 mg/ml protein.
3) Make up the cleavage buffer with the desired NV10 concentration, and use PD10 desalting columns to buffer exchange the target fusion protein into cleavage buffer containing NV10.
4) Continue with tag cleavage according to the standard protocol.
5) NV10 associates with the protein in solution and protects the cleaved native protein from aggregation and instability.





Removal of the maltose binding protein fusion partner (k-MBP) from thekinase, using Factor Xa, results in heavy aggregation and low yields of the native kinase. By adding NV10 to the cleavage buffer (20 mM Tris.HCl, 75 mM NaCl, 1 mM CaCl2, pH 6.5) aggregation can be significantly reduced, whilst the cleavage reaction remains unaffected.

Recommended Protocol for NV10 Removal

1) Bind the target protein to an ion exchange or affinity chromatography media under conditions appropriate to the target protein.
   In most cases the target protein will bind as normal to the resin of choice. If the binding is weaker than expected, the interaction with the solid phase can be enhanced by using release agents to weaken the interaction between the protein and NV10 polymer.
2) Wash the medium with a minimum of 10 column volumes of binding or wash buffer to remove NV10.
3) Elute the target protein using increasing ionic strength, pH gradient or a competing ligand (affinity chromatography).

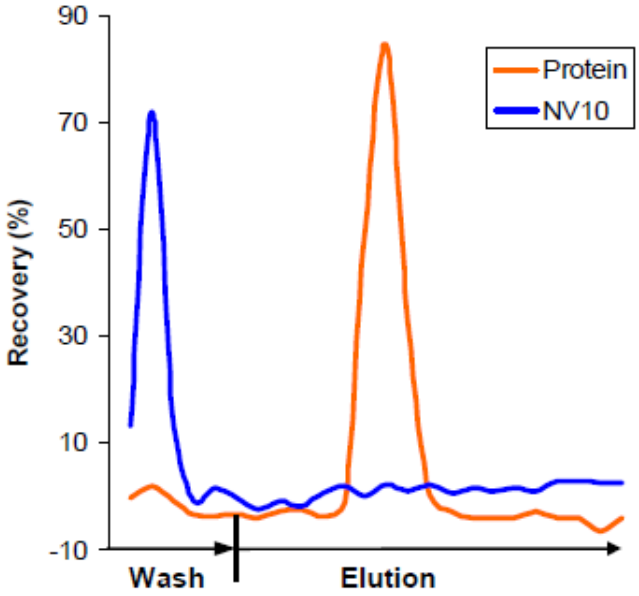


Figure 1: Typical profile illustrating separation of NV10 from target protein using IEx or affinity chromatography.

Frequently Asked Questions

Q 1 Can I make a concentrated solution of NV10?

A 1 NV10 can be dissolved up to a 50 mg/ml solution in any common aqueous buffer system. However, the NV10 crystallisation will occur faster in more concentrated solution (See Q 3).

Q 2 Is the NV10 solubility pH dependent?

A 2 The NV10 solubility is not pH dependent. NV10 can be dissolved in aqueous buffers with pH ranging from 3 to 11.

Q 3 Can I store a solution of resuspended NV10.

A 3a When NV10 is resuspended in an aqueous buffer the NV10 molecules will slowly crystallise. This will, in time, lead to a cloudy solution. As a rule of thumb a pure 1 mg/ml NV10 solution is stabile for at least one week at 4 °C. More concentrated solutions will appear cloudy more rapidly. The NV10 crystallisation process only occurs for pure NV10 solutions. Moreover, despite the undesirable cloudy appearance, a crystallized NV10 solution will retain its functionality.
A 3b When NV10 is resuspended in an aqueous buffer it is ideally used immediately for protein protection. However, solutions of resuspended NV10 can also be stored short term (1-7 days) at 4 °C and longer term (6 – 12 months) at -20 °C or -80 °C.

Q 4 Are any purification steps necessary before protein analysis?

A 4 A 1 mg/ml solution of NV10 is compatible with many analytical techniques.

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Protein Concentration Column Chromatography

Protein Concentration		***Column Chromatography***	
BCA Assay	✓✓✓	IMAC	✓✓✓
Bradford Assay*	✓✓	Ion Exchange	✓✓✓
UV spectroscopy	✓✓✓	Reversed Phase***	✓✓
Protein Structure		***Protein Activity***	
Biacore	✓✓	Cell Based Assays	✓✓✓
Circular Dichroism	✓✓✓	ELISA Assays	✓✓✓
Crystallography	✓✓	FRET assays	✓✓✓
Electrophoresis	✓✓✓		
Mass spectrometry**	✓✓✓		
NMR	✓✓		

* Use a blank containing NV10. NV10 will give a weak Bradford signal. The assay will show a reduced sensitivity for protein concentrations lower than 0.25 mg/ml. Use Expedeon's Bradford*ULTRA* Assay for higher sensitivity and less background interference.
** Standard C4 zip tip clean up recommended
*** Use of guard column recommended

It may be desirable to carry out protein purification steps before gel-electrophoresis, Bradford analysis and ELISA since more accurate results may be obtained.

Q 5 Which is the preferred method to remove NV10 from my protein solution?

A 5 The recommended purification method is ion-exchange chromatography, or immobilised metal affinity chromatography (IMAC) if the protein has a histidine tag. Hydrophobic interaction chromatography can be used as a secondary protein polishing procedure to remove trace levels of NV10.

Q 6 My protein only binds the chromatography resin weakly. Can I improve the binding?

A 6a To enhance the binding to the chromatography resin, release agents may be added to the sample. However, adding release agents will reduce the protein protection and may lead to higher protein losses on the column.
A 6b The actual protein properties may differ from theoretical protein properties. Try screening different ion exchange resins and buffers at different pH to find more suitable binding conditions.

Q 7 Can I use membranes, size exclusion chromatography or desalt resins to separate my protein from NV10 molecules?

A 7a No, these purification strategies are not recommended since NV10 has a large hydrodynamic radius and may therefore co-elute with your protein in size-based separations.
A7b To enable size-based purification strategies release agents can be used to weaken the NV10 protein protection. However, by doing so the protein will no longer be protected, which may result in protein losses.