Sticky Proteins: "Analysis and Discovery"

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JHUPO

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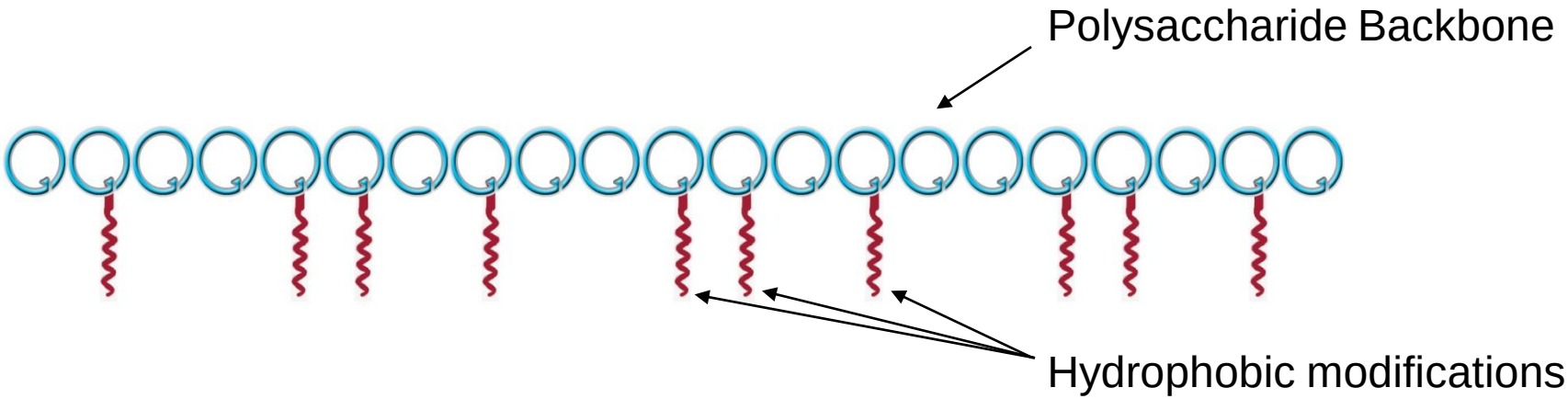
Expedeon - Introduction

Developing innovative technology to enable protein research and discovery

- Spin-out Cambridge University - Headquartered in Cambridge, UK with facilities in San Diego, US
- Innovative Solutions
  - Protein Solubility and stability
    - Nvoy Technology
    - PPS silent surfactant
  - Gelfree Fractionation
    - Enabling top down proteomics
    - Enhancing bottom up proteomics
    - Product characterisation
  - InstantBlue
    - Fast and fool proof protein staining
  - RunBlue Electrophoresis
    - Composite precast gel
  - Amintra Resins
    - COOL-tag resin: ligand Free Elution Consumable for protein researchs
    - Range of typical resins including Ni-NTA, GST, Protein A and protein G

NVoy Technology

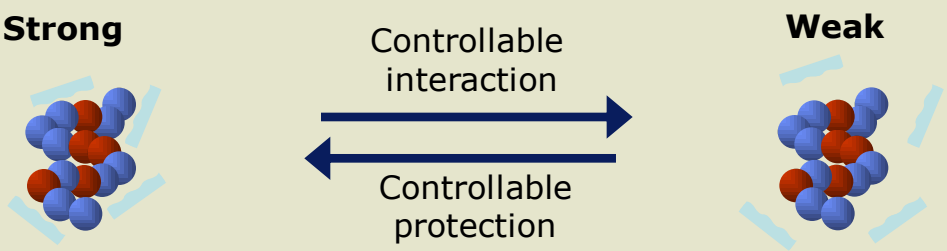


- **Specially chosen carbohydrate backbone with**
  - Linear
  - M_w = 5kDa (R_{hyd} ~ 18kDa) => Does not access binding sites
  - Multipoint, regio-specifc modification => Hydrophobic face
  - Uncharged, UV transparent and pH Stable (pH 2 to 11)
- **Dynamic interaction with protein**
  - k_d = 28 μM (GFP) & 12 μM (Hexokinase)
  - is easy to remove from protein sample
  - interaction can be controlled

NVoy Technology

Increased protein solubility

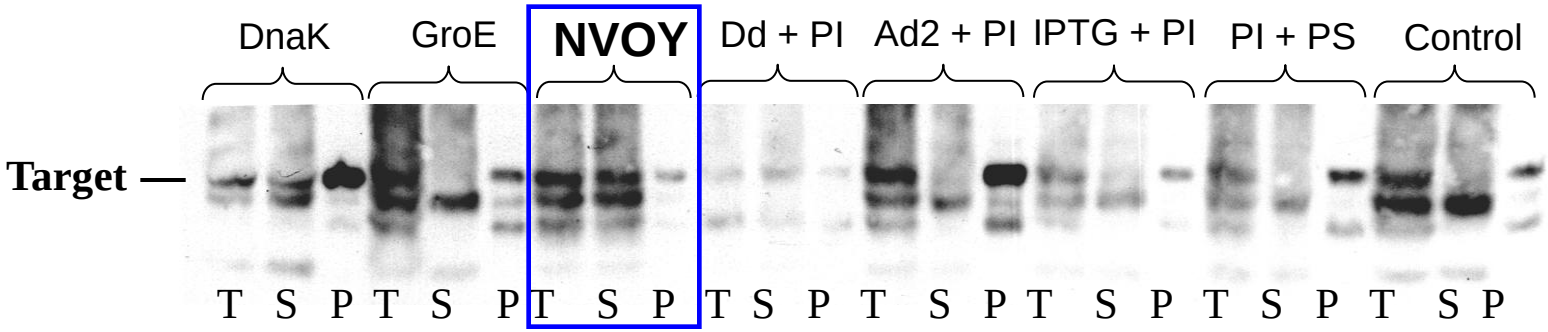
- Improved protein stability
- Reduced aggregation
- Process at high concentrations
- Retain protein structure and functionality



- Hydrophobic interaction: cited as the cause for protein aggregation in **~75%** of cases
- NVoy associates with surface exposed hydrophobicity and presents the hydrophobic backbone to the solvent.
- Prevents hydrophobic interaction between the target protein and
  - target proteins => prevents aggregation and promotes a heterogeneous sample
  - process surfaces (resin, membranes, etc.) => increasing yields
  - other proteins => increased purity & yield

Protein Expression

Cell Free



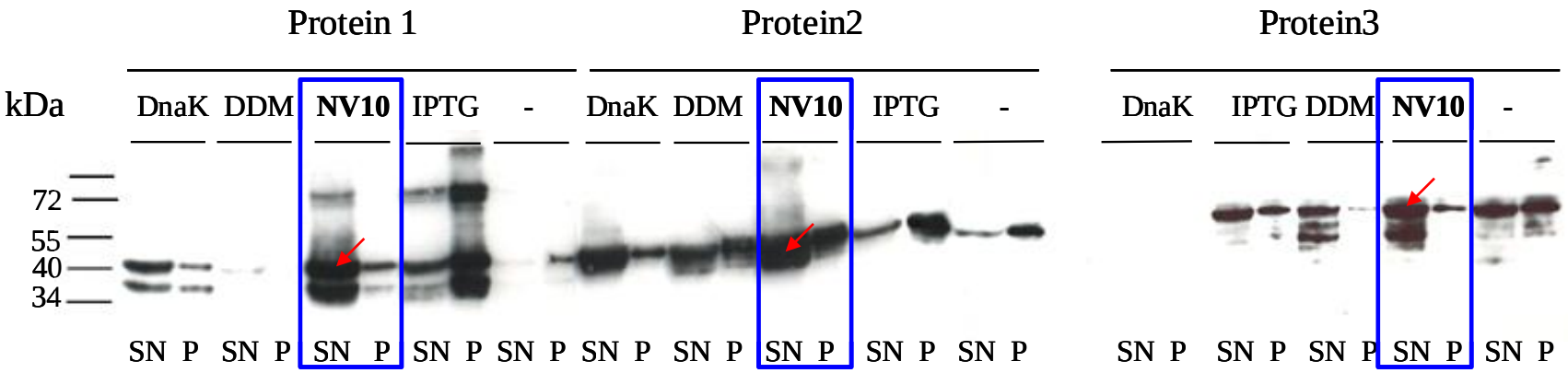
Dd = dodecahedra; PI = protease inhibitors ; Ad2 = Dodecahedra of adenovirus serotype 2 ; PS =pepstatin

Fusion protein of a Transcription Factor was expressed in a cell-free expression system. Different compounds were added to the cell lysate in order to optimise the protein yields. After 18 hours expression, the total lysate (T), soluble (S) and insoluble (P) fractions were analysed by western blotting

Data by courtesy of Dr Ana Villegas-Mendez, HumProTher Laboratory, France

Protein Expression

Cell Free



Fusions of antigenic murine proteins were expressed in a cell-free expression system. Different compounds were added to the cell lysate in order to optimise the protein yields. Soluble (S) and insoluble (P) fractions were analysed by western blotting

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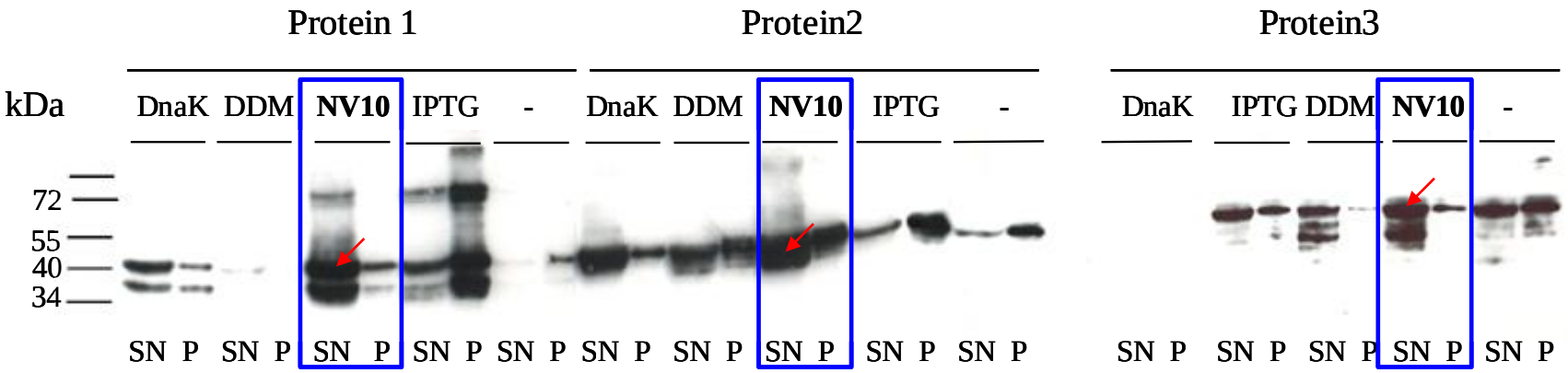
Protein Expression

Cell Free



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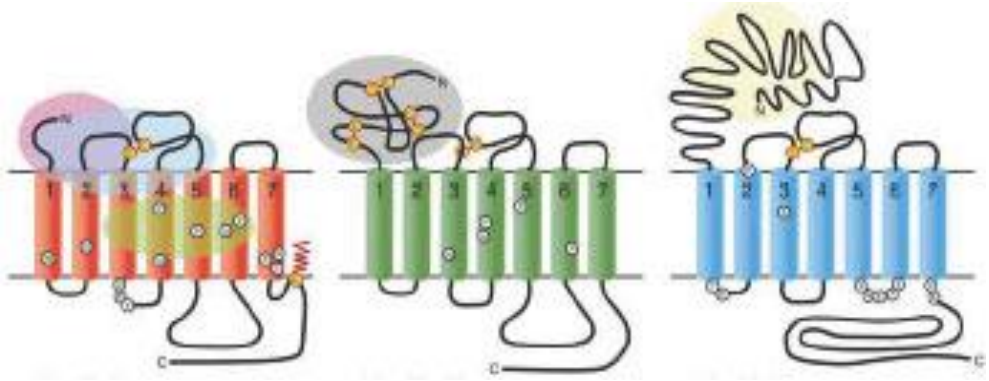
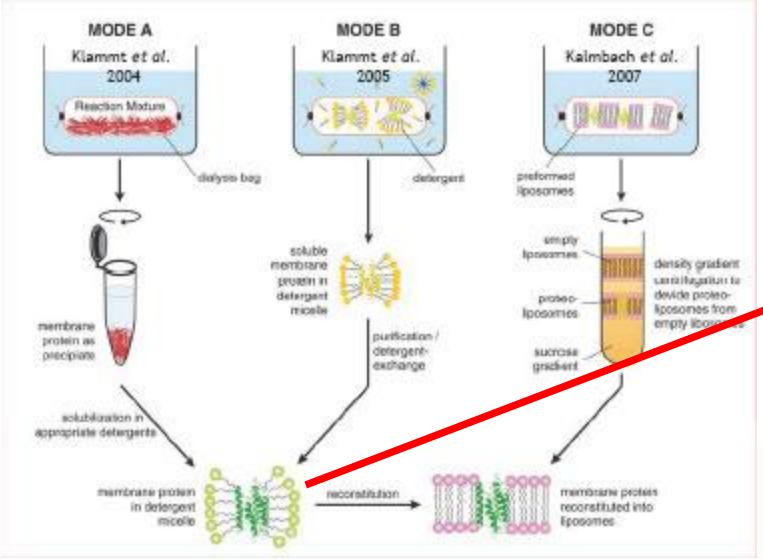
Membrane Proteins

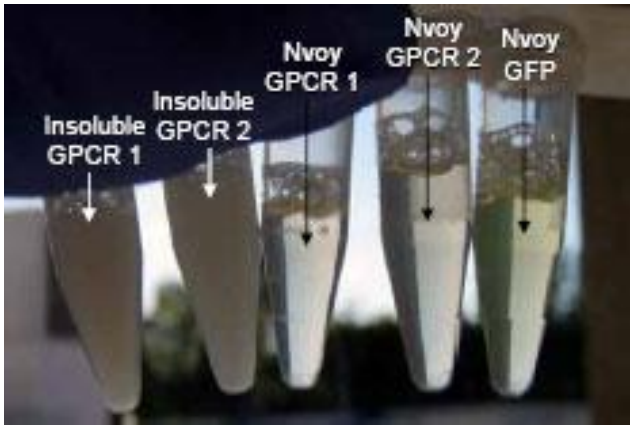
Cell Free Biosynthesis of GPCR's

- Family A and B GPCR's expressed CF Conventionally and with NVoy
- Found that NVoy works as in Mode B
- Concluded that NVoy does not interfere with CF expression
- NVoy enables soluble expression of active GPCR's (>10 nM ligand affinity)



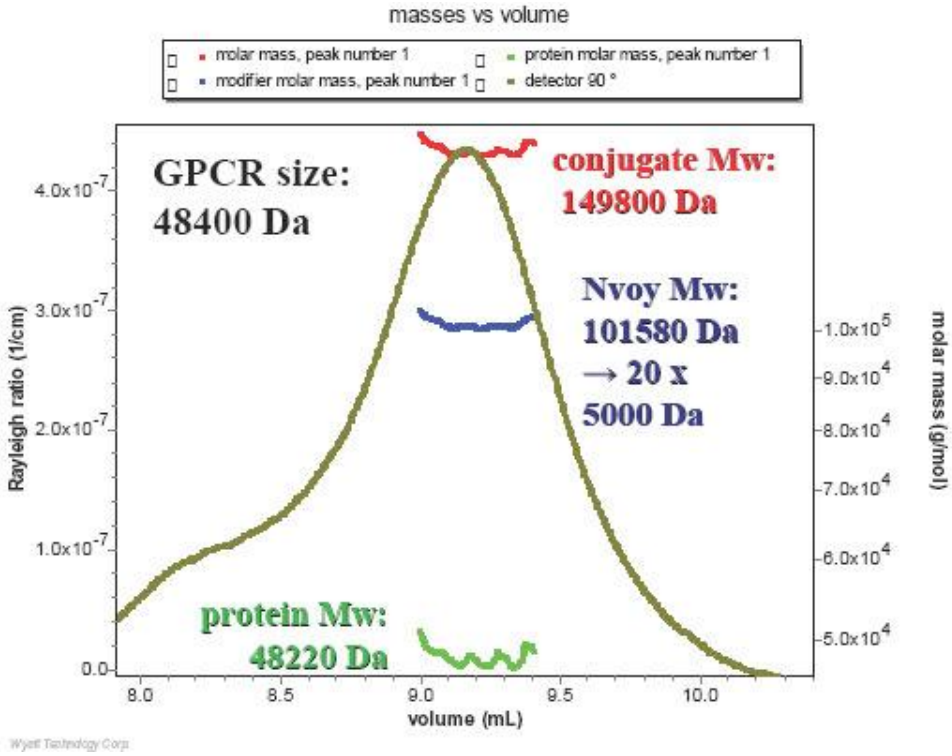
Family A- Rhodopsin Like | Family B – Secretin Like | Family C – Metabotrophic glutamate / Pheromone



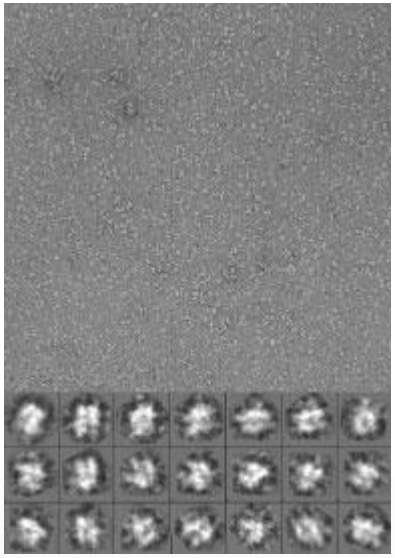


Membrane Proteins
Cell Free Biosynthesis

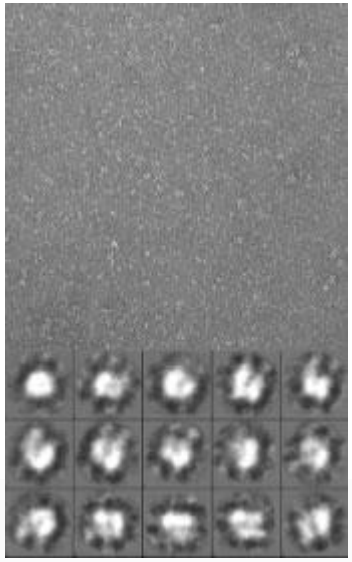
- Homogeneity of Cell-Free Produced Type 2 GPCR by HPLC and Single particle Analysis.



Family A GPCR



Family B GPCR

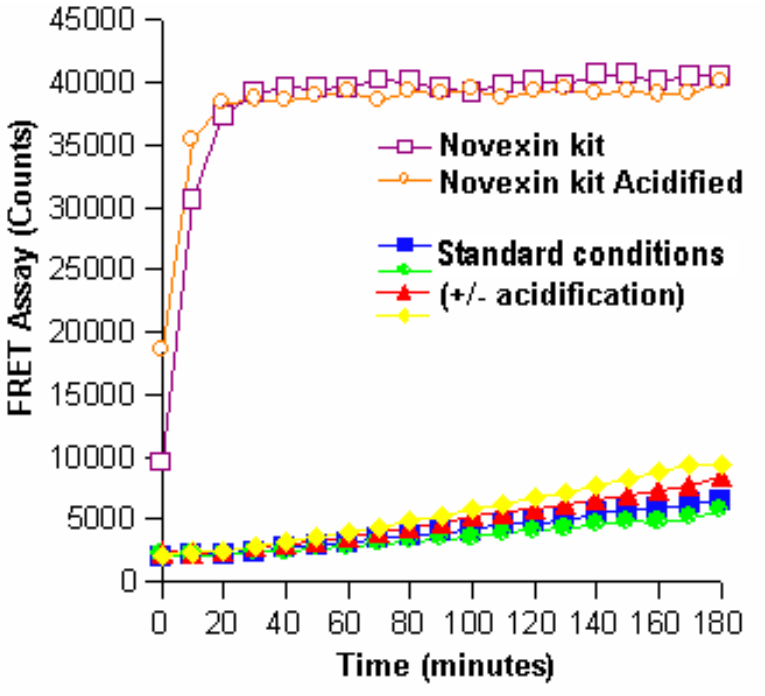


Full story in PNAS soon…

Protein Expression
Refolding

- Protozoan aspartic protease that forms inclusion bodies upon expression
- Refolding (Literature standard) results in poor yields and low protein quality

Using NVoy Improved yield and activity of refolded protein..



…and can be scaled-up to suit needs.

Scale	Volume	Total Activity	Ratio (Expected)	Ratio (Observed)
Small	**5**	**27625**	**1**	**1**
Large	**1000**	**6291563**	**200**	**228**

Ultrafiltration

Improving protein recovery

- Improve recovery of dilute protein solutions
- Enable concentration of aggregation prone proteins
- Enable preparation of supersaturated proteins solutions

Protein	% Recovery	
	- NVoy	+ NVoy
Antibody (10 ug/ml)	26%	98%
BSA (5 ug/ml)	46%	90%
Kinase	36%	100%
Membrane Protein	45%	98%

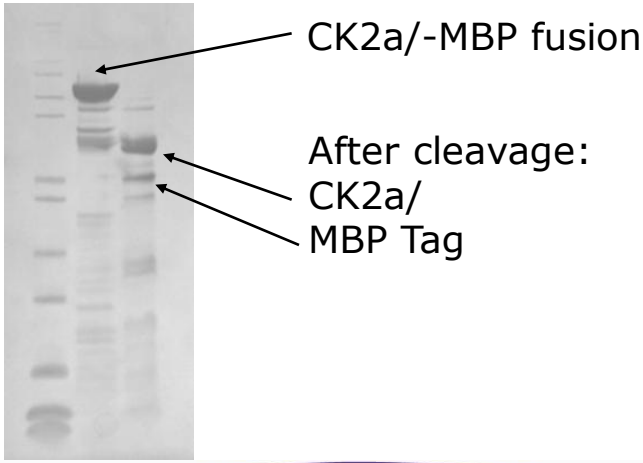
Data by various customers and other third parties

Fusion Protein Processing

Maintaining Protein solubility

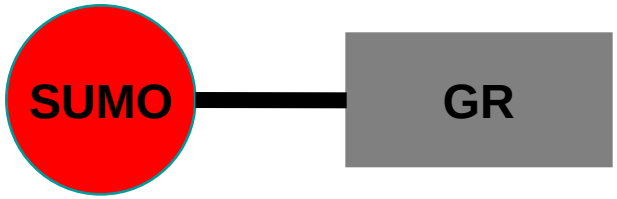
- Removal of the maltose protein fusion partner (k-MBP) from a kinase using Factor Xa.
- Production of CK2a/-MBP and then cleavage in the presence of CK2b to form holoenzyme complex CK2a/2b2
- Otherwise cleavage of MBP leads to aggregate formation

Time	% aggregation - NVoy	% aggregation + NVoy
1 hour	6%	0%
2 hours	29%	1%
3 hours	58%	1%
4 hours	100%	1%

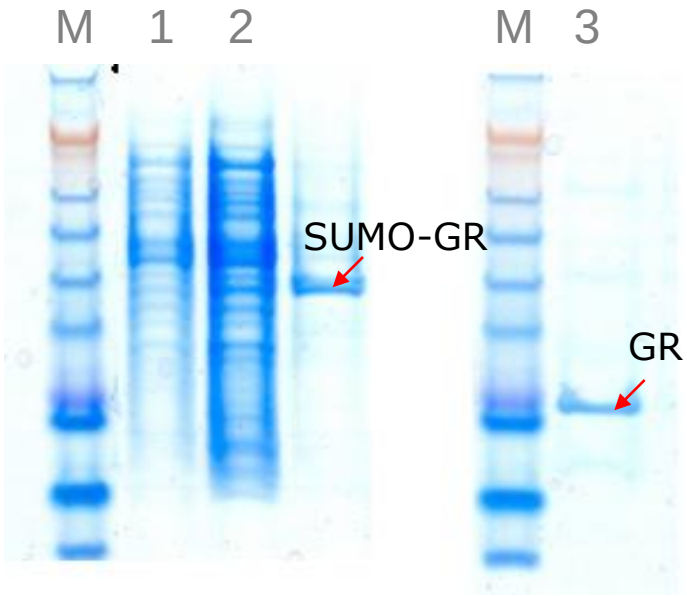


Fusion Protein Processing

Maintaining Protein solubility



- Fusion Protein
  - SUMO Tag (Ubiquitin-related affinity
  - Target protein: Glucocorticoid Receptor
- Pharmaceutical company producing GR as SUMO fusion but after cleavage of the tag a ligand is required to keep the protein soluble
- NVoy enabled
  - Stable and soluble cleaved GR without ligand
  - 100% cleavage in the presence of NVoy



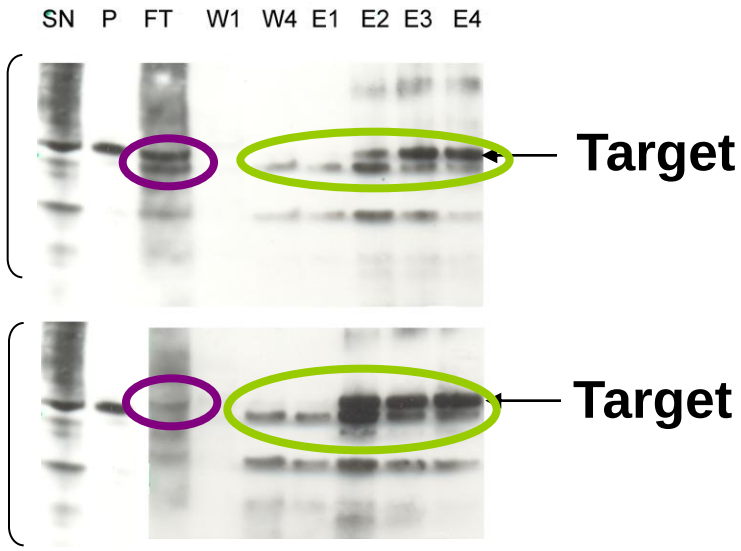
<u>Lane1&2</u> Cell pellet and lysate

<u>Lane 3</u> Cleaved soluble GR in 2% NVoy polymer

Protein Purification

Increased Yields & Recovery

- Transcription Factor
- Batch Binding to magnetic beads
- Improved binding to the beads
  - less target in Flow Through
  - higher recovey & yields



Data by courtesy of Dr Ana Villegas-Mendez, HumProTher Laboratory, France

Protein Purification

Increase Purity & Stability

- Large membrane associated protein (>160kDa) that requires co-factors for activity

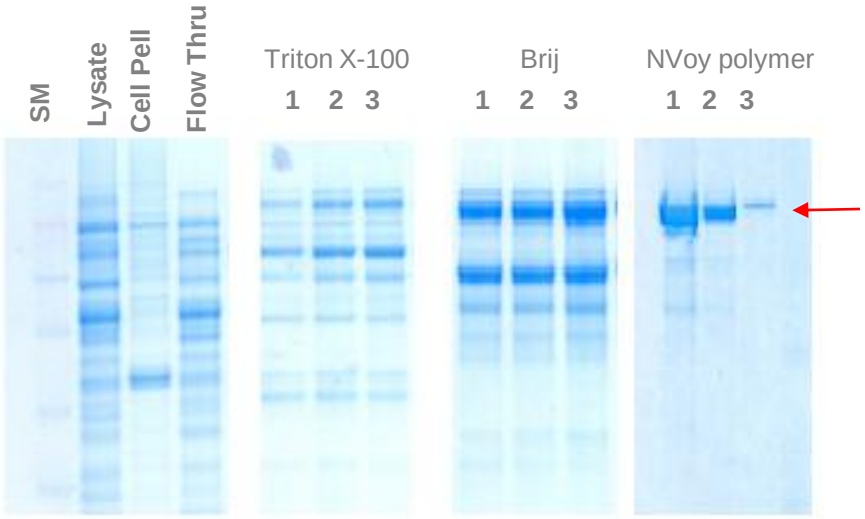
Using Detergents;

results in unstable enzyme preparations containing large number of contaminants

- At 4ºC loss of activity within 24h
- At -80ºC after one freeze/thaw cycle

Using NVoy Polymer;

- Cleaner protein preparations obtained
- Enzyme activity stable at 4ºC for over 4 weeks
- Enzyme activity stable at -80ºC for 4 freeze/thaw cycles

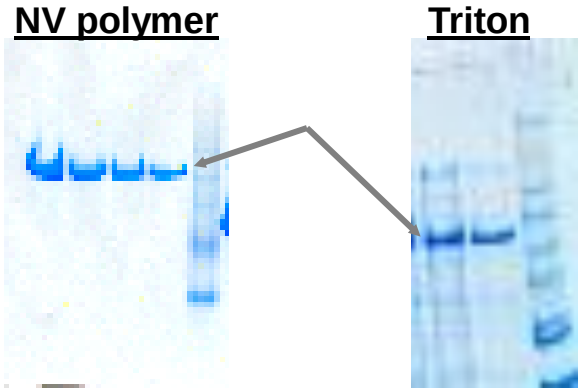


- Enzyme processed from *E. coli* lysates
- Cells cultured at 37ºC, induced with IPTG and cultured for further 72h.
- Cells harvested then lysed with buffer containing co-factors and NVoy polymer / detergents Lysate clarified by centrifugation and purified on ADP Sepharose using NVoy polymer

Protein Purification

Replacing Detergents

- Preparation of a cytosolic enzyme involved in DNA synthesis
- Enzyme required for screening and structural biology but normal production in detergents was not suitable for crystallography
- Protein processed in and purified with NVoy polymer in buffers gave increase in both the purity and yield of the protein.
- Specific activity of protein was similar to literature values for native enzyme



Protein Purification

Endotoxin Removal

Comparison of three methods:

1. Pierce Detoxi-gel
2. Non-ionic detergent (Triton X114) according to Reichelt *et al*., 2006
3. NVoy polymer

His-tag fusion	Control	Method 1 Detoxi gel	Method 2 Detergent	Method 3 NV polymer
Endotoxin (EU)	130,000	1,200	100	<1
Yield (%)	100	29	39	66
Activity (%)	100	37	25	77

Endotoxin is undetectable

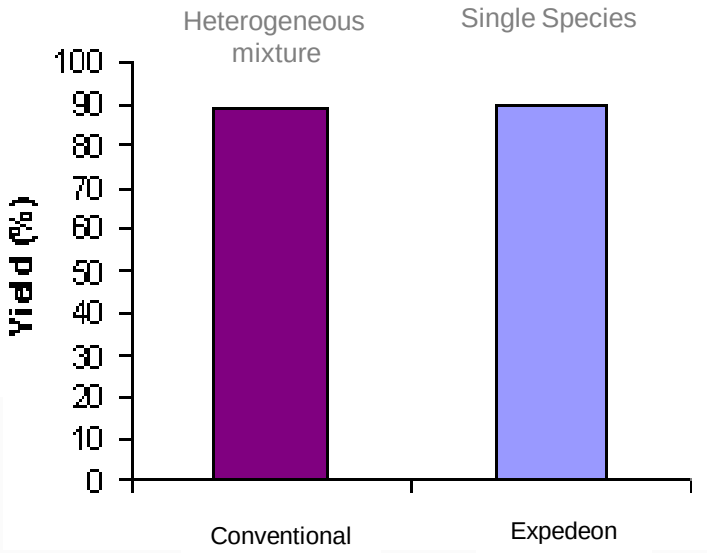
Yield is improved

Highly active

Protein Formulation

Improving monodispersity

- Bacterial protein produced in inclusion bodies, refolding screen gave good yield but heterogeneous mixture of proteins unsuitable for studies
- NVoy Refolding kit used as alternative to screen
- One set of experiments generated data comparable to screening and also single species of protein – project can be taken forward



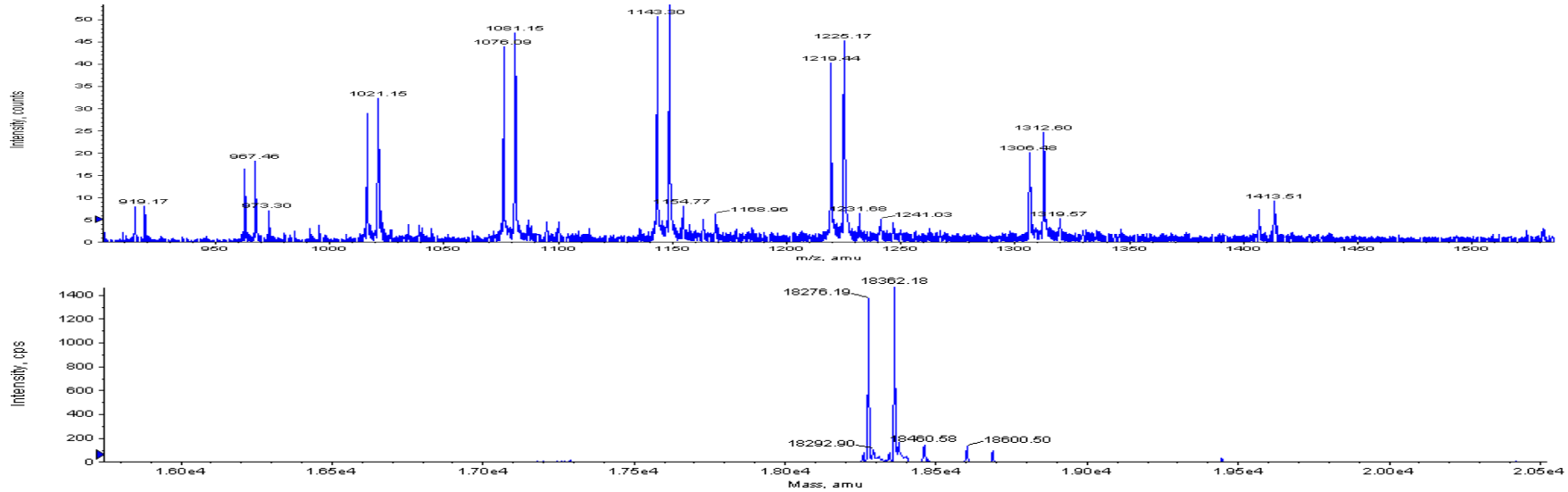
"extremely straightforward and easy to use whilst the results it generated were excellent"
" single species of protein with no loss of yield compared to the heterogeneous mixture of species"
"we will definitely use for further work with this protein and incorporate in our refolding studies with future proteins"

Data courtesy of Emergent Europe

Analytical Compatibility
Mass Spectroscopy

- Requires relatively high concentrations of protein (>5 μM) which can be problematic especially for larger proteins
- Technique does not readily tolerate commonly used stabilisers and solubilisers, e.g. detergents, glycerol and arginine

Electrospray Ionisation (ESI)

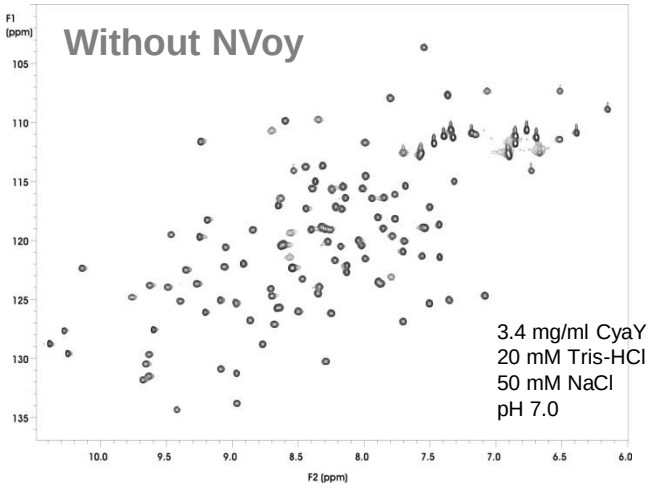


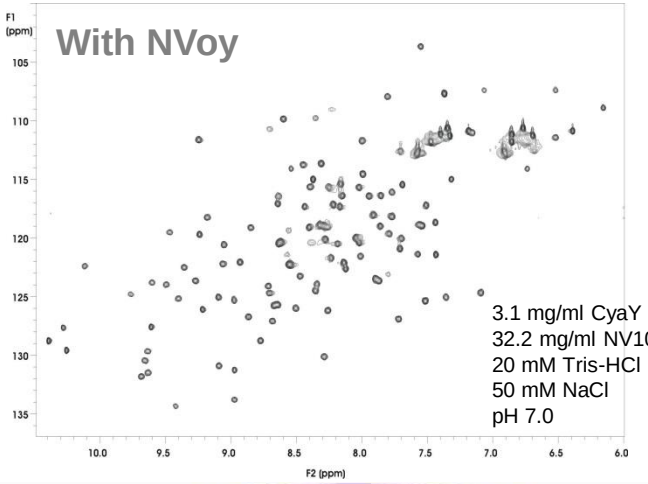
Protein ionised well with NVoy polymer. Deconvolution of spectra gives two protein species corresponding to the mass of β-lactoglobuline A and β-lactoglobuline B.

Analytical Compatibility

Nuclear Magnetic Resonance

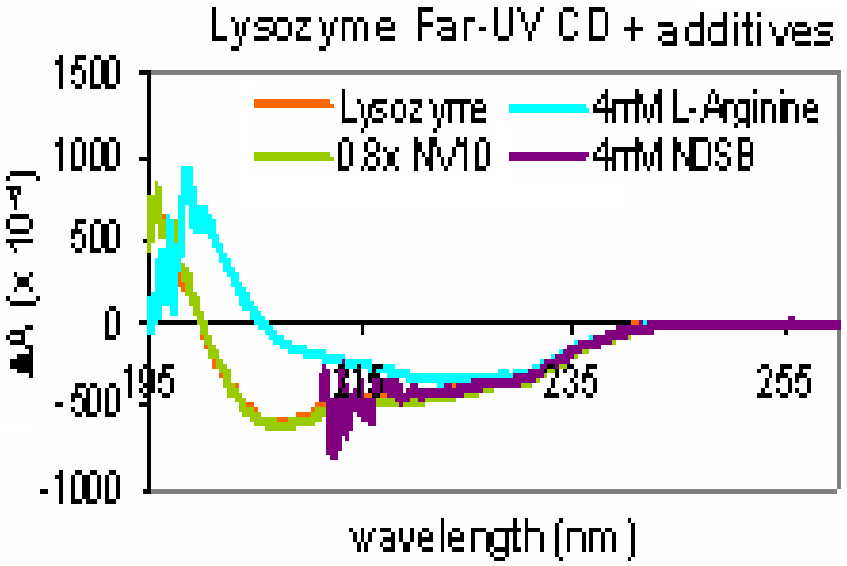
- Powerful technique to measure the structure, function and dynamics of proteins in solution.
- Aggregation caused by:
  - High protein concentration
  - High temp for several hours
  - Even low aggregation results in poor chemical shift dispersion and extreme line broadening, preventing 2D and 3D techniques
- NVoy polymer is invisible in 15N and 1H NMR enabling stable preparation of concentrated protein solutions.
- CyaY is a bacterial ortholog of the human mitochondrial protein Frataxin.
- Functional studies of this protein have utilised 15N-HSQC NMR spectroscopy (two dimensional NMR with one 1H and one 15N frequency) to clarify details of iron-binding sites.



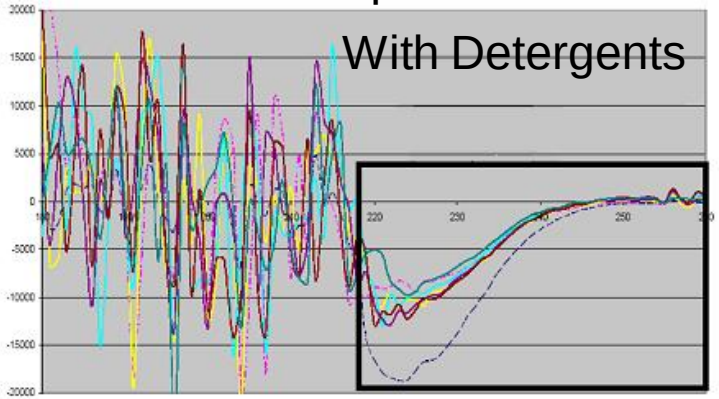


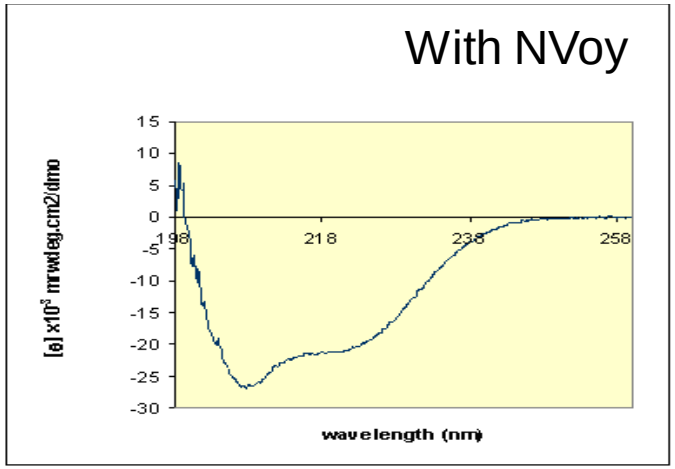
Analytical Compatibility
Circular Dichroism

- Estimates secondary and tertiary structure of proteins
- Confirms whether structure has been retained during protein processing,
- Structure frequently adversely affected by additives such as solubility enhancers and detergents.



EPO like protein





NVOY Summary

Key Benefits & Advantages

- Increase solubility & stability of proteins
- Retain protein functionality
- Minimise aggregation
- Enable analysis
- Simple & generic to use
- Replace detergents

The GELFREE® 8100 Fractionation System

Molecular Weight Fractionation with Liquid Recovery

www.expedeon.com

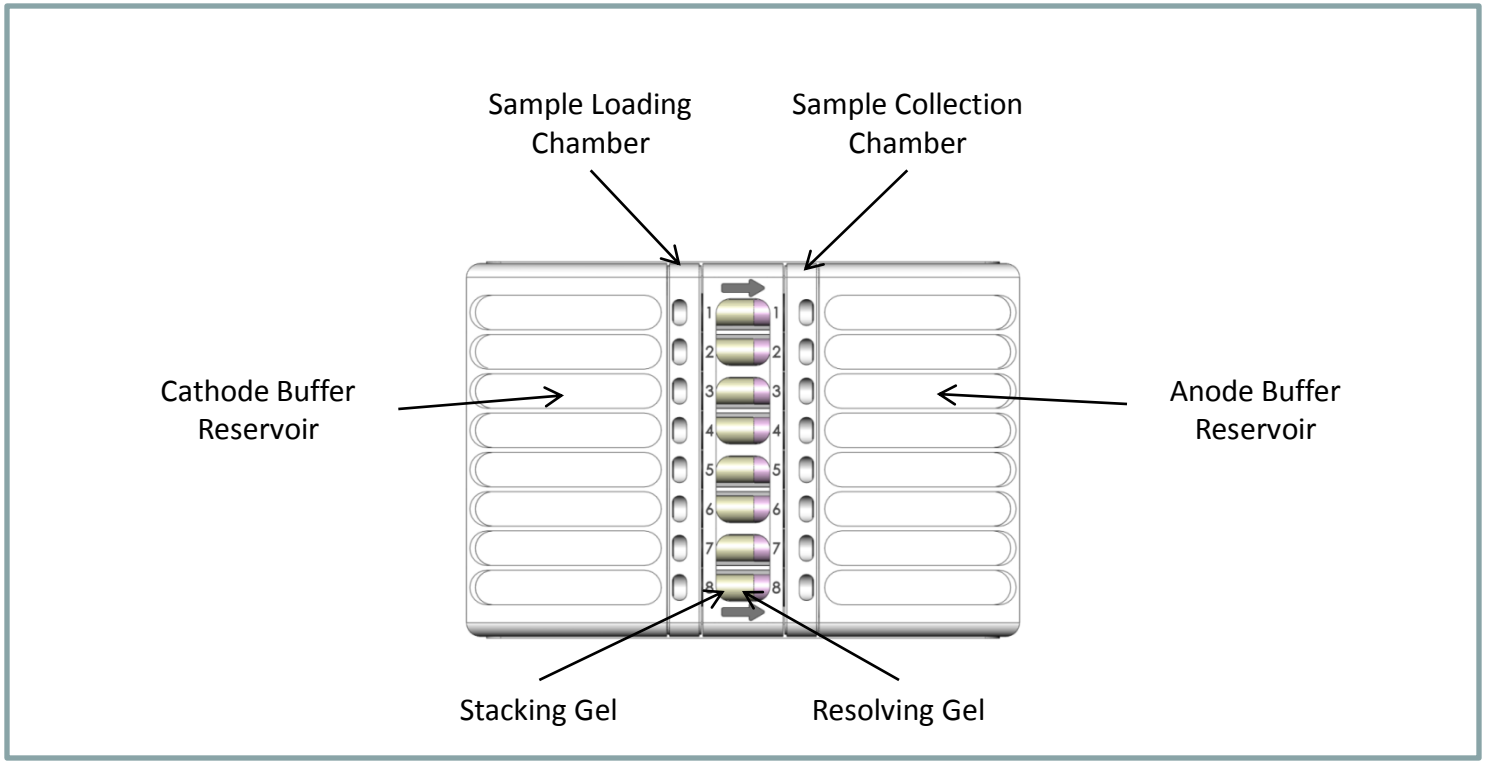
GELFREE

Key Benefits & Advantages

- Traditional electrophoresis, but in high-capacity tube format
- Fast, easy to use, bench-top device
- Intact protein MW fractionation from 3.5 – 500 kDa
- Programmable fractionation for isolating and purifying targeted proteins
- Liquid-phase recovery for in-solution digestion – no band cutting
- Up to eight samples processed in parallel
- High loading capacity (>5X more than a 1Dgel)
- High protein recovery (>80%); High reproducibility (<15%)
- Ready-to-use consumables

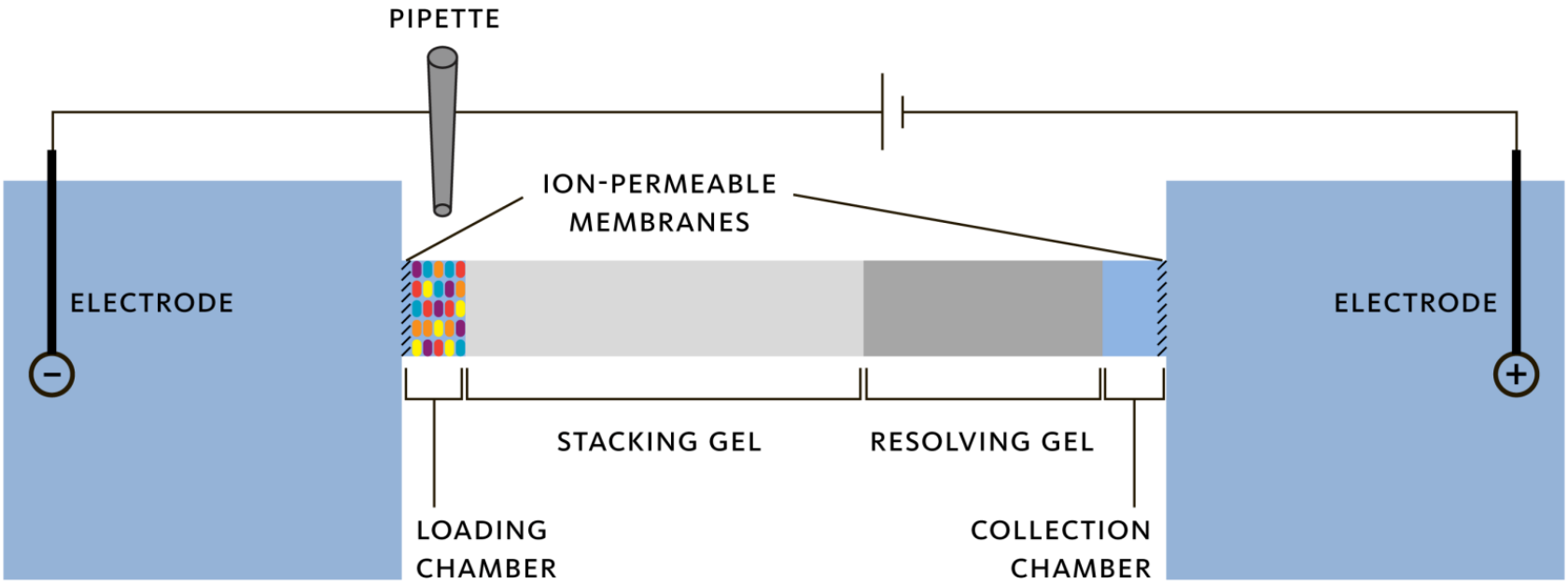
GELFREE

How it works – The Cartridge



GELFREE

How it works – 8100 platform



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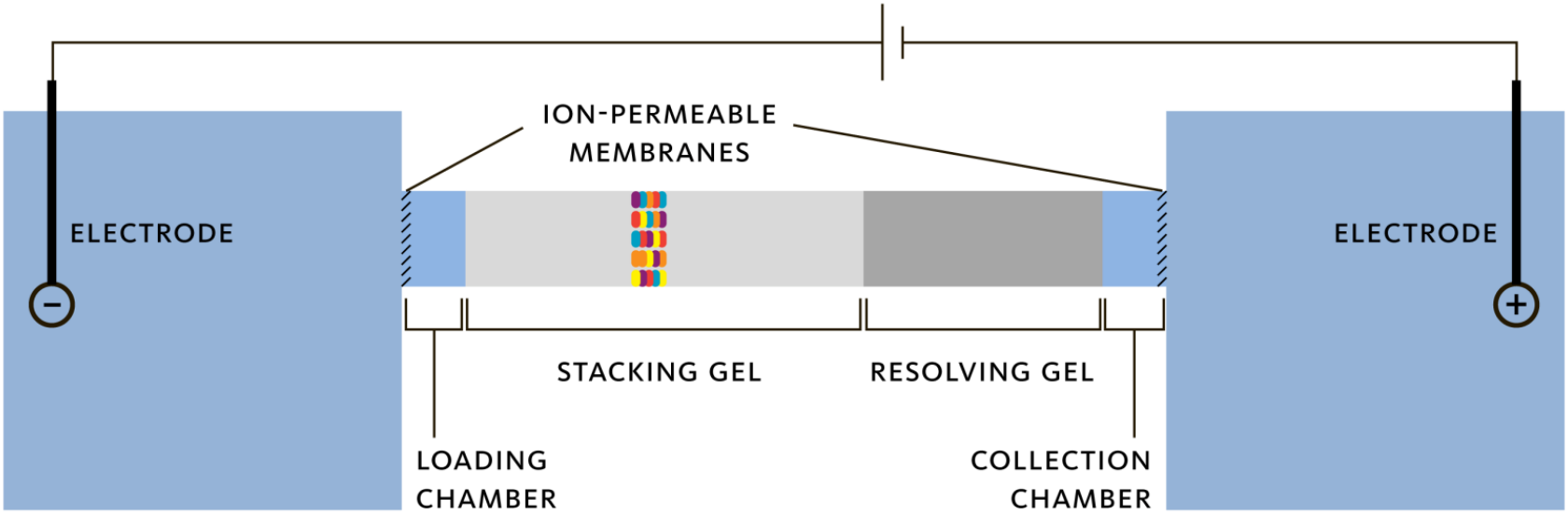

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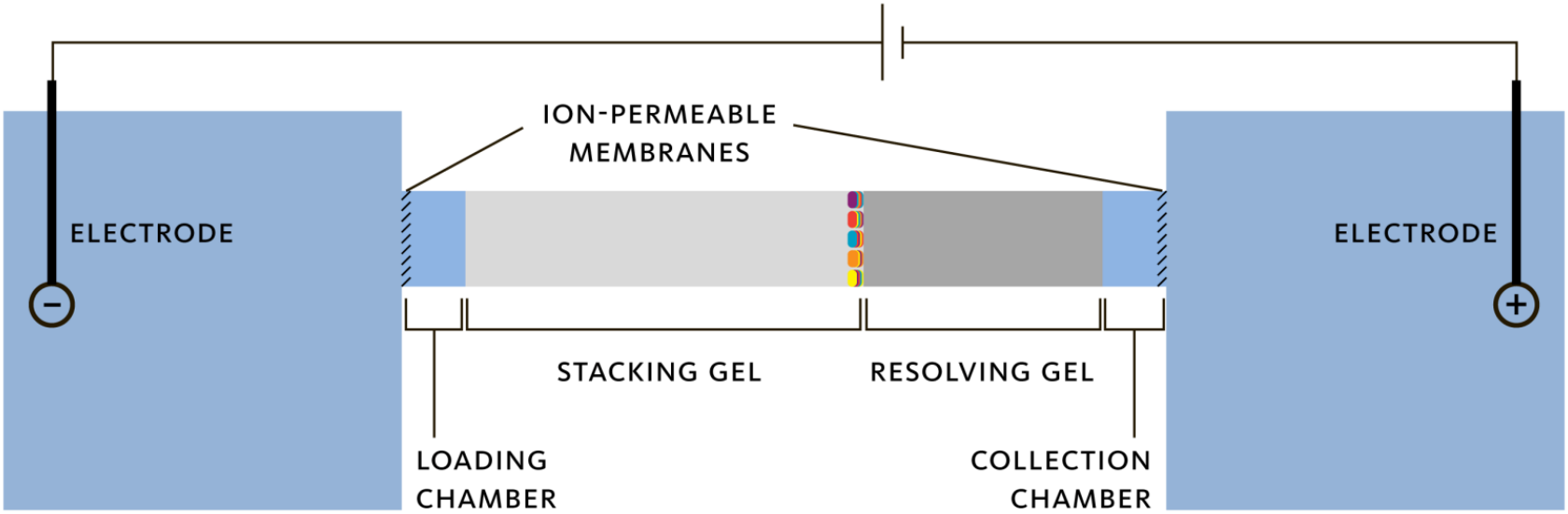

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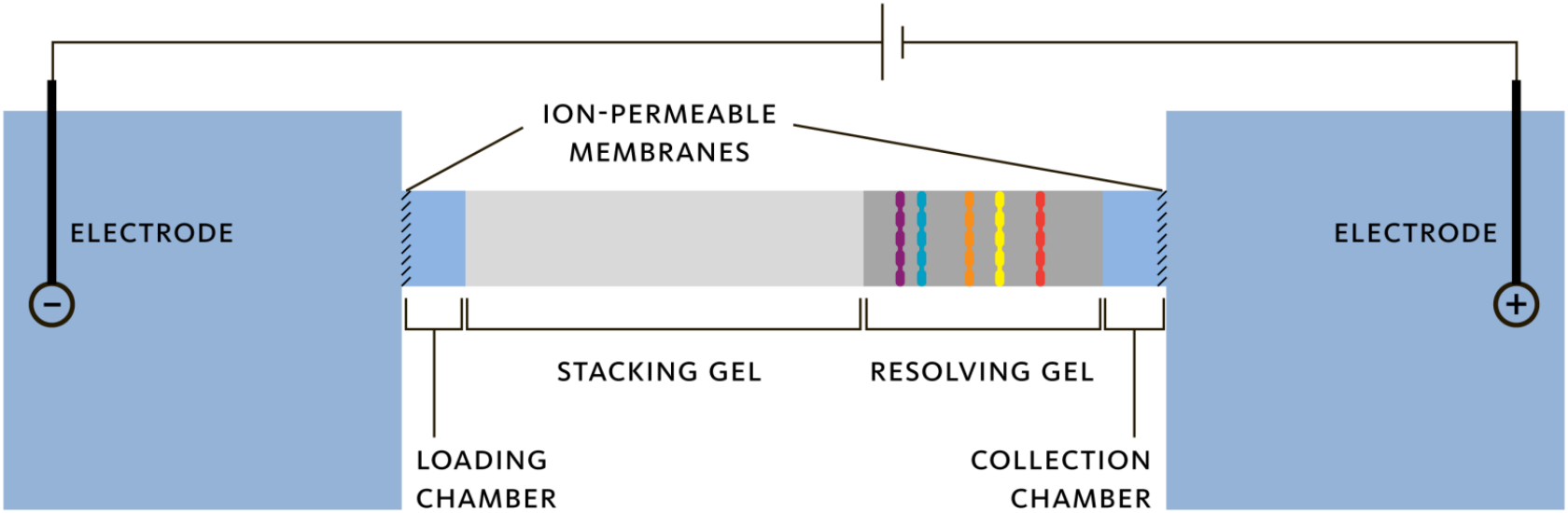

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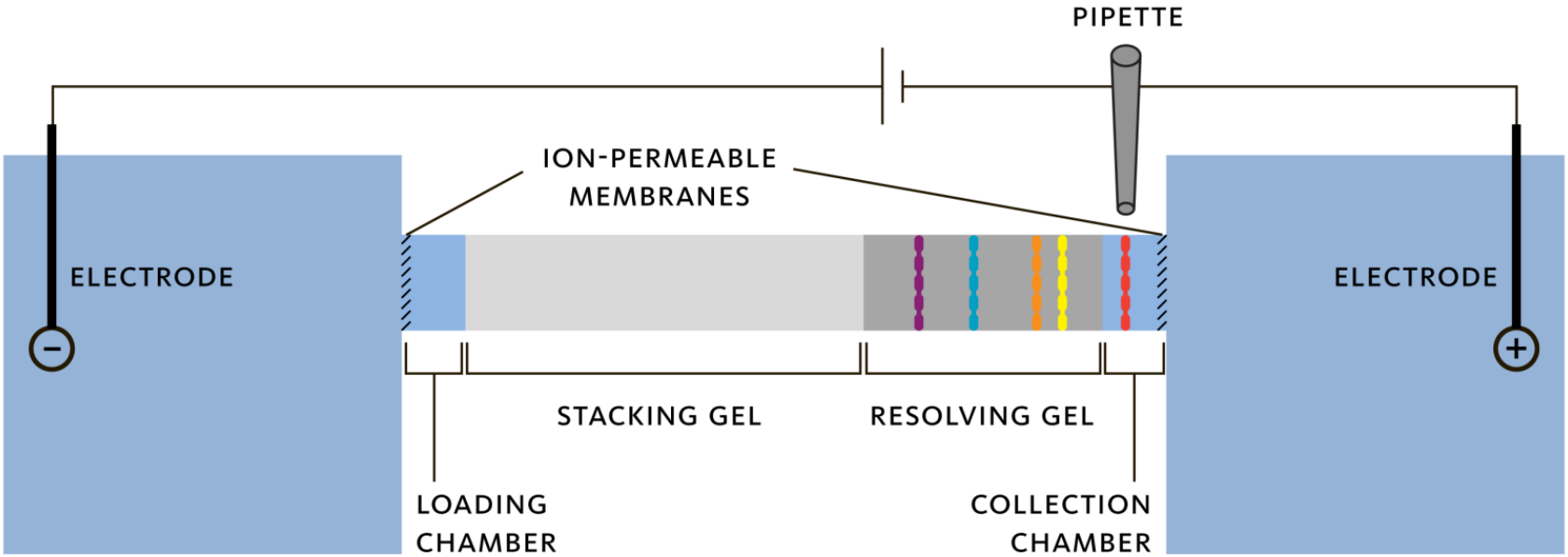

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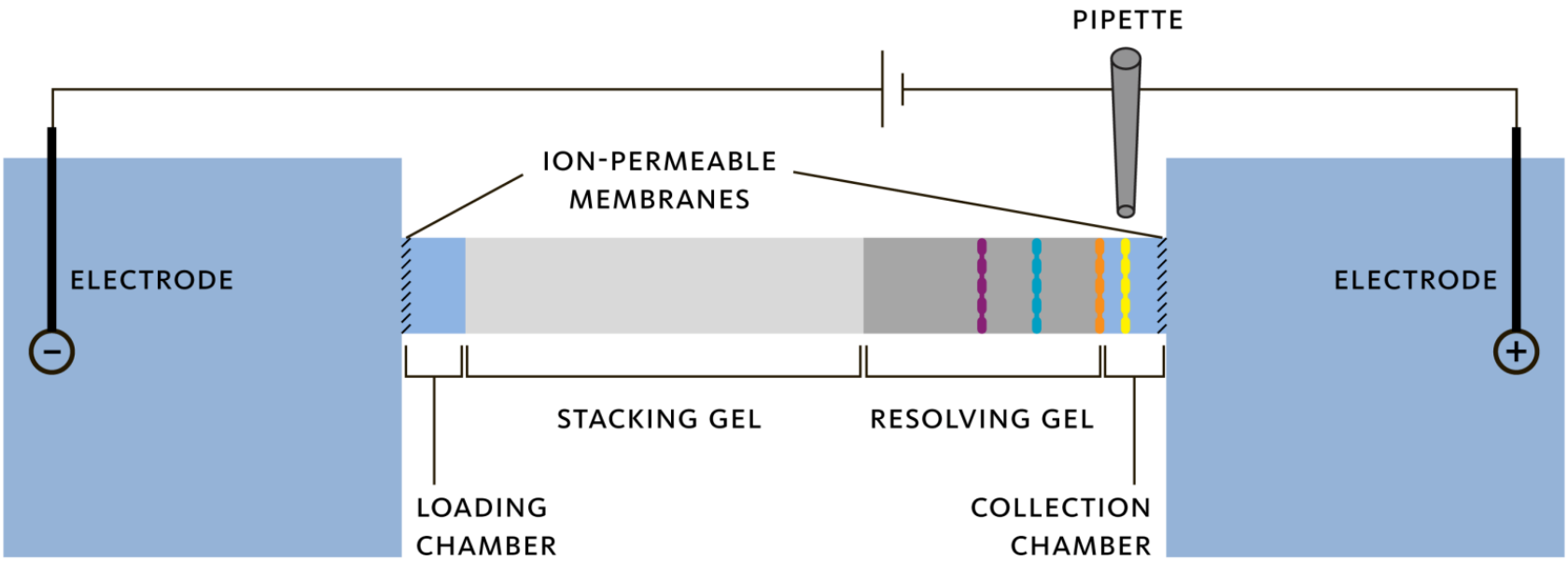


GELFREE
Applications

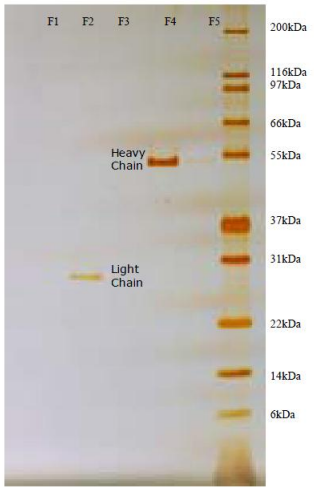
- Discovery Proteomics
  - Top - down: fractionation, PTMs
  - Bottom - up: fractionation, distinguishing isoforms

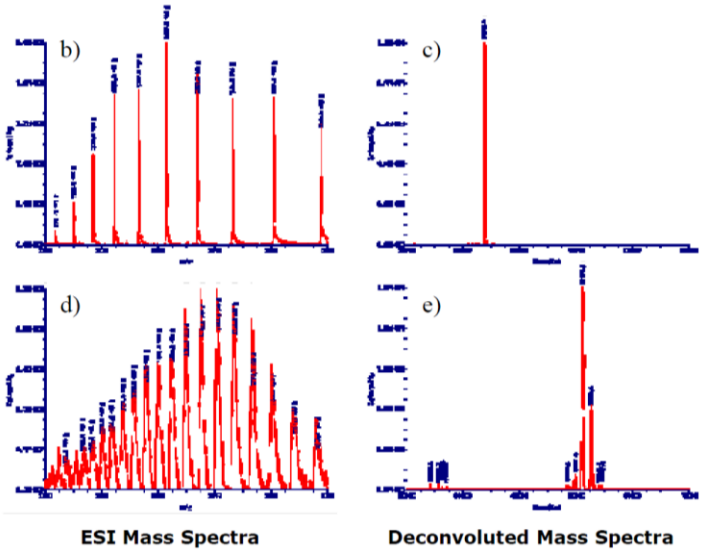
- Targeted Proteomics
  - Protein/peptide quantitation
  - Biotherapeutic characterization

GELFREE

Top Down: Intact Antibody Isolation

Analysis of intact mass of light chain and heavy chain mAb. Gelfree collection times were optimized for collection of the light and heavy chain mAb in distinct fractions. The optimized recovery of the light and heavy chain are demonstrated using 1D gel analysis Analysis of these fractions using ESI-MS is shown for the light chain (b-c) and the heavy chain (d-e). Heavy chain mass indicates presence of the G0F form of the mAb.





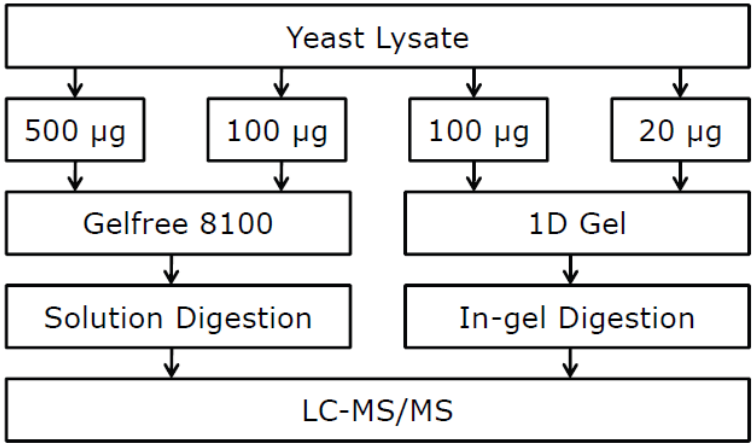
- simplified, universally applicable, method for isolation of antibodies and antibody fragments.
- can be optimized to ensure high recovery of the protein of interest.
- compatible with the intact analysis by ESI; likewise, the sample can be analyzed using bottom-up techniques.

GELFREE

Bottom Up: Gelfree vs 1D gel

EXPERIMENTAL DESIGN

This study compares traditional GeLC-MS/MS using a 1 mm precast 1D gel and in-gel digestion to the same fractions prepared using Gelfree 8100 and in-solution digestion. In short, a single yeast lysate was divided into aliquots for preparation using both techniques. Aliquots of 500 and 100 µg were fractionated using Gelfree 8100 while 100 and 20 µg aliquots were separated using 1D gel. Gelfree fractions were subjected to tryptic digestion in solution. Gel bands that correspond to identical molecular weight fractions for four of the Gelfree fractions (5-8) were excised from the 1D gel lanes and digested using in-gel tryptic digestion. Each sample set was analyzed using nanoLC-MS/MS on an ion trap mass spectrometer.



GELFREE

Bottom Up: Gelfree vs 1D gel

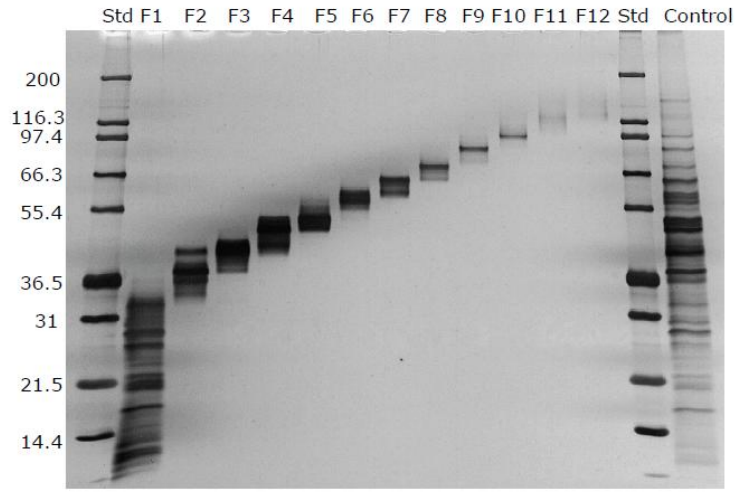


Figure 2: Fractionation of *S. cerevisiae* by Gelfree 8100. The lysate was fractionated into 12 fractions ranging in molecular weight from 3.5 to 150 kDa. The fractions were visualized using 1D gel electrophoresis, followed by silver staining. Fractions 5-8 were selected for comparison against GeLC. This figure shows 500 µg loading of Gelfree 8100.

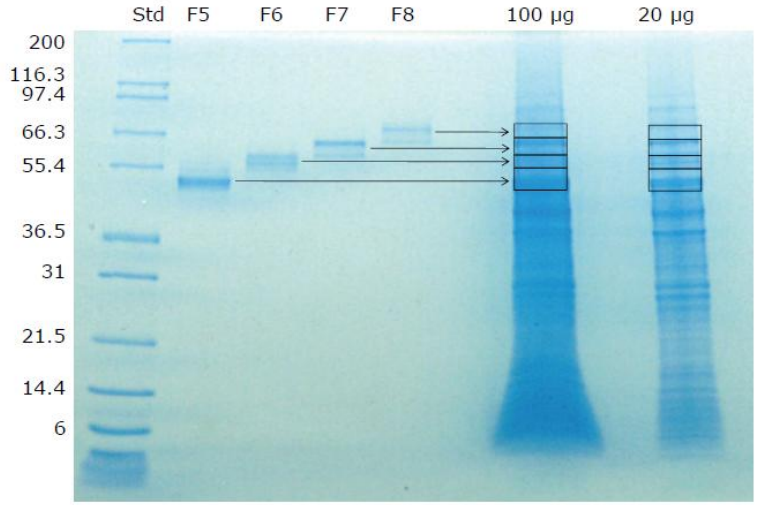
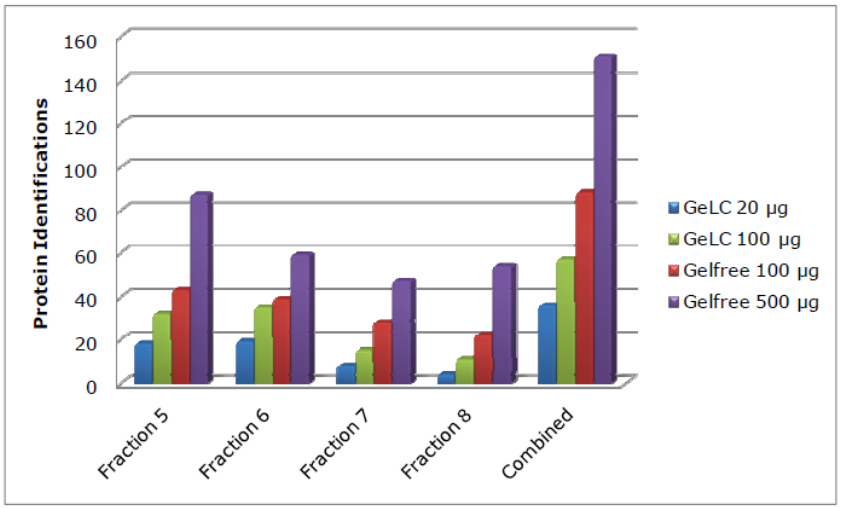
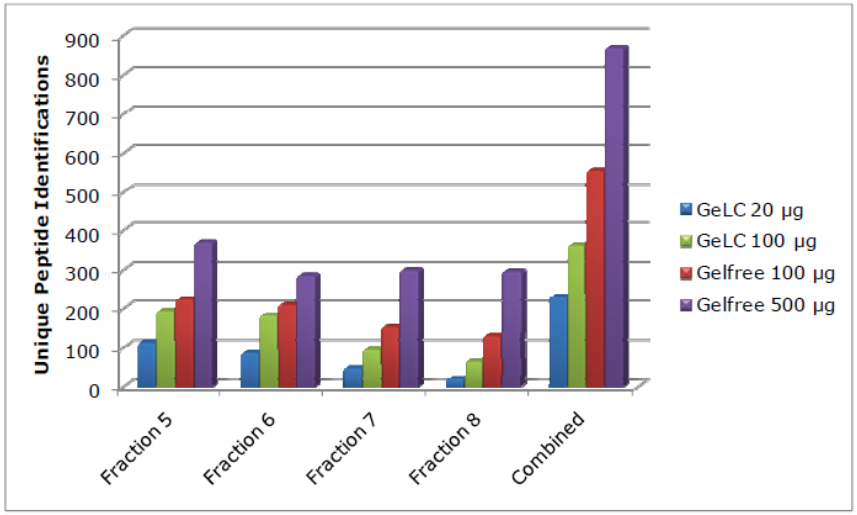


Figure 3: Preparation of *S. cerevisiae* for GeLC-MS/MS analysis. The lysate was separated using 1D gel electrophoresis. Aliquots from the Gelfree fractions were run in parallel to align with the unfractionated sample. The molecular weight ranges corresponding to each Gelfree fraction were excised and digested using standard protocols. The range of the four fractions chosen is approximately 15 kDa (53-68 kDa).

GELFREE

Bottom Up: Gelfree vs 1D gel

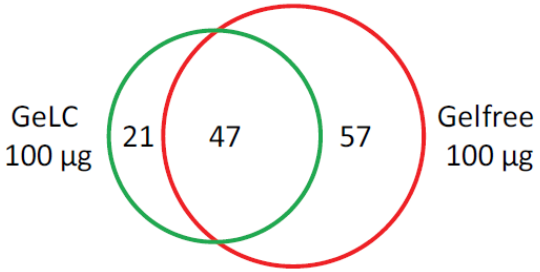


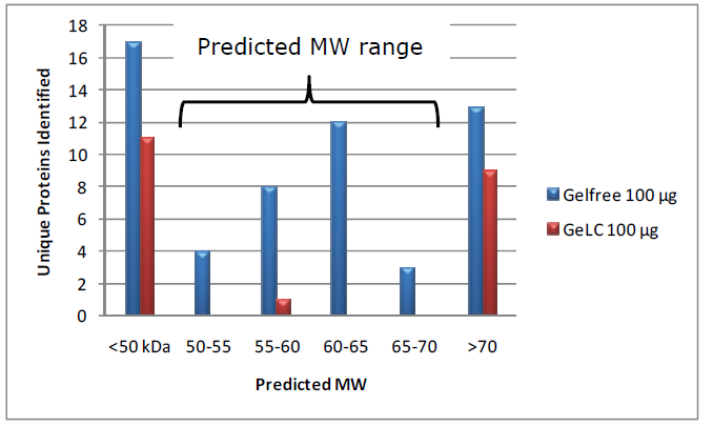


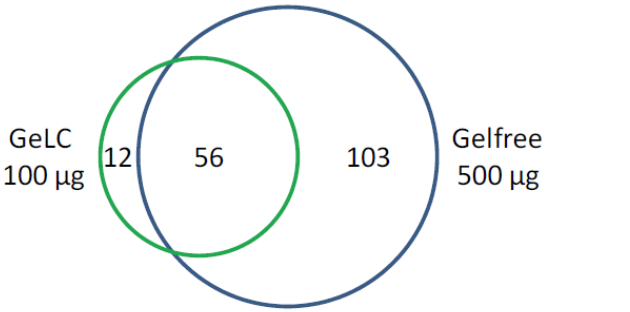
Comparison of the total number of valid proteins identified using Gelfree with those identified using conventional GeLC reveals significantly more proteins and peptides are identified using Gelfree 8100. This increase of approximately 50% can be directly correlated with both the higher loading capacity and the increase in the percentage of total protein recovered from Gelfree 8100.

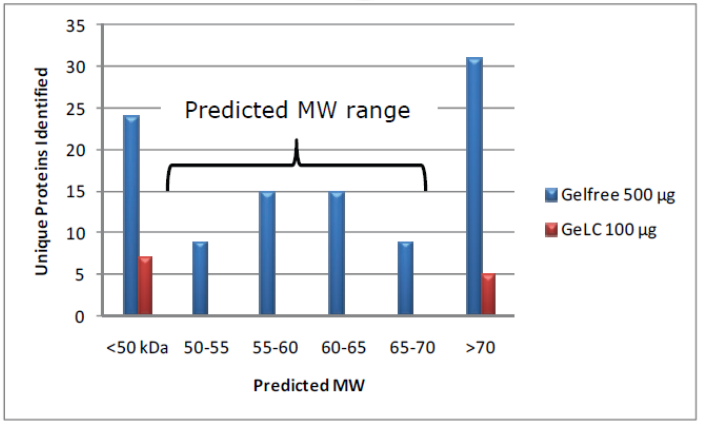
GELFREE

Bottom Up: Gelfree vs 1D gel





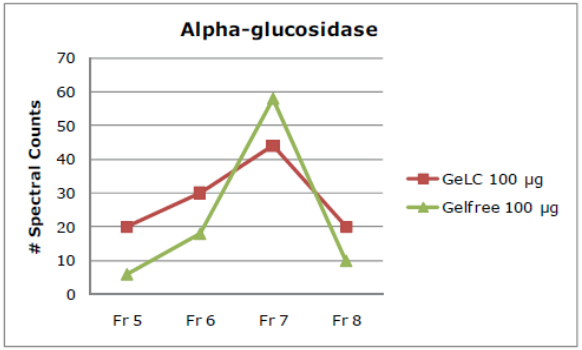


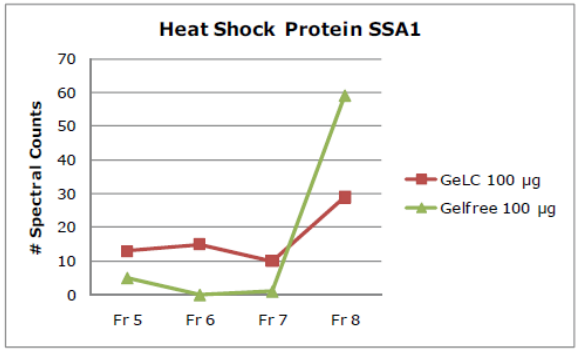


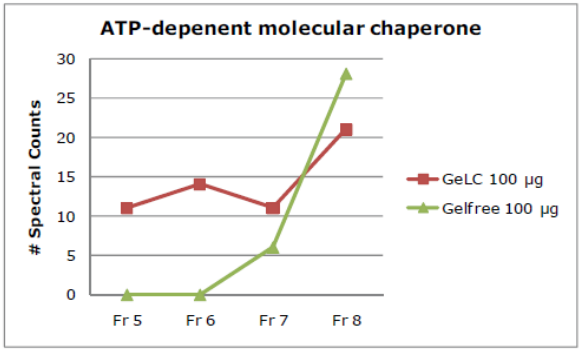
Venn diagrams show the complementarity of Gelfree and GeLC. Further analysis of the unique proteins identified using each technique reveals that GeLC only provides unique protein identifications outside the selected mass range due to poor resolution or inaccurate excision of bands. In contrast, Gelfree increases the number of valid protein identifications in all ranges.

GELFREE

Bottom Up: Gelfree vs 1D gel







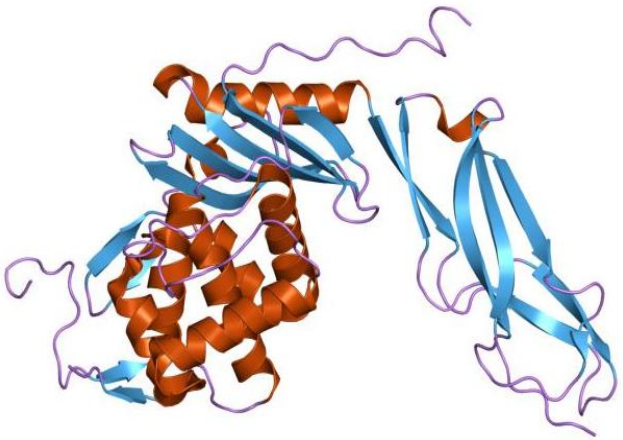
High loading capacity of Gelfree provides increased resolution relative to conventional 1D gels. Examination of spectral counts of identified proteins across all fractions reveals that proteins are spread across fewer fractions, reducing redundancy of protein identifications across fractions at equivalent protein loading.

Amintra: COOL-TAG

Ligand free elution

What is the COOL-TAG?

The COOL-TAG is a monomeric, soluble 28.5kDa engineered fragment of a Penicillin Binding Protein.

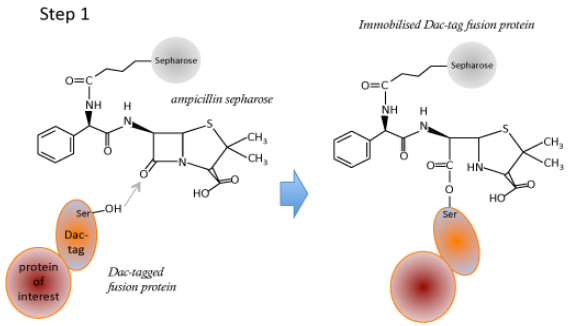


Amintra: COOL-TAG

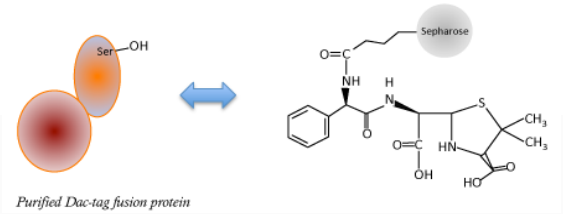
Ligand free elution

How does it work?

The COOL-TAG binds rapidly and efficiently to ampicillin sepharose at room temperature by forming an ester bond via a serine residue.



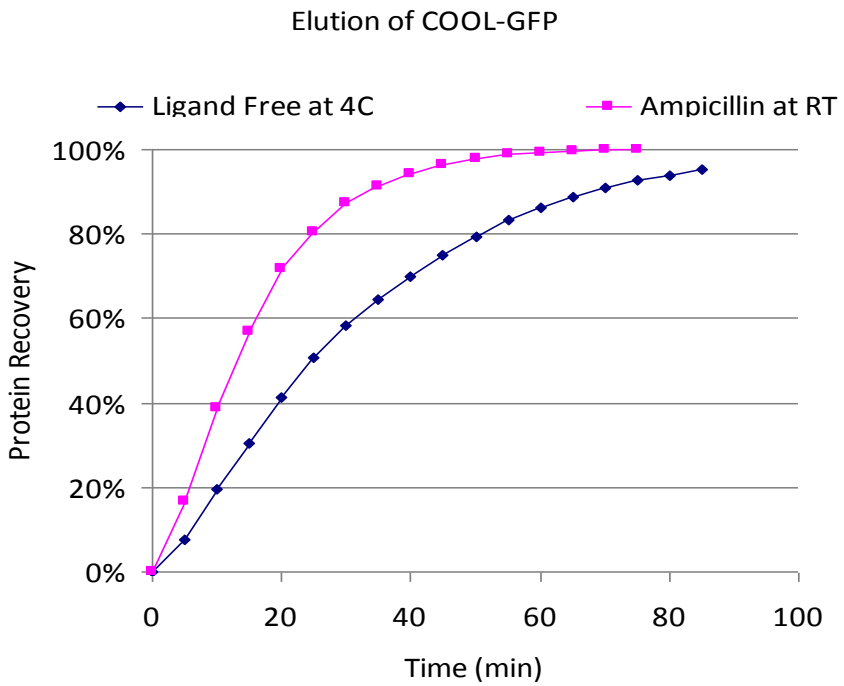
The target protein can then be eluted at 4C or via competitive elution with ampicillin.



Amintra: COOL-TAG

Key Benefits

Ligand Free Elution at 4C



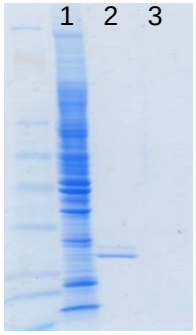
cool-tagged protein can be eluted either at 4C or storing the sample on ice or competitively with ampicilin. Ampicilin mediated elution is slighty faster. Protein recovery is excellent for both methods

Amintra: COOL-TAG

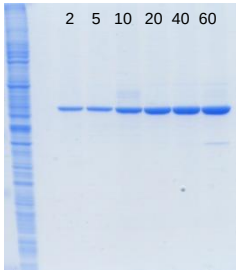
Key Benefits

High Specific affinity

Unlike other tags, only COOL-TAGGED proteins bind the resin. As demonstrated by the pulldown of 2mg HEK 293 protein extract with 20µl different resin. Ni-Sepharose (lane 1) pulls down many contaminants, GST-sepharose (lane 2) non-specifically binds GST and carbonyl reductase, Ampicilin sepharose (lane 3) does not bind contaminants



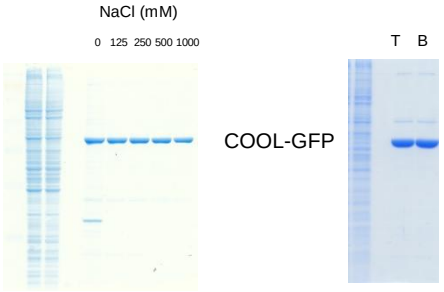
Rapid Binding



cool-tagged target protein rapidly binds to ampicillin sepharose. Saturation is achieved after 60 minutes. Up to 10mg/ml protein can be bound to the resin.

High Compatibility

COOL-TAG technology is compatibile with salt concentrations up to 1M, works in your buffer of choice at pH 7.5 and is suitable for use with non-ionic detergents such as triton 0.2% X-100 (T) or 0.06% Brij35 (B).



2mg HEK 293 protein extract with 20µl resin

Thank You for your Attention

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