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Ministero dell'Università e della Ricerca

Italiadomani
PIANO NAZIONALE DI RIPRESA E RESILIENZA

VITALITY

Sviluppo di una piattaforma per proteine ricombinanti a potenziale uso terapeutico

Università degli Studi di Urbino
17 dicembre 2024

VITALITY

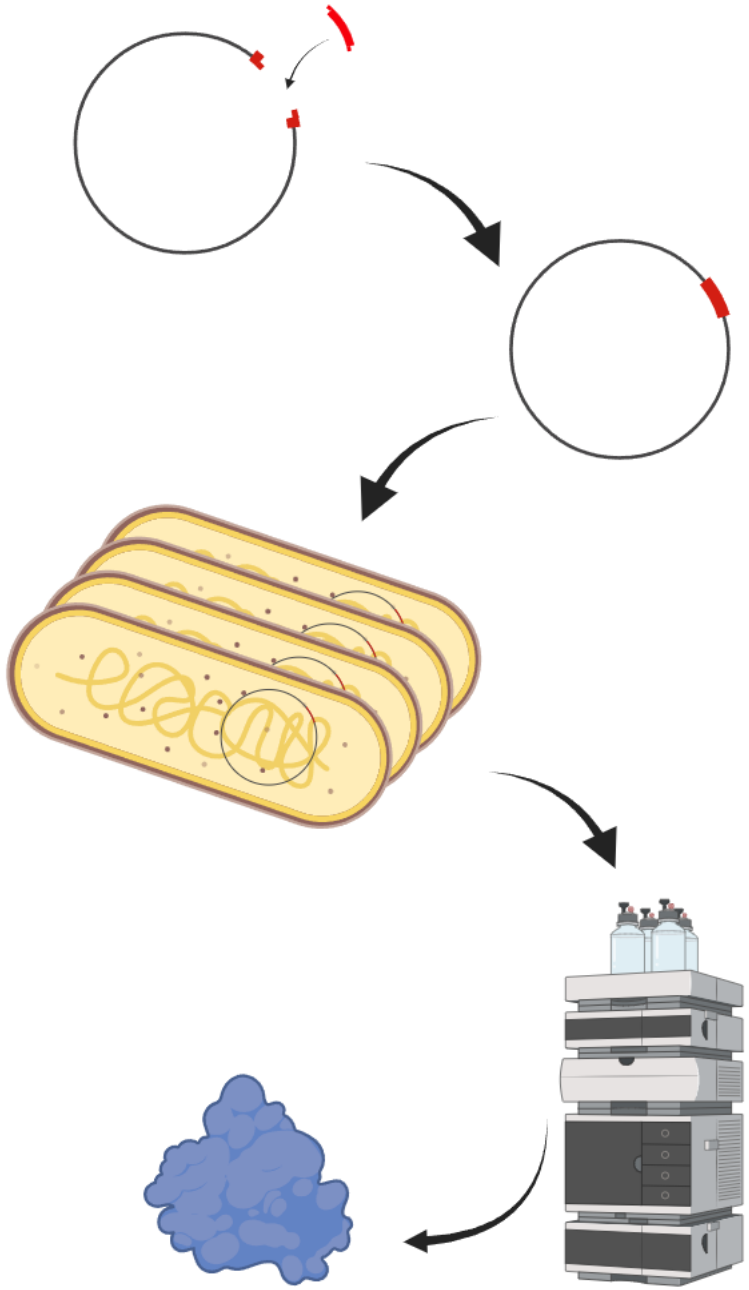
How is a recombinant protein produced?

The protein-coding DNA sequence is inserted into a plasmid

The plasmid is transferred into host cells

Host cells produce the protein

The protein is purified from the host cells

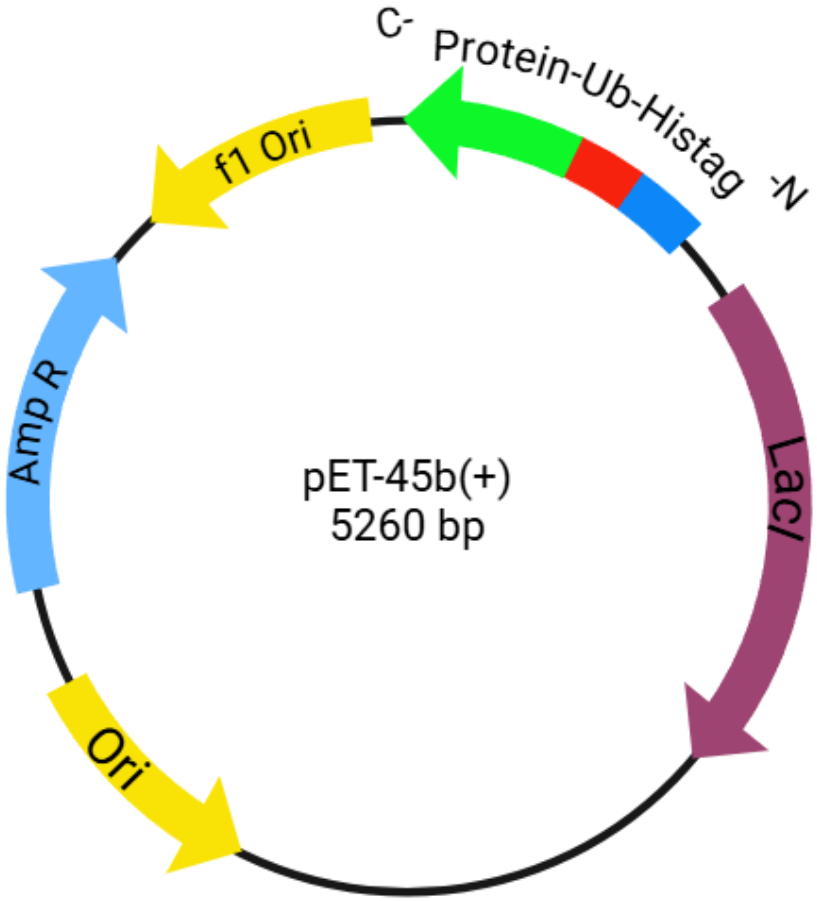


Our production strategy:

A pET-45b plasmid is transformed into Escherichia coli BL21(DE3) cells

Cells are grown and protein epression is induced through Introduction of IPTG into the culture

Cells are harvested and processed for purification



Our production strategy:

Our recombinant proteins are expressed with a His-Ubiquitin tag as a fusion partner

The Histidine portion of the tag allows purification of the recombinant protein through IMAC

the ubiquitin portion allows easy removal of the entire tag

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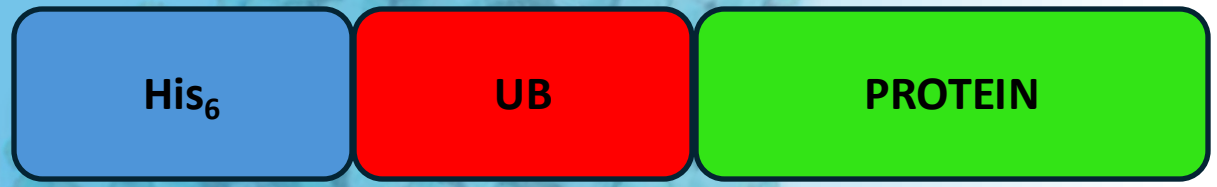
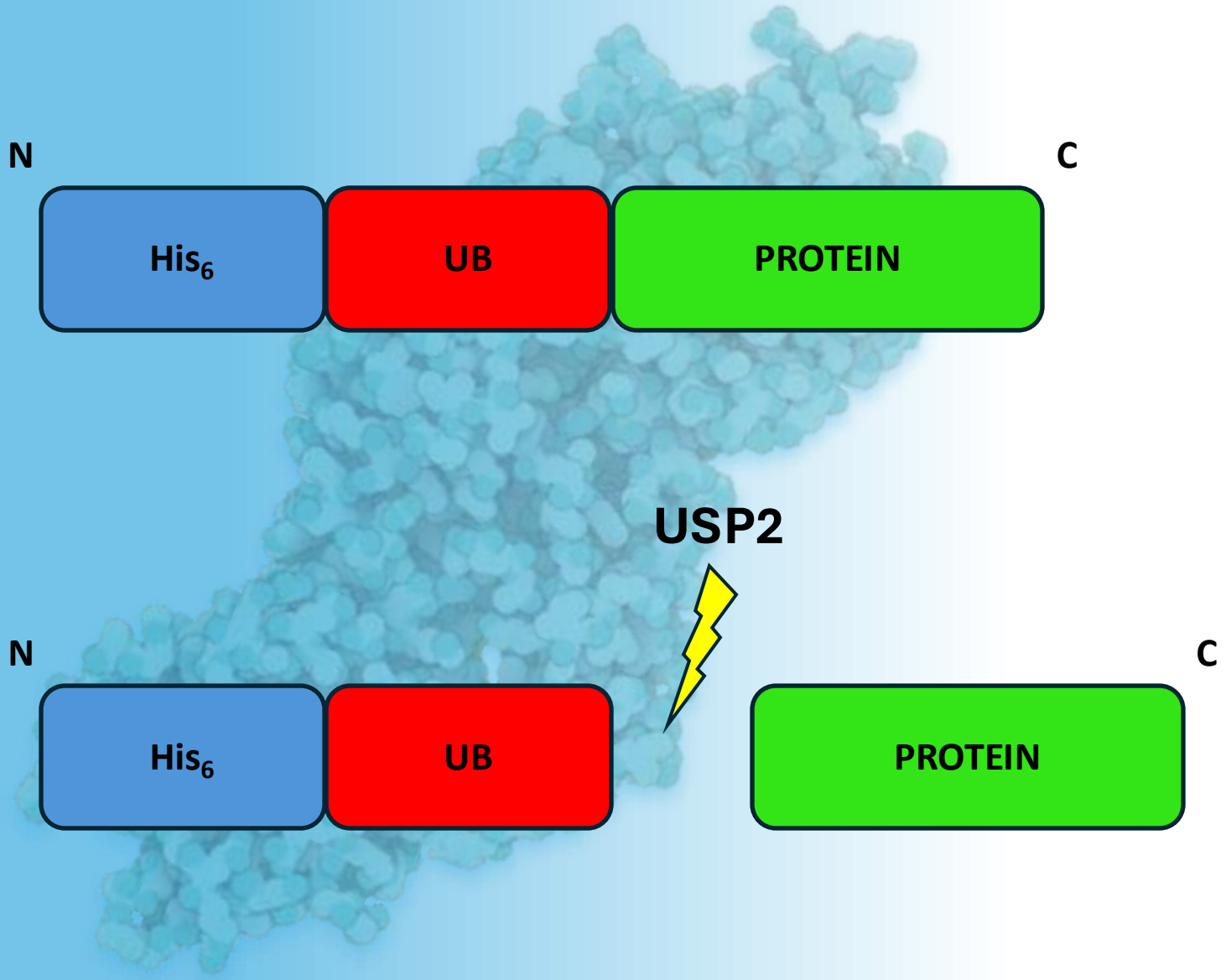


His-Ubiquitin tag removal

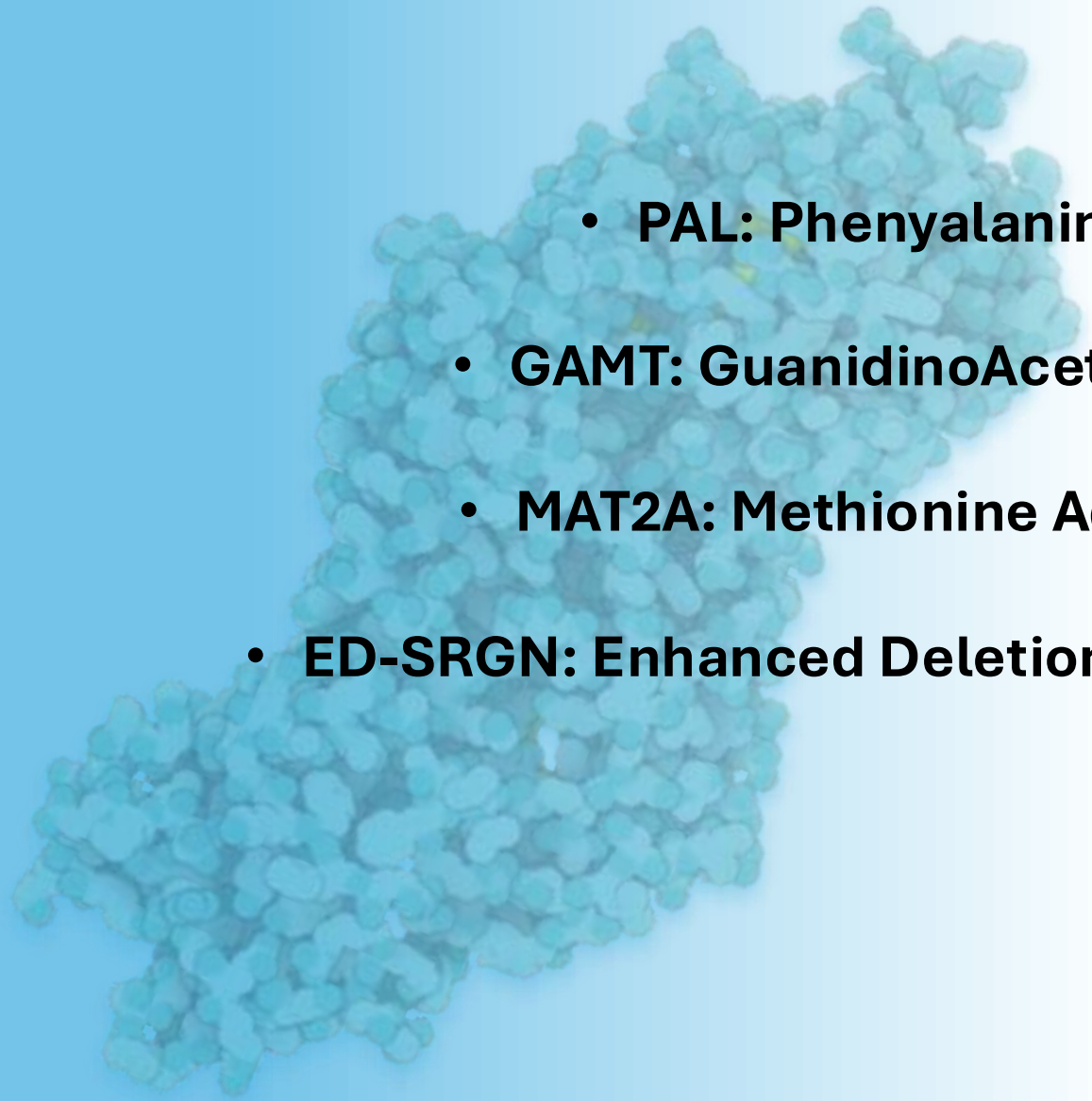


The ubiquitin portion of the tag allows easy removal of the entire fusion partner

The His-Ubiquitin tag is removed through digestion with USP2

The resulting protein is now free of exogenous portions

Our Main recombinant enzymes

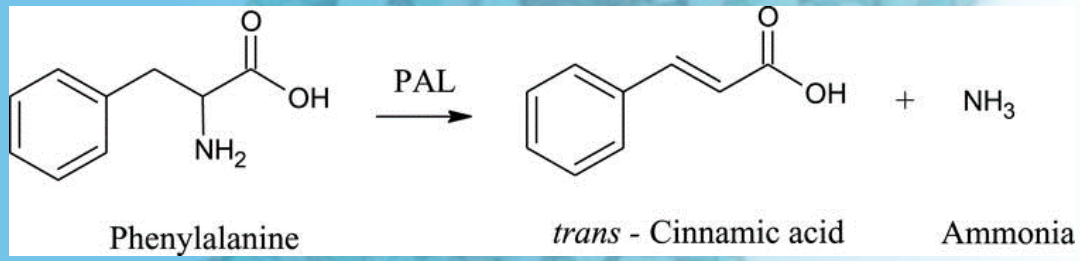


- PAL: Phenyalanine Ammonia Lyase
- GAMT: GuanidinoAcetate MethylTransferase
- MAT2A: Methionine AdenosylTransferase 2A
- ED-SRGN: Enhanced Deletion Single RNA-Guided Nuclease

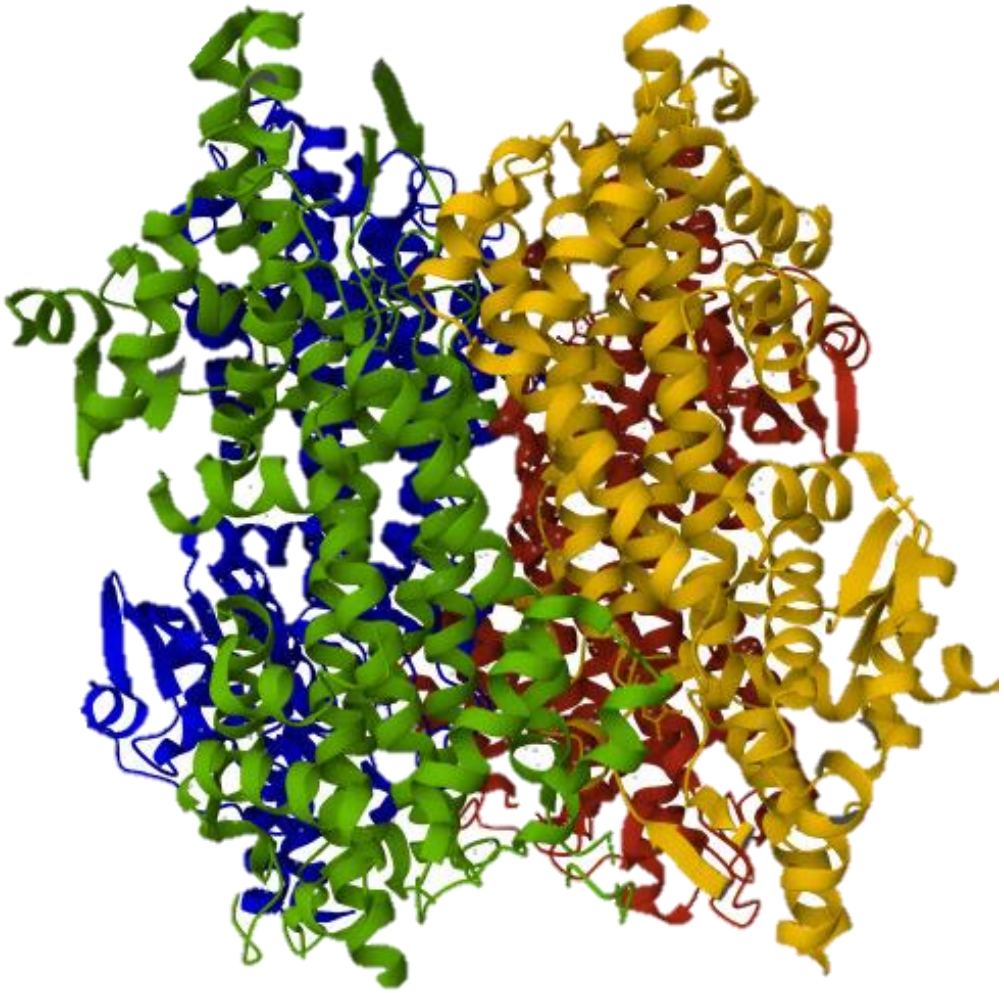
PAL
Phenyalanine Ammonia Lyase

- sequence derived from the cyanobacterium *Anabaena variabilis*

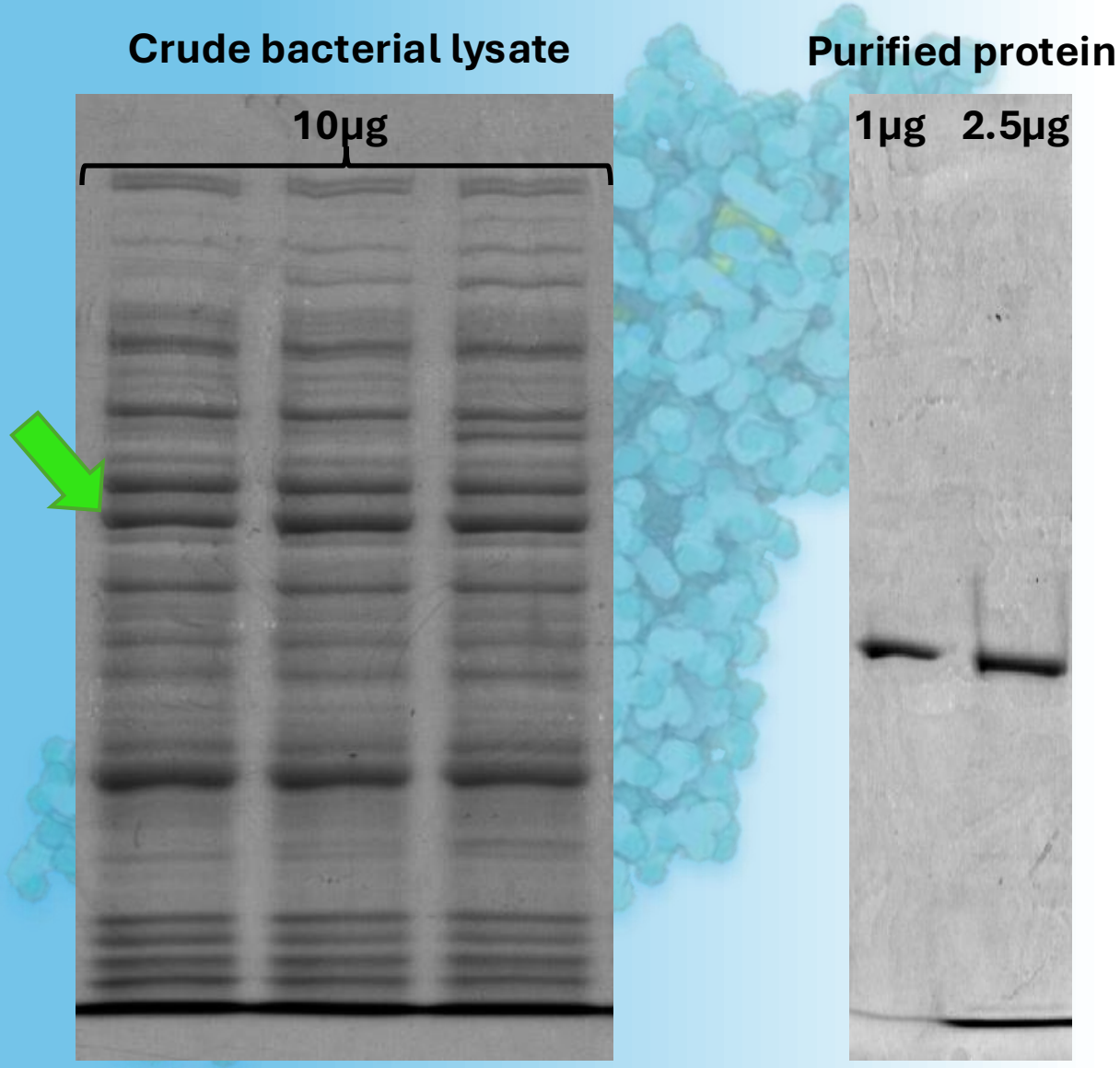
- converts L-Phenylalanine into *trans*-cinnamic acid and ammonia



- potential treatment for Phenylketonuria



PAL
Phenyalanine Ammonia Lyase



PAL
- The finished product is very pure

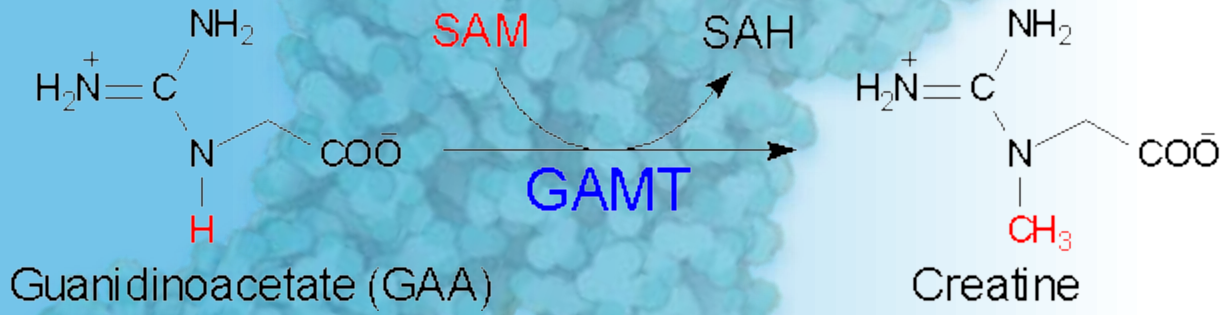
- Exhibits sufficient enzimatic activity

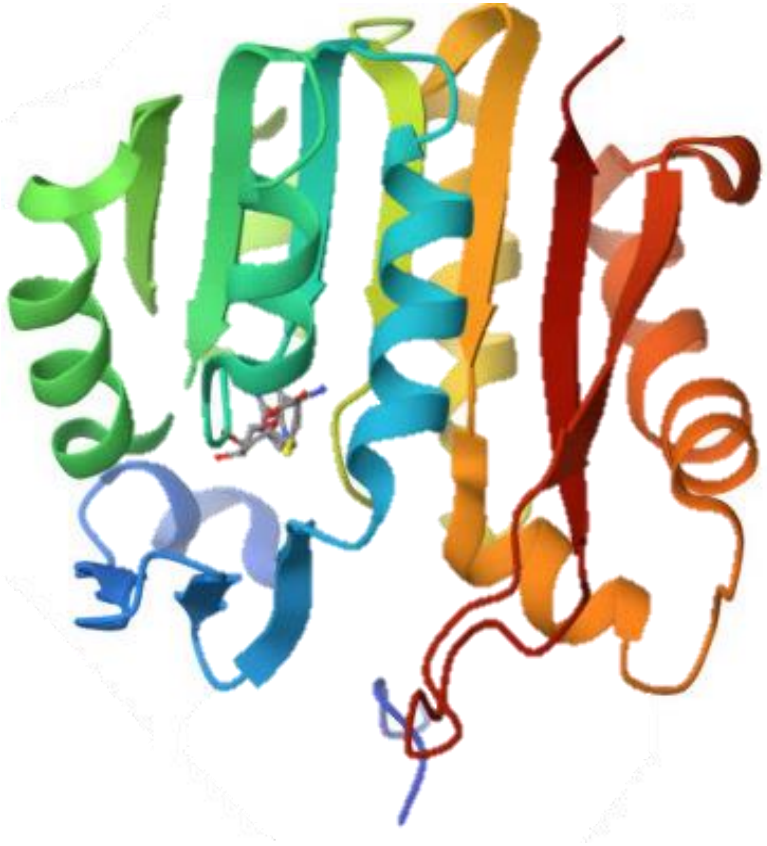
Our current efforts are pointed towards reducing endotoxin levels

GAMT
GuanidinoAcetate MethylTransferase

- originally derived from the human gene, 4 aminoacids have been mutated to increase stability and solubility

- converts Guanidinoacetate and S-Adenosyl-Methionine into Creatine and S-Adenosyl-Homocysteine



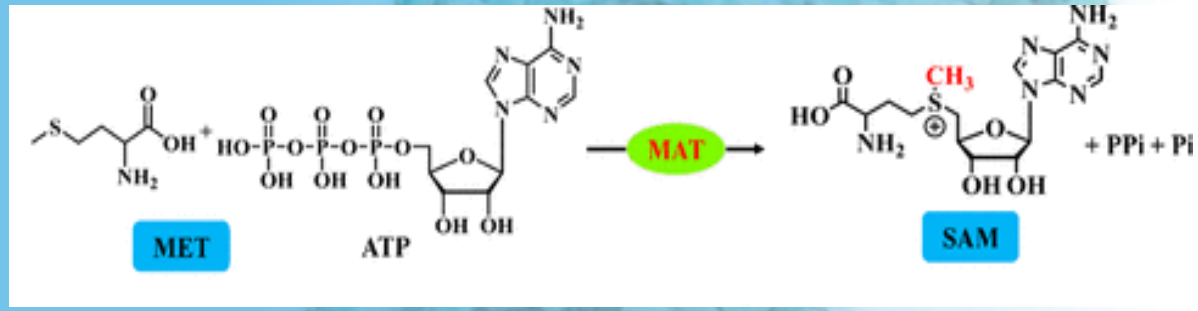


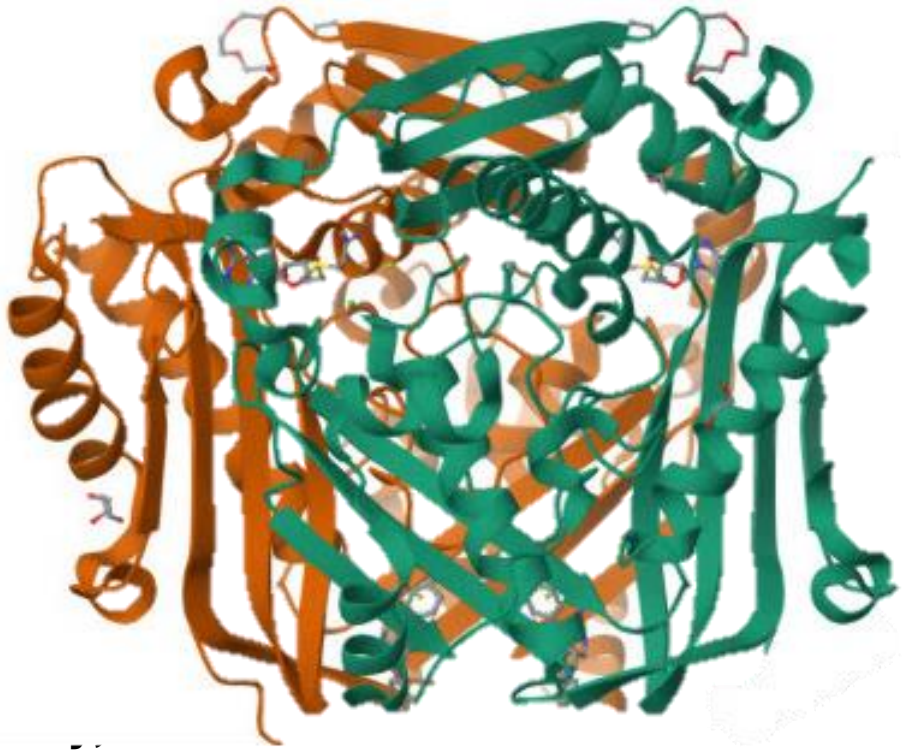
- to be administered in conjunction with MAT2A

MAT2A
Methionine AdenosylTransferase 2A

- derived from the human MAT2A sequence, with no modifications

- Forms S-Adenosyl-Methionine from Methionine and ATP.



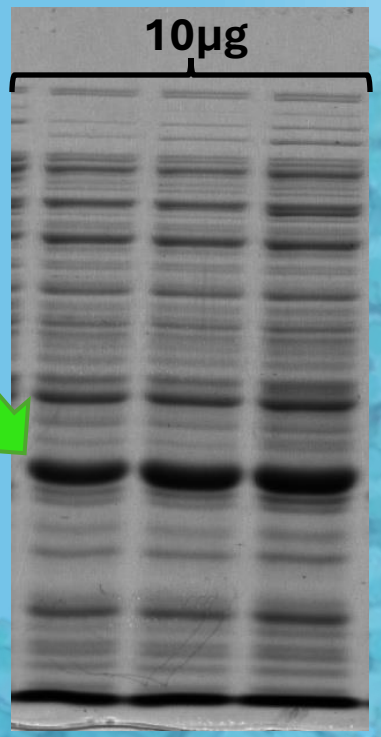


- to be used in conjunction with GAMT as a therapy for GAMT-defici
MAT2A supplies SAM, which GAMT needs to produce creatine

Guanidinoacetate + S-Adenosyl-Methionine ⟶ Creatine + S-Adenosyl-Homocysteine

GAMT
GuanidinoAcetate MethylTransferase

Crude bacterial lysate



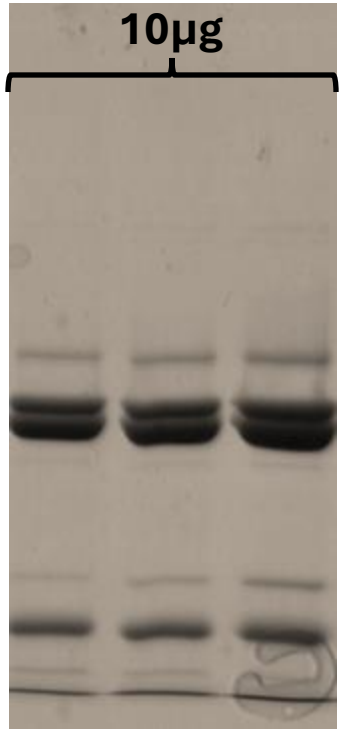
Purified protein

GAMT
- Very pure
- Sufficient enzymatic activity

MAT2A
Methionine AdenosylTransferase 2A

Crude bacterial lysate

Purified protein



MAT2A
- Some residual contaminants
- Enzymatic activity is lost during IMAC purification

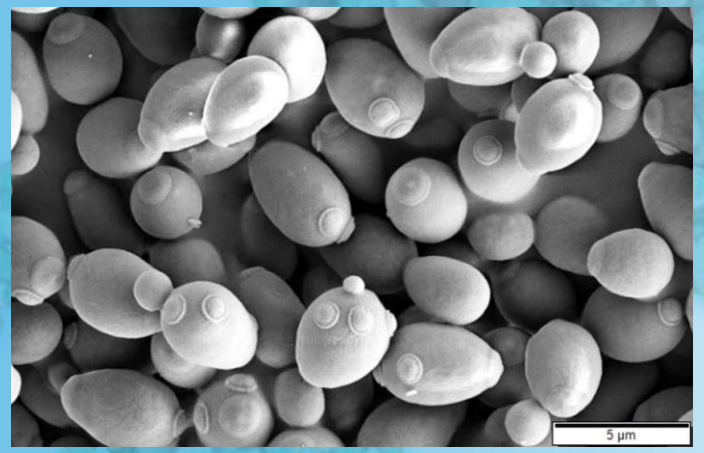
As MAT2A is inactive when expressed in bacteria, production has been moved to *Saccaromyces cerevisiae*

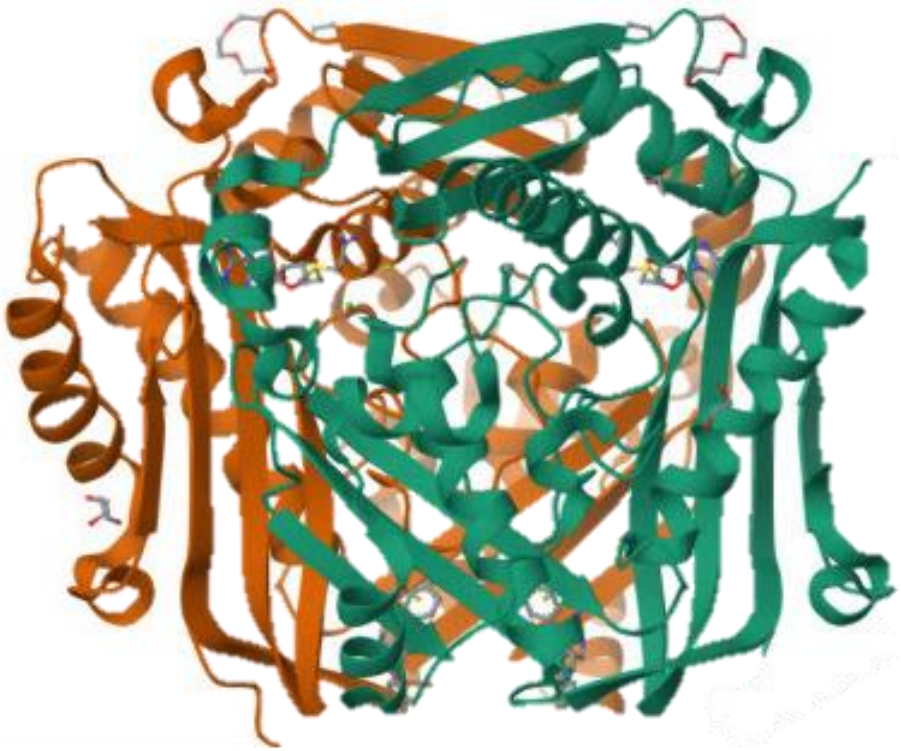
Advantages over Escherichia Coli:

- no contamination from bacterial Endotoxins

- capable of post-translational modifications

- cost-effective and safe





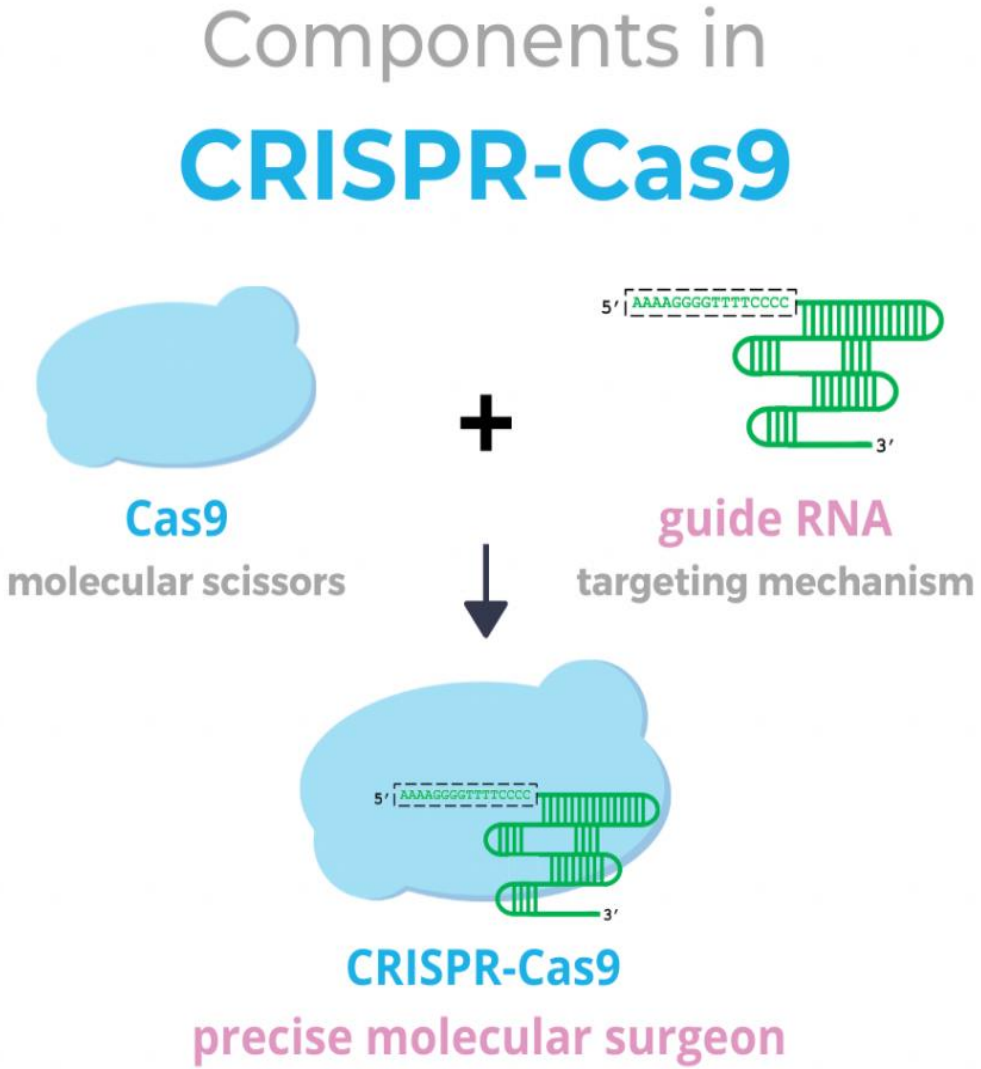
Thanks to:
Dott. Gianluca Morganti
Dott.ssa Federica Biancucci
Prof. Michele Menotta

SRGN

Single RNA-Guided Nuclease

- Derived from the acclaimed CRISPR/Cas9 genome editing system

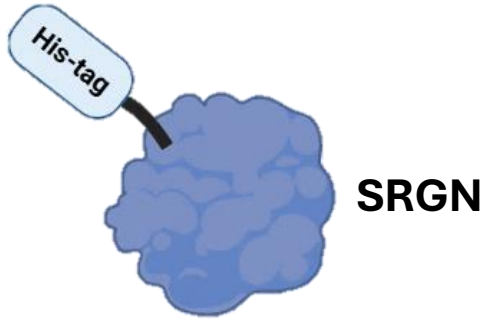
- when coupled with a custom-designed gRNA, the protein is capable of cleaving a specific DNA target sequence



Thanks to:
Pietro de Angeli, Ph.D.

His-SRGN

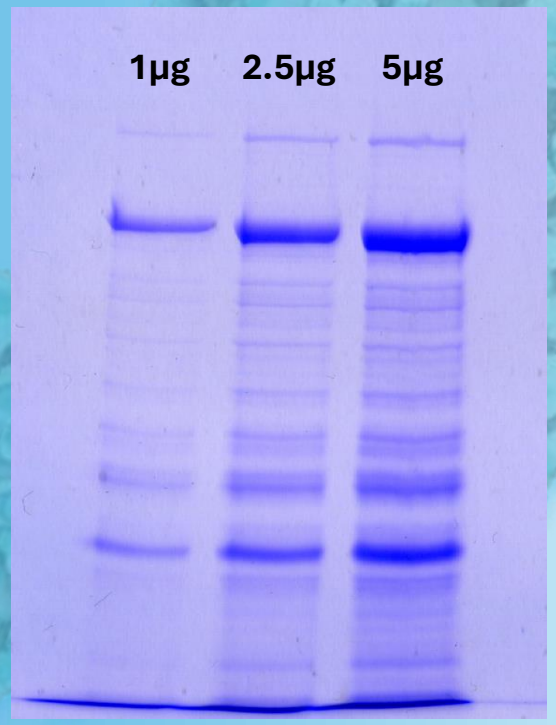
Single RNA-Guided Nuclease



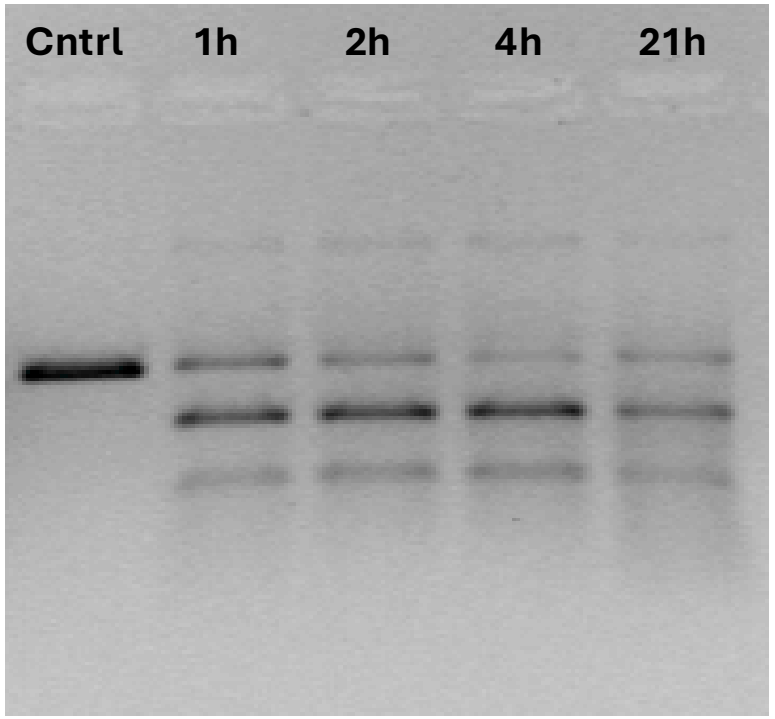
the purified protein still presents contaminants, but low yield limits purification options

preliminary tests on His- SRGN showed effective *in vitro* cleaving activity

Purified SRGN

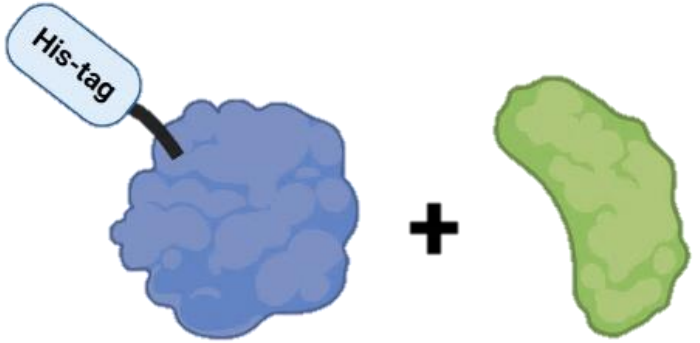


In vitro digestion assay



His-ED-SRGN

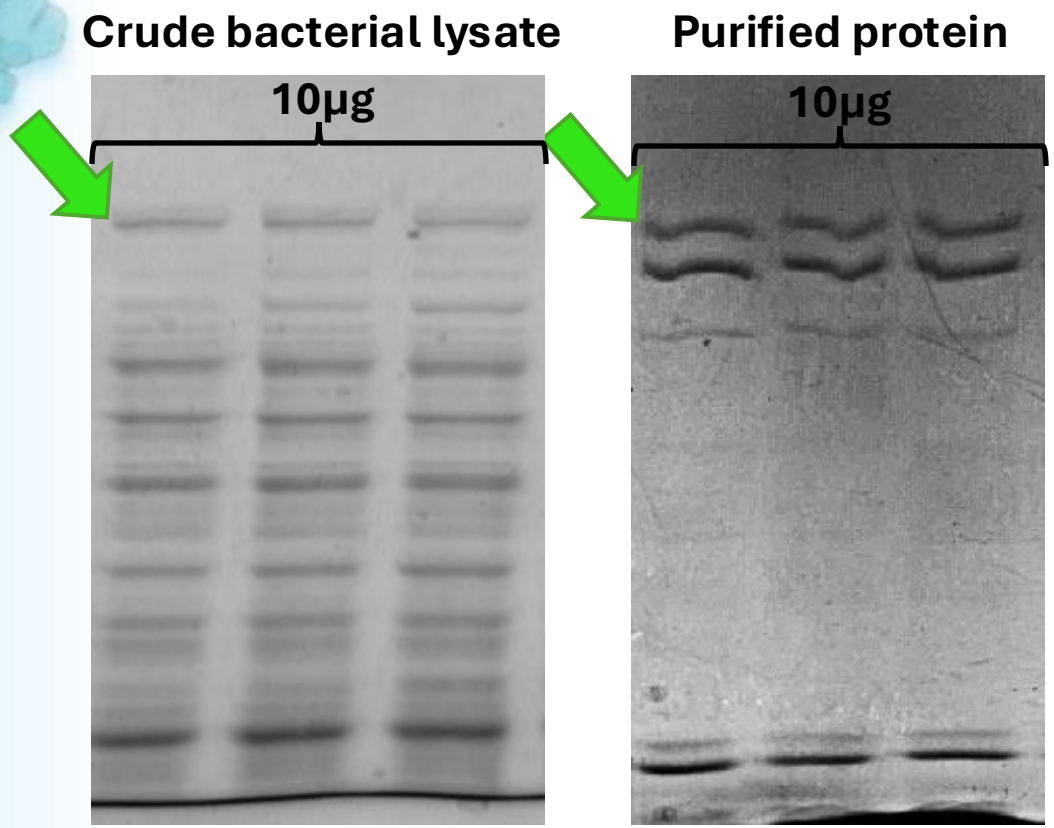
Enhanced Deletion Single RNA-Guided Nuclease



- The addition of the TREX2 domain permits the targeted degradation of specific DNA sequences

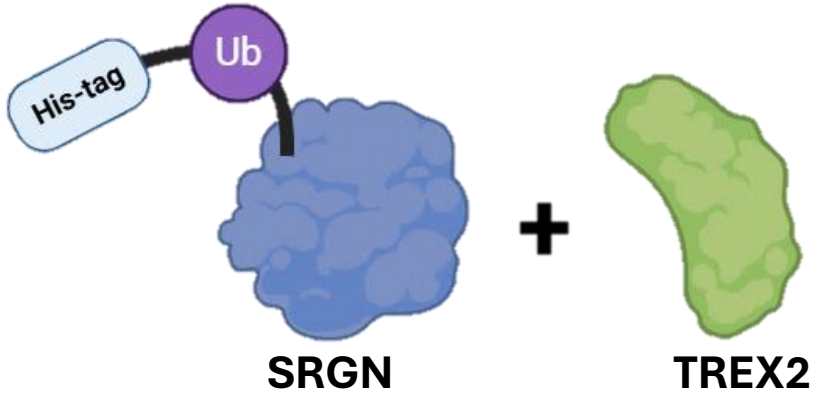
- ED-SRGN expression is very cumbersome for bacterial cells;

- addition of a second purification step resulted in less contaminants, although yield remains very low

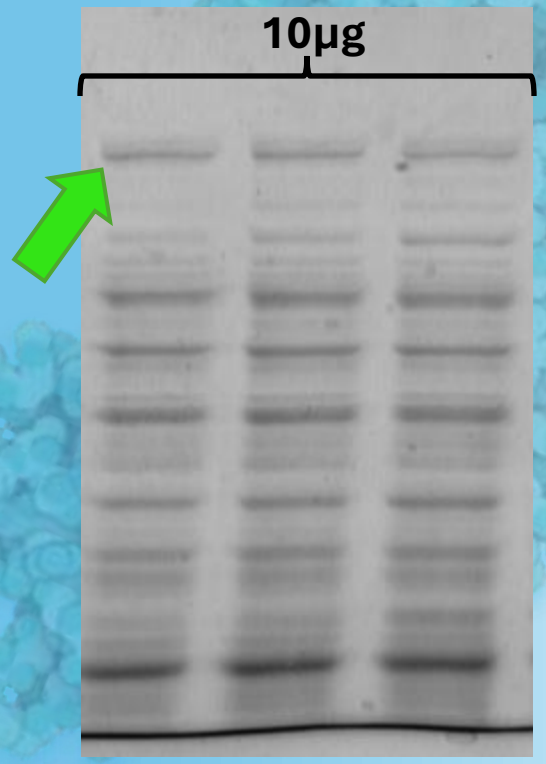


His-Ub-ED-SRGN
To increase protein yield:

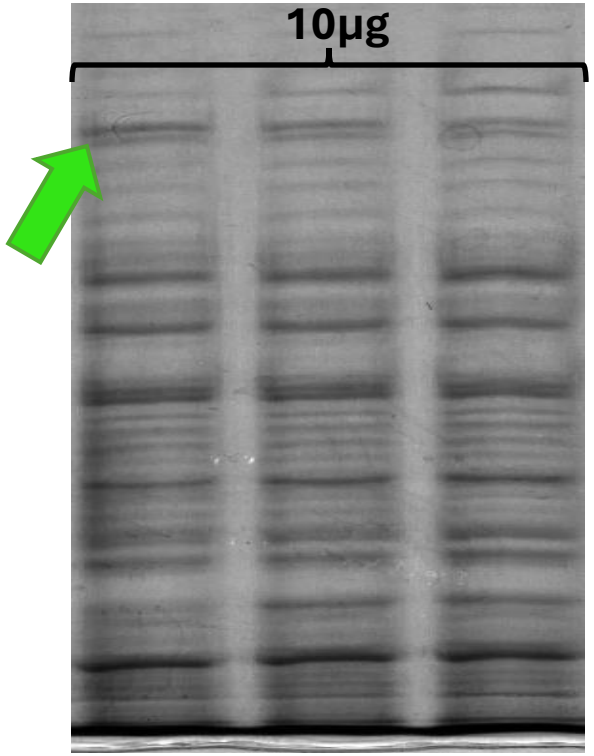
- A ubiquitin molecule has been added



His-EN-SRGN

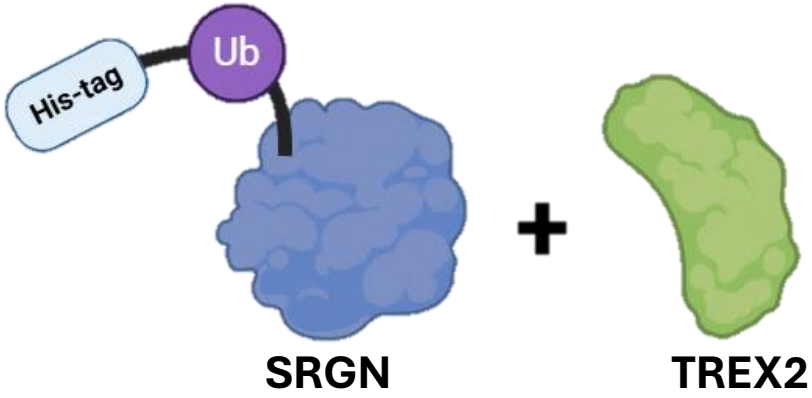


His-Ub-EN-SRGN

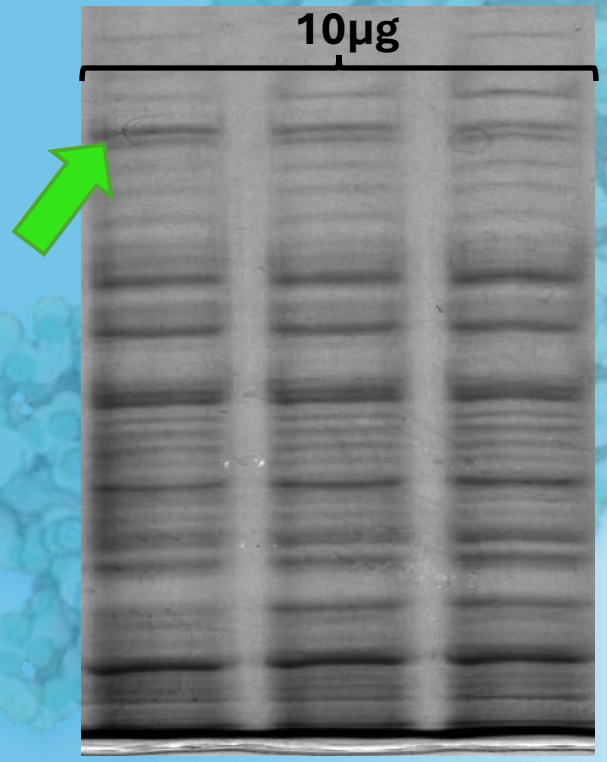


His-Ub-ED-SRGN
To increase protein yield:

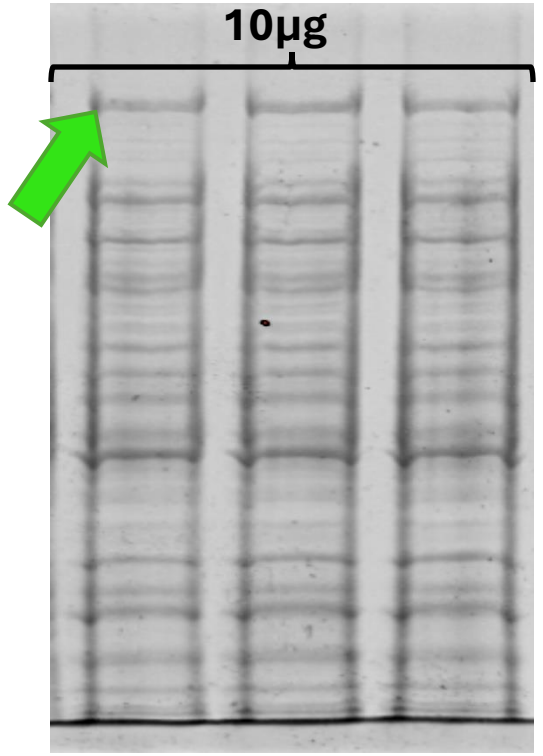
- overnight induction at lower temperature and IPTG concentration



+37°C, 1mM IPTG, 5-hour induction



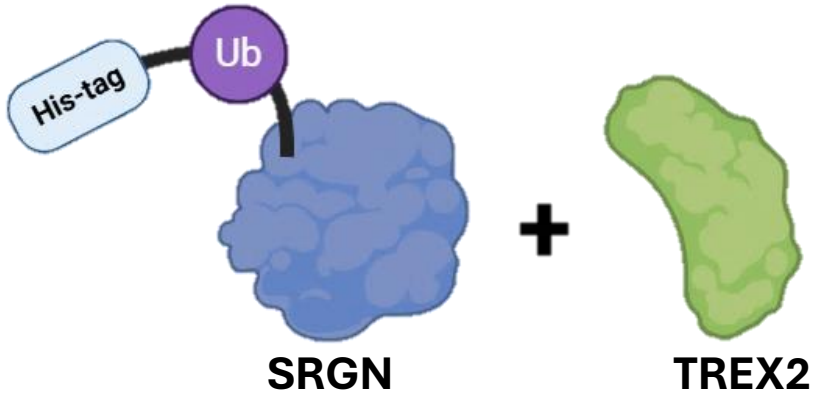
+25°C, 0.5mM IPTG, 5-hour induction



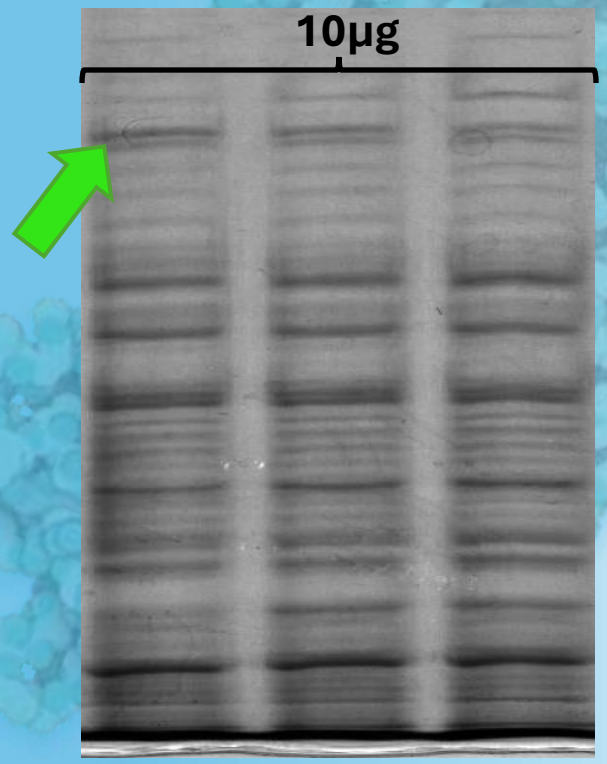
His-Ub-ED-SRGN
To increase protein yield:

- in silico analysis has revealed significant % of rare codons in the protein sequence

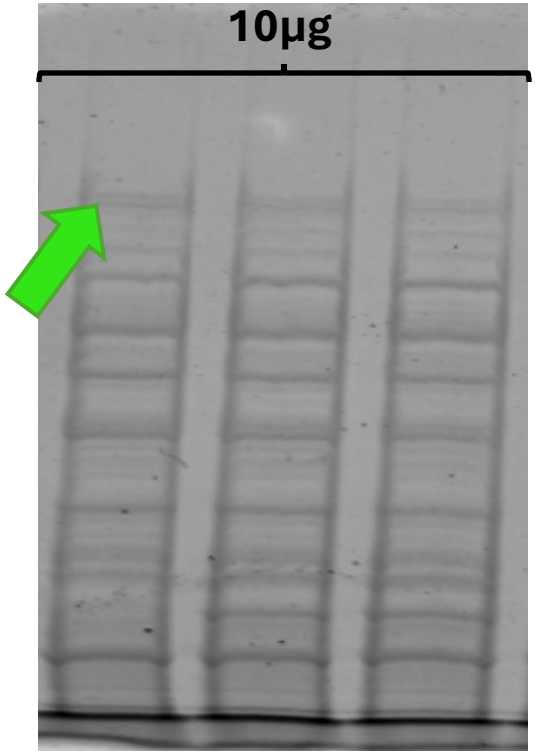
- expression in BL21(DE3) pRIL cells



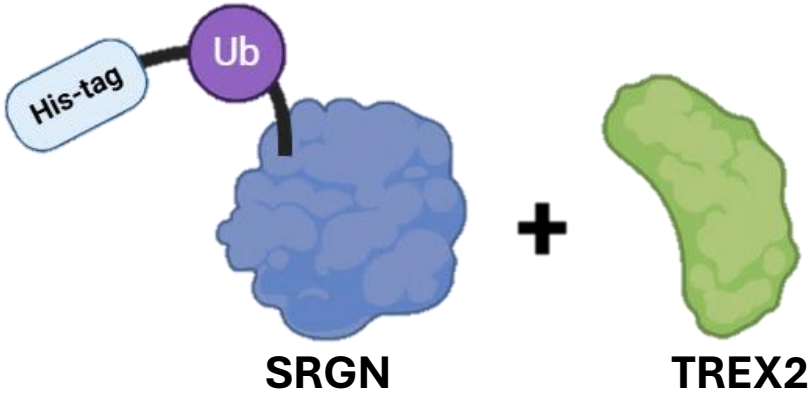
BL21(DE3)



BL21(DE3) pRIL



His-Ub-ED-SRGN
To increase protein yield:



- A ubiquitin molecule has been added

- overnight induction at lower temperature and IPTG concentration

- expression in BL21(DE3) pRIL cells

Switch to eukaryotic cell lines

Thank you for your attention!

Special Thanks to:

- Prof. Mauro Magnani
- Prof.ssa Luigia Rossi
- Dott.ssa Sara Biagiotti
- Dott.ssa Francesca Pierigé
- Dott. Mattia Paolo Aliano
- Prof.ssa Marzia Bianchi
- Dott. Pietro de Angeli
- Dott.ssa Federica Forte
- Dott.ssa Tania Vanzolini
- Dott. Tomas di Mambro
- Dott. Gianluca Morganti
- Dott.ssa Federica Biancucci
- Prof. Michele Menotta
- The 11A Lab Posse

VITALITY

IMAC: Immobilized Metal Affinity Chromatography

Cell lysate =
His-Ub-Protein + contaminants

HisTrap column

Ni^{++}

Ni^{++}

Ni^{++}

The cell lysate is loaded onto the HisTrap column

IMAC: Immobilized Metal Affinity Chromatography

His-trap column

Ni^{++} His-Ub-Protein

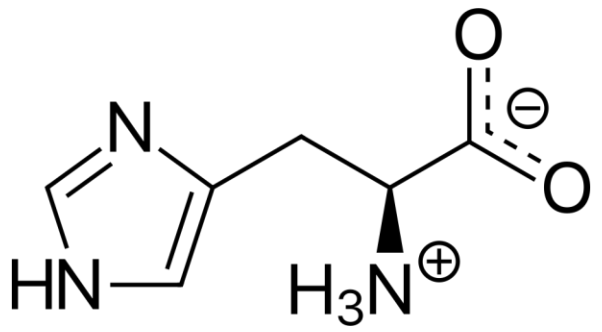
Ni^{++} His-Ub-Protein

Ni^{++} His-Ub-Protein

Contaminants

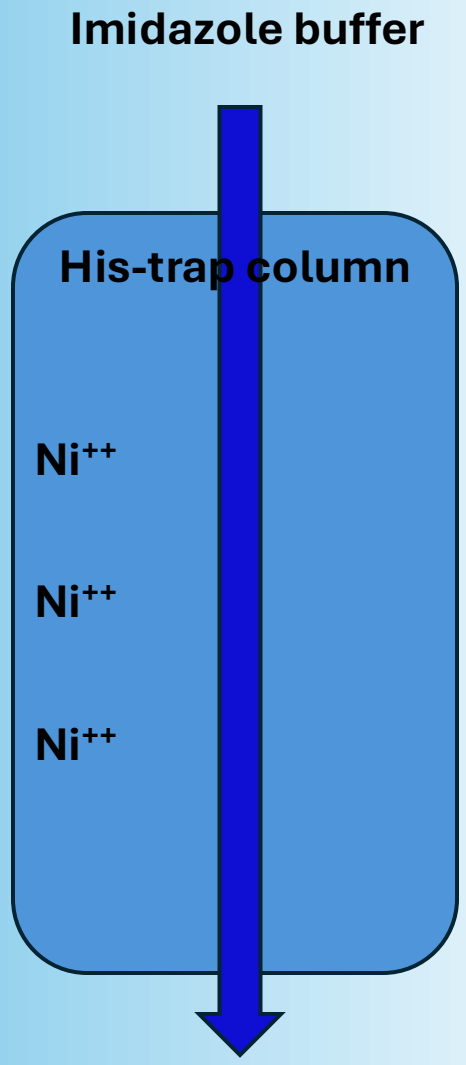
The histidine portion of the recombinant protein binds the nickel ions attacched to the column resin

The contaminants are eliminated

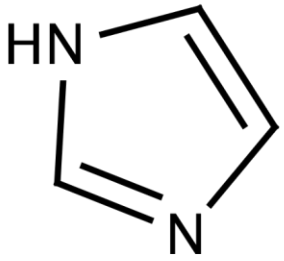


Histidine

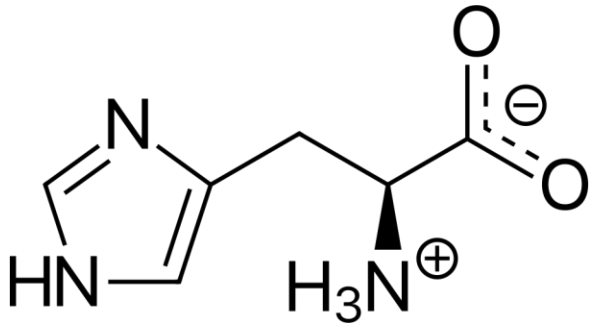
IMAC: Immobilized Metal Affinity Chromatography



The recombinant protein is detached from the resin through an imidazole buffer



Imidazole

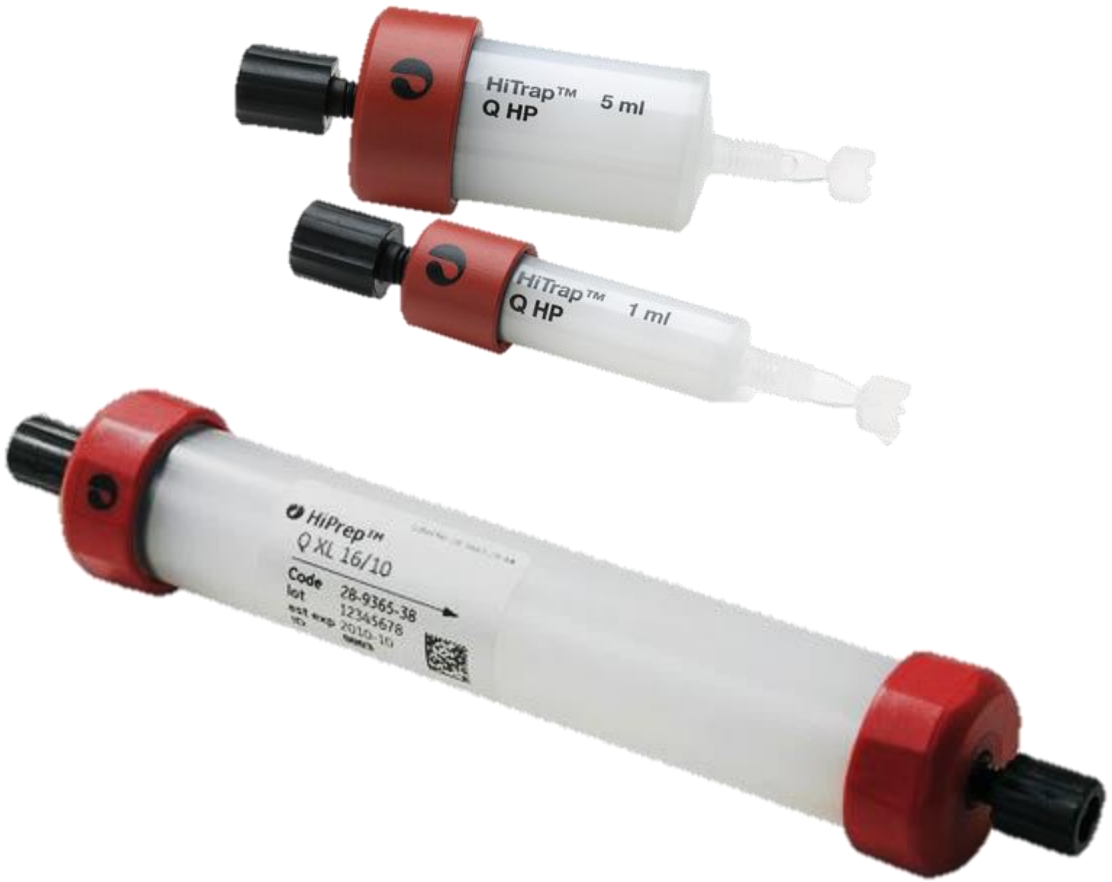


Histidine

Further processing

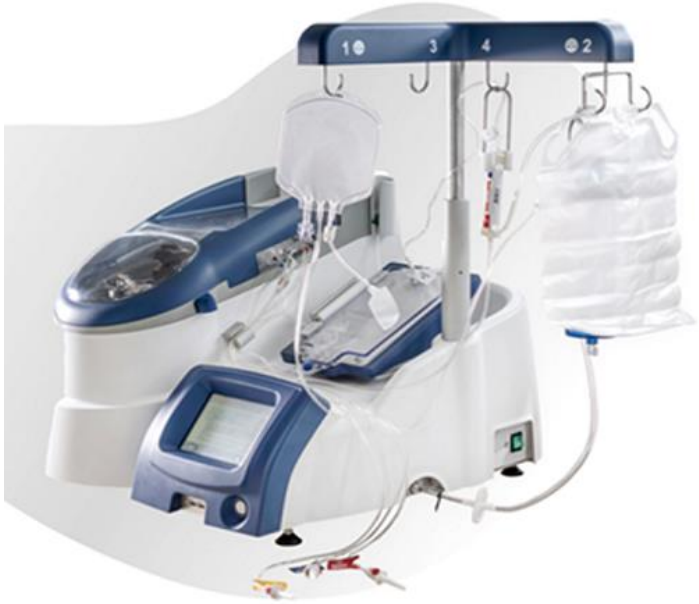
The recombinant protein can be purified further to eliminate remaining contaminants

To this end, additional chromatography techniques are employed, such as ion exchange and size exclusion chromatography



Erythrocytes as preferred delivery method

Enzyme loading into erythrocytes, which are later reinfused into the patient



Advantages:

- erythrocytes provide a “safe” environment

- longer half-life, which translates into fewer admistrations

- fewer adverse affects, such as immune activation