# SCREENING AND OPTIMIZING CONDITIONS FOR CRYSTALLIZATION OF HUMAN RECOMBINANT PROTEINS PARTICIPANTS IN POST-TRANSLATIONAL MODIFICATIONS

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**Abstract:** The association between the two proteins Nuclear protein localization protein 4 and Ubiquitin recognition factor in ER Associated Degradation 1, abbreviated as (hNpl4-Ufd1), which is specific to the human ubiquitin-proteasome system, plays an important role in many aspects of cellular functions, including cell replication, protein degradation, and protein transport. The structure and function of these proteins (Npl4-Ufd1) have been detailed in numerous studies. Although much interest has been raised about the structure and function of Npl4-Ufd1 from the past to date, only a study by Nguyen et al. (2022) described the detailed structure of this complex protein in humans. The study also shows that the screening and crystallization process plays an important role, having a decisive influence on the quality of the observation and structural analysis of hNpl4-Ufd1. In this study, we want to present the process of finding out the conditions to create single and complex crystals of two proteins Ufd1 - Npl4, and then optimizing the environmental conditions to be able to conduct molecular structure analysis using X-ray diffraction.

*Keywords: Protein crystallization, Screening, Optimizing.*

## 1. Introduction

Since the early 2000s, studies on protein family members related to many functions in cells, including cell cycle regulation, protein degradation, and protein transport have been published [1]. This group of proteins belongs to the group of multifunctional proteins related to ATPases (AAA-family members) through the ability to bind to adaptor proteins, the most researched and mentioned representative is p97/VCP with a specific directly involved in protein degradation is NPL4-Ufd1 [2]; [3] and continuously since then, the structure and function of these proteins have always been a topic that many research groups have focused on to understand more about the structure and functions that it participates in religious activities [4]; [5]; [6]; [7]; [8]; [9].

There are many commonly used methods to determine the structure of proteins such as X-ray Crystallography, X-ray Free Electron Lasers, NMR Spectroscopy, 3D Electron Microscopy, and Integrative modeling. X-ray crystallography is the most powerful

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and common method used to observe and analyze the molecular structure of a substance or compound (macromolecules) including proteins. The most important problem to be able to do this is to create crystals of the substance or compound that needs to be analyzed, which in this research is protein crystals. Creating protein crystals has been done using different methods for more than 150 years. Competition for time, or in other words, the person who creates the crystal first is the one who succeeds and all those who follow later are the ones who fail. Our team also experienced failure because we were unable to create crystals of the protein we needed to research.

In addition, creating crystals is just the beginning, a small step towards success, because many proteins can form crystals but cannot be analyzed by crystal X-ray diffraction. Typically structures that are considered significant and reliable are analyzed and observed at 1 to 3 Angstroms (1-3 Å). To achieve this, the crystal needs to be large enough, hard enough, stable, and capable of diffraction. In addition to the above factors, many other factors affect the resolution of the crystal structure like the skill of the person performing the analysis, analysis time, analysis conditions, crystal molecular structure, etc. These factors are beyond the scope of our study.

These show the importance of creating crystals as well as optimizing conditions to create good crystals capable of analysis, to achieve the ultimate goal of the research, which is to observe and analyze in detail the molecular structure of proteins that we need to pay attention to.

## 2. Materials and research methods

### *2.1. Material*

The recombinant protein was used by expression in bacterial cells and purified to the final concentration required by the test. The expression and purification process is presented in detail in the author's study [7].

The crystallization medium was purchased from Hampton Research and Quiagen.

Chemicals used for optimization were purchased from Sigma Aldrich.

### *2.2. Research Methods*

The principle of the method is based on the evaporation and precipitation of the solution. The prerequisite is to create a supersaturated state of the solution, as shown in Figure 1. Supersaturation is a non-equilibrium condition in which the molecules exceed the saturation limit, below a certain point special chemical and physical conditions but must still be present in the solution.

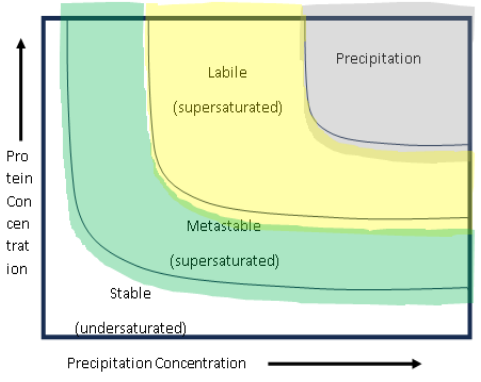


**Figure 1. The principle of protein crystallization.**

To create a supersaturated state, we must understand the principle of protein crystallization. It depends on the type of precipitant, the concentration of the

precipitating agent, the pH of the buffer solution, the type of buffer, and the temperature of the experiments to be able to create satisfactory crystals, after screening to find the environment that creates precipitation we need to optimize the conditions suitable for create the crystal [10].

### *2.3. Experiment design*

Screening protein concentration at the following values: 0.1 mM, 0.2 mM, 0.25 mM, 0.5 mM, and 1 mM. The concentration value of each protein is calculated based on the sequence and length of amino acids through the Expasy calculator (Expasy.org).

The process was conducted on 3 objects: Ufd1 and Npl4 protein alone and a complex of Ufd1 - Npl4 by mixing the two proteins in a 1:1 ratio to reach a concentration of 1mM.

Conduct screening conditions and precipitating agents using screening kits including h-Index I, PEG Rx I& II, PEG Ion I & II, Q-Agien Classic Suit, Emeral Wizard I&II, H- Salt Rx 1&2. The total design is 6 plastic discs for each sample concentration and for each subject.

Select the screening environment based on the following criteria: Precipitating agent, crystal formation time, pH value of the solution, size, and stability of the crystals.

The selected temperature and humidity will be room temperature $25^0$C and humidity < 60%.

Using the Sitting Drop Vapor Diffusion method for screening crystal formation conditions. By taking 1 µl of the test sample at each concentration above and mixing it with 1 µl of the medium at the location of the sample well, each condition is repeated 2 times and then covered with specialized tape.

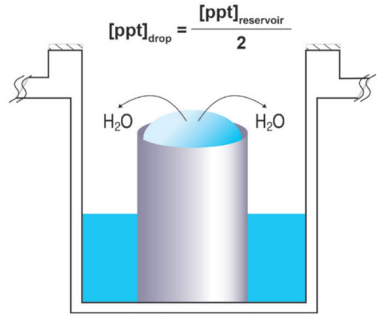


**Figure 2: Sitting Drop Vapour Diffusion method (Source: Hampton Research. USA)**

The Hanging Drop Vapor Diffusion method to optimize conditions. By pipetting 2 µl of the test sample at the optimal concentration, and mixing it with 2 µl of the optimized media solution on a glass slide. Invert and mount on top of the medium well with gel to seal, then place in the specialized cabinet above.

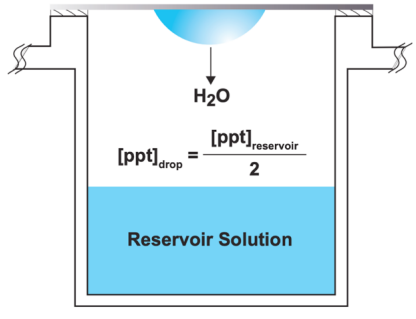


**Figure 3: Hanging Drop Vapour Diffusion method (Source: Hampton Research. USA)**

Samples will be placed in the cabinets. During the observation process, you need to pay attention to the observation time and environmental temperature.

Check the crystal's stability using a suitable-sized loop.

## 3. Results

With 5 sample concentrations and 6 environmental kits, a total of 90 plates

were made for the subjects to be studied, corresponding to 2880 environmental conditions used for screening.

We were not successful in crystallizing individual Ufd1 proteins, but for Npl4 and the Npl4-Ufd1 complex we observed crystal formation and growth under a variety of environmental conditions.

After 4 weeks, at position 71 of the SaltRx-HR2 kit, small, fine crystals appeared, less than 1 µm in size. After further observation until week 7, the size of the crystals appeared and reached from 5 µm to 10 µm with monoclinic shapes, (Figure 4) with a sample concentration of 0.5 mM of the Npl4 complex. -Ufd1 [7].

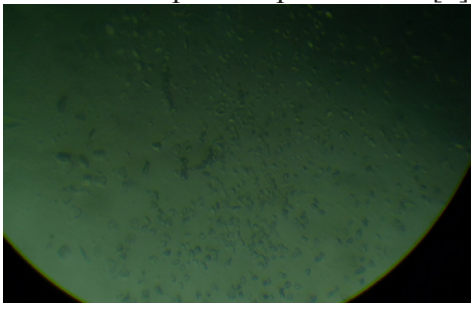

**Figure 4: Condition: SaltRx-HR2 -Number 71: 1.5M Lithium sulfate monohydrate, 0,1M Bis-Tris propane pH 7.0.**

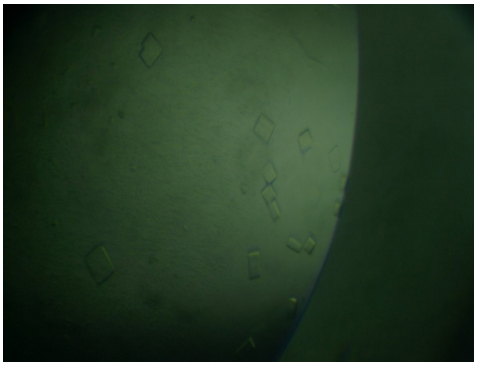

**Figure 5: Condition: Qiagen - Protein complex suite; Number 5, 0.1M MES pH6.5, 15% (w/v) PEG 550 MME**

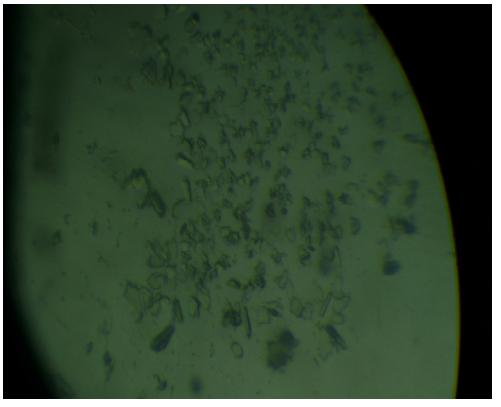

**Figure 6: Condition: H. Index; Number 69, 0.2 M Ammonium Sulfate, 0.1 M Tris pH 8.5, 25% PEG 3350.**

At Number 5. Qiagen - Protein complex suite medium, large crystals with clear shapes and angles of Npl4 protein crystals appeared after 3 days (Figure 5) [7] and at H.Index medium; Number 69 also appeared crystalline with the same protein concentration of 0.5 mM (Figure 6).

Check the environmental conditions of these wells and optimize the conditions.

By varying the parameters: pH, precipitate, and precipitate concentration. Use the Hanging drop method. After 2 weeks from the date of optimization, the crystals appeared very large and clear, with clear edges, and transparent. Optimal environmental conditions for crystals (Figure 3): 1.5M Lithium sulfate monohydrate, 0.1M Bis-Tris propane pH 7.0 (Figure 5), With magnetic crystals (Figure 4) the environmental conditions were optimized to 0.1M HEPES pH 6.5, 16% (w/ v) PEG 3350, 5% (v/v) MPD (Figure 8), and optimal environmental conditions for (Figure 6) are 0.3 M Ammonium Sulfate, 0.1 M Tris pH 8.5, 25% PEG 4000 (Figure 7).

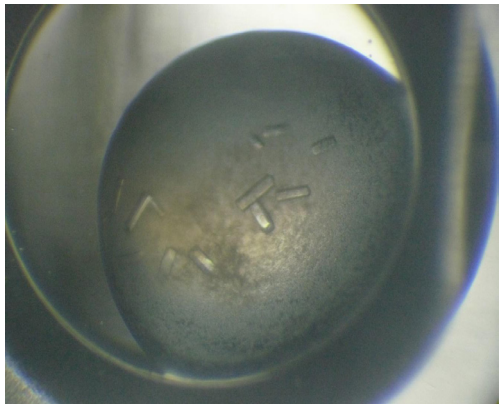

**Figure 7: Condition: 0.2 M Magnesium acetate tetrahydrate, 25% PEG 3350, Tris-pH 8.5. [7]**

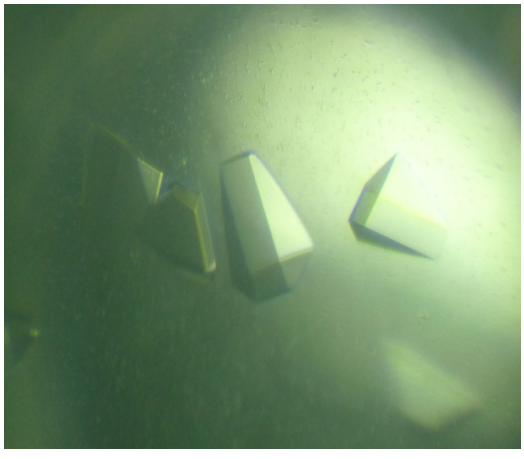

**Figure 8: Condition: 0.1M HEPES pH 6.5, 16% (w/v) PEG 3350, 5% (v/v) MPD. [7]**

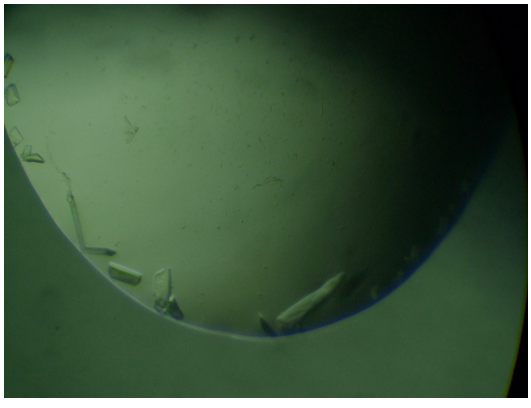

**Figure 9: Condition: 0.3 M Ammonium Sulfate, 0.1 M Tris pH 8.5, 25% PEG 4000.**

## 4. Discuss

Observing the screening process, samples at concentrations of 0.1 mM, and 0.2 mM appeared cloudy and clear drop. This proves that the sample concentration is too low, not enough to create a supersaturated, undersaturation environment.

At the condition of 1 mM, the heavy drop phenomenon appears, and the concentration of the sample and the precipitating agent is too high, leading to the strong precipitation phenomenon (Figure 1).

The pH parameters of the environment show that the appropriate conditions to form crystals are the pH range of weak acid pH = 6.5 (Figure 8) or weak base pH = 8.5 (Figure 7). At these value ranges, the protein is not denatured and retains its structure to form crystals. The neutral pH value pH = 7 is not optimal possibly may be because it is close to the PI value of the protein.

The precipitating agent and the concentration of the precipitating agent also have a direct effect on crystal formation. By adding high molecular weight substances Polyethylene Glycol 3350 or 4000. The shape and size of protein crystals are optimized through the results stated above. These macromolecular compounds can retain water as well as slow down the evaporation of the environment, minimizing protein denaturation.

In addition, the characteristics of these macromolecular substances are high viscosity, creating a favorable environment for proteins to arrange and bind together tightly, slowly but sustainably; like a soft framework surrounding the crystal, this could be used to explain the success we experienced.

Testing the stability of the crystals, the results showed that the optimized crystals were stable and capable of being used for X-ray diffraction testing.

## 5. Conclusion

We successfully crystallization of Npl4 monomers and the Npl4-Ufd1 complex but not the Ufd1 protein may be due to the amino acid sequence of the monomers containing too many hydrophilic residues for crystallization leading to instability in the tertiary structure. When two proteins bind together, the bonds make the complex stable and create favorable conditions for crystal formation.

The pH value and type of precipitating

agent greatly affect the shape and size of the crystal. Especially for the Npl4-Ufd1 complex, the initial precipitating agent, which was a low-concentration salt, was completely replaced by a solution with high molecular structure PEG 3350 and MPD.

Our study has presented specifically and in detail the process of screening and optimizing the crystallization conditions of the Npl4-Ufd1 protein complex. Achieve the objectives of the research.

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