

## Production and Isolation of Magnetic Protein Crystals in HEK293T Cells

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**[Abstract]** Advances in protein engineering have enabled the production of self-assembled protein crystals within living cells. Our recent publication demonstrates the production of ftn-PAK4, which is a ferritin-containing crystal that can mineralize iron and become magnetic when isolated. We have developed an optimized protocol for the production and isolation of PAK4-based crystals. The crystals are first grown in low-passage HEK293T cells, released using a lysis buffer containing NP-40 and DNase, and collected under careful centrifugation conditions. Our protocol maximizes the purity and yield of crystals and is quick and straightforward.

**Keywords:** Protein Crystal, Isolation, ftn-PAK4, “In cellulo”, Crystal

**[Background]** Recent works have reported the production and isolation of “*in cellulo*” crystals through the heterologous expression of proteins in living cells. These crystals have varied applications, such as cargo delivery (Ijiri *et al.*, 2009) or x-ray structure determination (Baskaran and Ang, 2015). The properties of the crystals vary, but they are generally quite large relative to the cell, ranging from 1-2  $\mu\text{m}$  up to hundreds of  $\mu\text{m}$  in size (Schönherr *et al.*, 2015). In our recent work, we modified inka-PAK4 crystals to create ftn-PAK4, which is a ferritin-containing crystal that can mineralize enough iron to be attracted to a nearby permanent magnet (Li *et al.*, 2019).

The production and isolation of intact protein crystals poses several unique experimental challenges. Because the crystals are so large, purification methods such as gel electrophoresis cannot be used. Likewise, because the crystals are protein-based, harsher lysis conditions such as SDS will disassemble the crystals. However, clean suspensions can be critical for downstream applications. For example, excessive debris in an inka-PAK4 suspension can trap auto-oxidized iron and stick to crystals, generating spurious magnetic attraction results.

Here, we present a protocol for the production, isolation, and iron loading of ftn-PAK4 and inka-PAK4 crystals. It is possible to achieve results using only deionized water as the lysis buffer, but we present several optimizations that significantly improve crystal yield and minimize unwanted debris. These considerations should inform future work on other protein crystals, both for their production and isolation as well as their functional applications.

## **Materials and Reagents**

1. Pipettes and Pipette Tips
2. 1.5 ml Eppendorf centrifuge tubes
3. Glass slides
4. 6 Well Tissue Culture Plate, Tissue Culture Treated, Flat Bottom (Fablab, catalog number: FL7105)
5. HEK 293T cells (< 10 passages) (ATCC, catalog number: CRL-3216)
6. DMEM High Glucose without Sodium Pyruvate (Gibco, catalog number: 11965092)
7. Penicillin/Streptomycin (Gibco, catalog number: 15140122)
8. Heat Inactivated Fetal Bovine Serum (Gibco, catalog number: 10438034)
9. Lipofectamine 2000 (Invitrogen, catalog number: 11668030)
10. Opti-MEM Reduced Serum Medium (Gibco, catalog number: 31985070)
11. GFP-PAK4, ftn-PAK4, and inka-PAK4 plasmids
12. HEPES Buffer, 1 M, pH 7.2-7.5 (Gibco, catalog number: 15630130)
13. NP-40 (Sigma, catalog number: I8896, also known as Igepal CA-630)
14. Deoxyribonuclease I (DNase) (Worthington, catalog number: LS002007)
15. Earle's Balanced Salt Solution (EBSS) (Life Technologies, catalog number: 14155-063)
16. Ferrous Ammonium Sulfate Hexahydrate (FAS) (Sigma-Aldrich, catalog number: FX0245)
17. D-Mannitol (Sigma-Aldrich, catalog number: M4125)
18. Concentrated Hydrochloric Acid (Fisher Scientific, catalog number: A144-500)
19. Potassium Ferrocyanide Trihydrate (MP Biomedicals, catalog number: 0215256080)
20. Lysis buffer (see Recipes)
21. DNase stock solution (see Recipes)
22. Iron loading stock solution (see Recipes)
23. Prussian Blue stain (see Recipes)

## **Equipment**

1. Centrifuge (Eppendorf, model: 5804)
2. Rocker (VWR Scientific Rocking Platform, Model 100)
3. Microscope with Brightfield and Fluorescence Imaging (Leica, model: DMI 6000B)

## **Procedure**

### **A. Transfection**

1. Prepare cells

Plate HEK293T cells at approximately 60% confluency in a 6-well culture plate. Ensure that the cells have not been passaged more than 10 times before transfection.

*Note: Cells can be plated any time before transfection, including immediately beforehand, as long as the density is 60% at the time of transfection.*

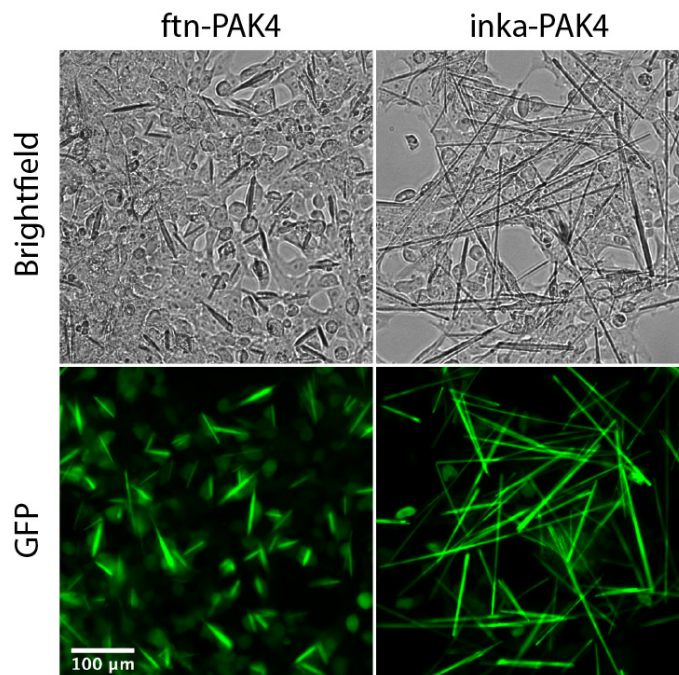
## 2. Transfection

Transfect HEK293T cells using Lipofectamine 2000 according to the manufacturer's instructions.

- Use 2  $\mu$ g of total plasmid, and 6  $\mu$ l of Lipofectamine 2000 per well, for a 1:3 plasmid to reagent ratio.
- To produce ftn-PAK4, use 1  $\mu$ g of ftn-PAK4 plasmid and 1  $\mu$ g of inka-PAK4 plasmid. To produce inka-PAK4, use 2  $\mu$ g of inka-PAK4 plasmid. The plasmid concentration is not important, only the total mass of DNA.
- To aid in crystal visualization through fluorescence microscopy, include 0.2  $\mu$ g GFP-PAK4 plasmid in the plasmid mix. It is not necessary to increase the amount of Lipofectamine used.

## 3. Wait for crystal growth

The first crystals should appear 24 h after transfection. While inka-PAK4 crystals should be immediately visible, ftn-PAK4 crystals may not be obvious after 24 h. To maximize crystal yield and size, maintain cells in culture for 72 h after transfection before isolation (Figure 1).



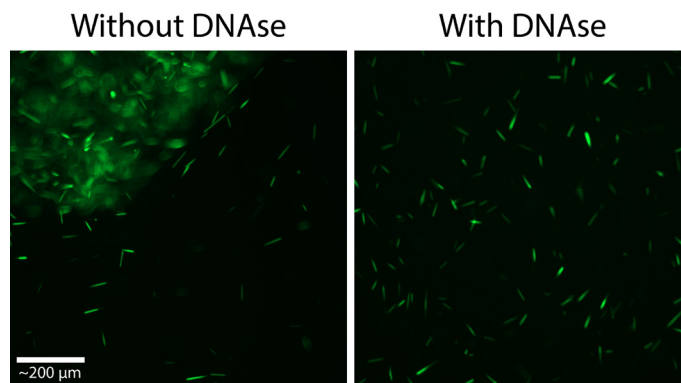
**Figure 1. HEK293T cells containing ftn-PAK4 and inka-PAK4 crystals, with GFP-PAK4, 72 h after transfection. These crystals are ready for isolation.**

## B. PAK4 isolation

### 1. Lyse Cells

Remove the media from the cells, and gently rinse once with 1 ml room-temperature sterile deionized water. Add 500  $\mu$ l lysis buffer and 10  $\mu$ l DNase solution directly to the cell culture well, and place on a rocker at 50 tilts/min at room temperature for 40 min.

The presence of DNase in the lysis buffer is critical to high yields. Without DNase, a sticky pellet will form from the lysed cells' DNA, which will trap most of the crystals (Figure 2).



**Figure 2. A ftn-PAK4 isolation without, and with DNase.** In the DNase-free condition, many of the crystals have been trapped in a sticky pellet, substantially reducing the concentration of free crystals.

### 2. Centrifuge

After rocking, transfer the lysed cell solution to 1.5 ml Eppendorf centrifuge tubes. Centrifuge at 400 rcf for 1 min in a swinging-bucket centrifuge. Discard the supernatant and resuspend the pellet in 500  $\mu$ l of 0.1 M HEPES buffer.

*Note: The supernatant will still contain crystals, which can be collected using a second centrifugation.*

### 3. Verify Isolation

Check crystal yield by pipetting a few  $\mu$ l of the suspension onto a coverslip and looking at it under the microscope. Isolated crystals should be visible through the eyepiece using a 10x objective under both brightfield and GFP illuminations.

## C. Iron loading and assay

### 1. Add Iron

Prepare a solution of FAS (50 mM, 100x) and mannitol (500 mM, 100x) in sterile deionized water. Add 1  $\mu$ l of each stock solution to a 100  $\mu$ l aliquot of the crystal suspension. Incubate at room temperature for exactly 15 min.

*Notes:*

- a. *The final concentrations are 500  $\mu$ M FAS and 5 mM mannitol. The mannitol reduces the autooxidation of the iron.*



## **Notes**

1. The passage number of the cells used is a critical variable in crystal yield and purity. High-passage cells will grow fewer, smaller crystals, and crystal suspensions from high-passage cells will contain significantly more debris.
2. After iron loading, if the mannitol is not removed from the solution before PB stain, it will react with the PB stain to create a precipitate over several min. This precipitate doesn't interfere with staining as a brief check, but it will interfere with clean imaging.
3. When imaging the PB stained crystals, it is important to use brightfield microscopy and not phase contrast, as phase contrast will distort colors.
4. Some variables are more important than others.
  - a. The following variables are highly sensitive to variation: cell passage number, centrifugation speed and time, iron loading stock concentrations, and iron loading duration.
  - b. The following variables are less sensitive to variation: Lysis duration and rocking speed, and PB stain concentrations and duration.
  - c. While the presence of mannitol helps to obtain cleaner results, its presence is not necessary to achieve iron loading of ftn-PAK4.

## **Recipes**

1. Lysis buffer
  - 0.1 M HEPES Buffer
  - 1% v/v NP-40
  - Store at room temperature
2. DNase stock solution
  - 12,500 Units/ml
  - Dissolved in EBSS
  - Store at -20 °C
3. Iron loading stock solution
  - 1 ml MilliQ water
  - 19.6 mg Ferrous Ammonium Sulfate (500 mM stock)
  - 91 mg Mannitol (500 mM stock)
  - Note: Do not store—make fresh each time.*
4. Prussian Blue stain
  - 1 ml MilliQ water
  - 50 µl Concentrated Hydrochloric Acid
  - 5 mg Potassium Ferrocyanide
  - Note: Do not store—make fresh each time.*

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## **Competing interests**

The authors declare that there are no competing interests or conflicts of interest.

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