# Coupling counterdiffusion and micro-seeding techniques to increase protein crystallization succeed

**Hernández-Hernández Ma. Angeles**[1], *González-Ramírez Luis A.* [1], *F. M. Baldock Peter*[2] , *A. Briggs Richard*[2], *A. Kolek Stefan*[2], *D. Shaw Stewart Patrick*[2] , *Gavira Jose A.*[1]*

[1]LEC, IACT (CSIC-UGR). Ed. Instituto López Neyra. P.T. Campus de la Salud. 18100. Granada, Spain.
[2]Douglas Instruments Ltd., East Garston, Hungerford, BERKS, RG17 7HD, UK.

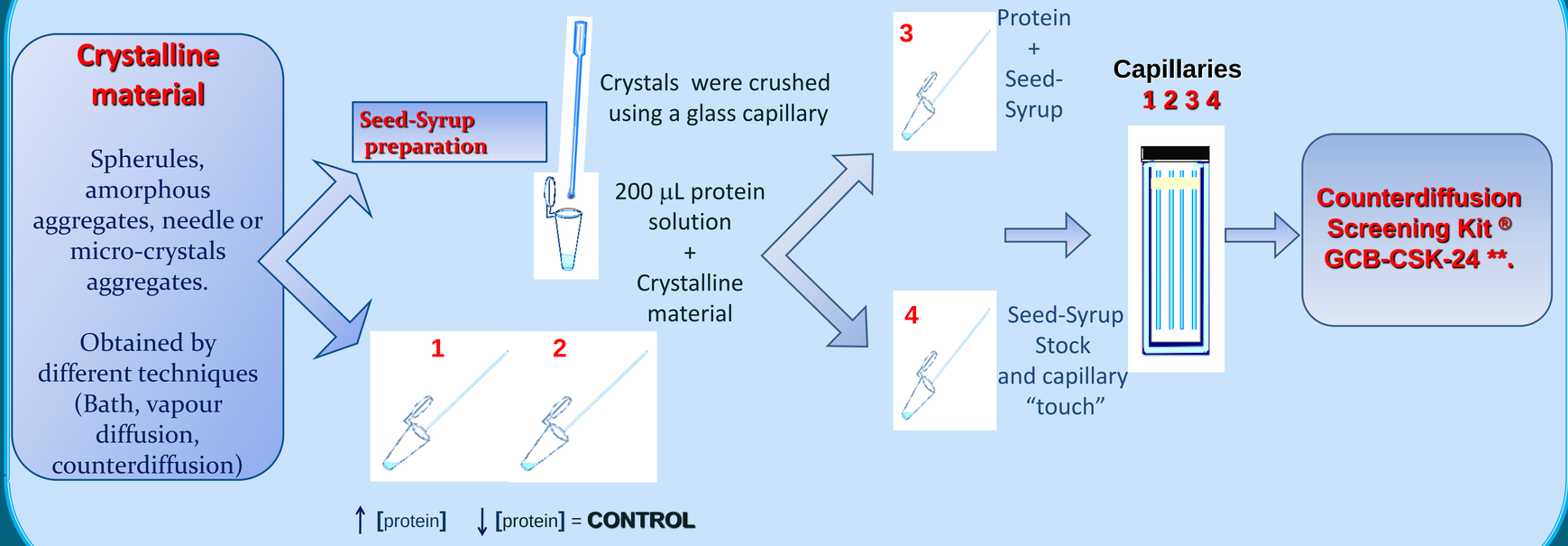
## INTRODUCTION

Distinct methods have being studied to improve quality and size of protein crystals. Protein crystallization in capillaries by counterdiffusion technique has been used routinely for crystal optimization. On the other hand, seeding techniques has open a possibility to improve protein crystal quality and size by carrying on new crystallization experiments on the metastable region of the phase diagram where only growth can occur.

Ideally, seeding should be implemented using batch technique to avoid subsaturation conditions that could dissolve the seed or, high supersaturation conditions as can be obtained in vapour diffusion technique. Counterdiffusion technique allows the introduction of seeds into a system where the mass transport is controlled by diffusion in which supersaturation increase slowly enough to permit the growth of several seeds minimizing cross influence, mixing and conventions effects.

In this work we present the proof of this concept producing optimized crystals of several model proteins by the combination of counterdiffusion and micro-seeding techniques.

## EXPERIMENTAL




## RESULTS

**TABLE 1. Crystallization conditions for initial protein micro-seeds preparation and the final condition to study micro-seeding.**

| *Protein* | *Protein concentration and seed preparation conditions* | *Micro-seeding conditions* |
|---|---|---|
| *Glucosa Isomerase* | 20 mg/ml, Batch experiment using 100 mM Hepes pH 7.0 and 0.9 M Ammonium sulfate. | 30 mg/mL<br>10 mg/mL<br>10 mg/mL + seeds<br>10 mg/mL + dipped in seeds |
| *Ribonuclease-A* | 240 mg/mL, Counterdiffusion technique, precipitating solution **C21**:** 6 M Na-Formate. | 240 mg/mL<br>50 mg/mL<br>50 mg/mL + seeds<br>50 mg/mL + dipped in seeds |
| *Catalase* | 36 mg/mL, Vapor diffusion technique using the *crystallization mushroom*. Precipitating solution 10% w/v PEG-4000. | 40 mg/mL<br>7 mg/mL<br>7 mg/mL + seeds<br>7 mg/mL + dipped in seeds |
| *Myoglobin* | 80 mg/mL, Counterdiffusion technique, precipitating solution **C1**:** 30% PEG 4K, 0.2 M Mg Chloride, 0.1 M Tris-HCl pH 8.50 | 80 mg/mL<br>5 mg/mL<br>5 mg/mL + sedds<br>5 mg/mL + dipped in seeds |
| *α-Lactalbumin* | 72 mg/mL, Hanging drop technique. Precipitating solution **C23**:** 0.82 M K-Phosphate, 0.82 M Na-Phosphate, 0.1 M Na-Hepes pH 7.50. | 30 mg/mL<br>5 mg/mL<br>5 mg/mL + sedds<br>5 mg/mL + dipped in seeds |
| *Thaumatin* | 30 mg/mL, D5 from Jena Bioscience Membrane screen 3; 1.5 M Lithium Sulfate, 100 mM HEPES Sodium Salt, pH 7.5 | 90 mg/mL<br>10 mg/mL<br>10 mg/mL + sedds<br>10 mg/mL + dipped in seeds |
| *Xylanase* | 36 mg/mL, No.45 from Molecular Dimensions Structure Screen1 ; 4.0M Sodium Formate | 56 mg/mL<br>36 mg/mL<br>36 mg/mL + sedds<br>36 mg/mL + dipped in seeds |
| *Trypsin* | 30 mg/mL, D6 from Jena Bioscience Membrane screen 3; 2.0M Sodium Chloride, 100mM Sodium Formate | 90 mg/mL<br>30 mg/mL<br>30 mg/mL + sedds<br>30 mg/mL + dipped in seeds |
| *Concanavalin* | 5 mg/mL, B8 from (Molecular Dimensions) JCSG plus screen; 10% w/v PEG 8000, 100ml Tris-HCl, pH 7.0, 200mM Magnesium Chloride | 15 mg/mL<br>5 mg/mL<br>5 mg/mL + sedds<br>5 mg/mL + dipped in seeds |

****Counterdiffusion Screening Kit ® ref. GCB-CSK-24. See the last part appendix the composition of the 24 conditions.**

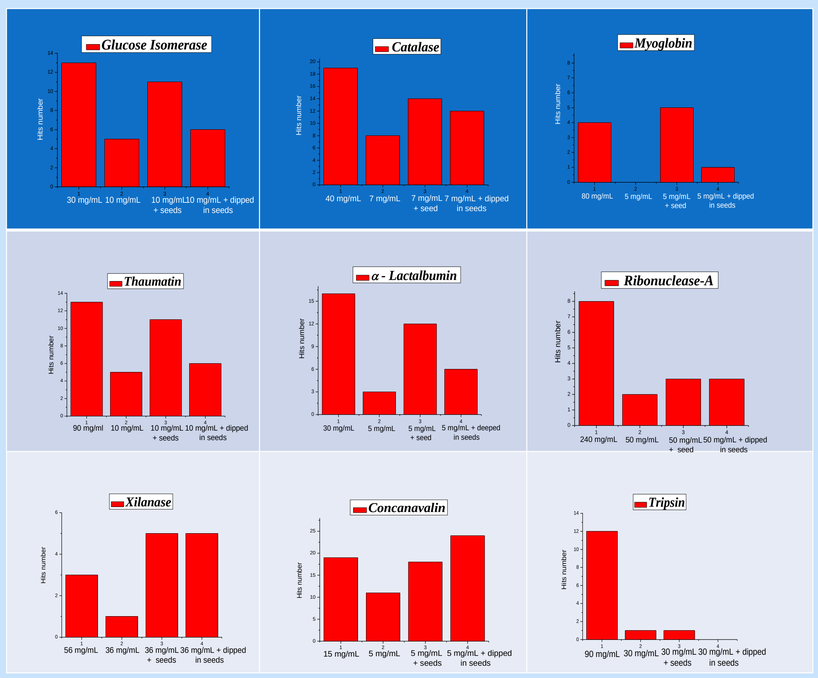



**TABLE 2. Number of hits in the protein crystallization study in capillaries by combination of counterdiffusion and micro-seeding techniques using different model proteins.**

**NOTE:** The last capillary correspond to the filled with the proteins solution and after dipped in the Seed-Syrup Stock solution also named as "touch".

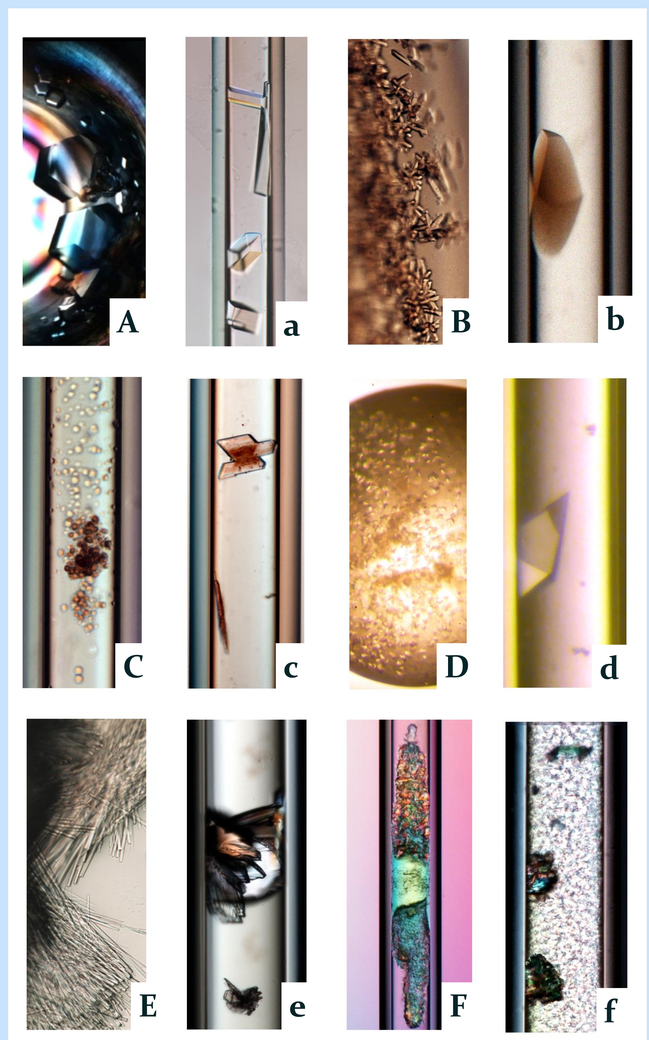



***Figure 1.*** **Protein crystals microphotographs. Capital letters show the seeds used, small letter crystals after seeding. A) Glucosa Isomerase** crystals obtained by batch 20 mg/mL and 100 mM Hepes pH 7.0 and 0.9 M Ammonium sulfate, **a) Glucosa Isomerase** crystals after seeding 10 mg/mL condition C2** **B) Catalase** crystallized at 36 mg/mL by vapor diffusion technique and 10% w/v PEG-4000, **b) Catalase** at 7 mg/mL condition C8**, **C) Myoglobin** obtained at 20 mg/mL condition C1**; **c) Myoglobin** crystallized at 5 mg/mL condition C1**; **D) Thaumatin** 30 mg/mL and precipitated with D5 from Jena Bioscience Membrane screen3; 1.5 M Lithium Sulfate, 100 mM HEPES Sodium Salt, pH 7.5; **d) Thaumatin** 30 mg/mL and obtained in condition C7**; **E) α-Lactalbumin** crystallized at 72 mg/mL by Hanging drop technique and 0.82 M K- Phosphate, 0.82 M Na-Phosphate, 0.1 M Na-Hepes pH 7.50.; **e) α-Lactalbumin** crystallized at 5 mg/mL. C13**; **F) Ribonuclease-A** obtained at 240 mg/mL condition C21**; **f) Ribonuclease-A** crystallized at 50 mg/mL condition C21**.

One of the goals of this study was to increase the number of hits during the screening in capillaries by counterdiffusion using the minimum Kit (24 conditions) using micro-seeding technique. Our results show that both protocols, mixing the seed with the protein solution and touching the seed syrup, increase the number of hits when compare with the low concentration protein solution (90%). In three cases (33%) the number of hits using seeds was higher than those obtained with high protein concentration solution and in one case we got crystals only when seeds were included.

## CONCLUSIONS

For the first time, it is reported the crystallization of several model proteins using micro-seeding by the counterdiffusion technique. Overall, most of the protein studied (90%) increase the number of hits in which the nucleation occurs even when the capillary only touches the "seeds syrup".

The most effective protocol was mixing the protein solution with the seed-syrup, which produced an homogeneous distribution of seeds along the capillary.

It is well known that the initial seed may influence definitively the quality of the final crystals. However in this work the use of (in appearance) not so good quality crystals, such as the case of spherulites of Myoglobine, was done on purpose resulting in the growth of faceted crystals.

**Seeds from the initial crystalline-precipitate can be used to set-up a minimum counter-diffusion screening experiment, even at low protein concentration, to find new crystallization conditions and to produce crystals of improved visual quality.**

**E-mail**: gavi@lec.csic.es hernanh@ugr.es ** triana@trianatech.com

## REFERENCES


- A. McPherson, in: Crystallization of Biological Macromolecules, Cold Spring Harbor Laboratory Press, New York, 1998 (Appendix section).
- Enrico A. Stura and Ian A. Wilson, (1990). Analytical and Production Seeding Techniques. METHODS: A Companion to Methods in Enzymology. Vol. 1, No. 1, August, pp. 38-49.
- J.M. García-Ruiz, (2003). Counterdiffusion Methods for Macromolecular Crystallization. Methods in Enzymology, Vol. 368 130-154.
- R.J. Read and J.L. Sussman (eds.): Evolving Methods for Macromolecular Crystallography, (Succeeding with seeding: some practical advice**,** Terese Berfors) 1–10 © 2007. Springer
- T. Bergfors, (2003). Seeds to crystals, Journal of Structural Biology 142, 66–76.
- E. Saridakis,K. Dierks, A. Moreno, M. W. M. Dieckmannd and N. E. Chayen. (2002). Separating nucleation and growth in protein crystallization using dynamic light scattering. Acta Cryst. D58, 1597-1600.


## ACKNOWLEDGEMENTS

This work was supported by the OptiCryst project of the VI European Framework Program and the Andalusian Regional Government, Spain, project RNM 5384. This research is a product of the "Factoría Española de Cristalización", a project Consolider- Ingenio 2010 of the Ministerio de Innovación y Ciencia (MICINN) of Spain