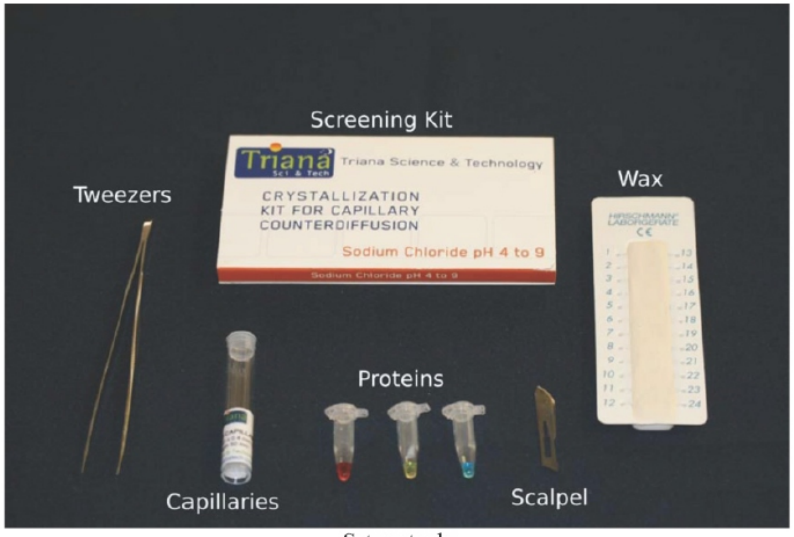
PROTOCOL 1: CD using pre-filled GCB-Domino

Counterdiffusion using the commercial pre-filled GCB-Domino.

The pre-filled GCB-Domino can be used for the initial screening or for crystal improvement.

While the ***GCB-CSK*** is intended only for initial screening (with capillary of 0.1 mm inner diameter), the ***CCD*** can be use for both, initial screening and crystal improvement.



Set-up-tools

How to prepare the macromolecular solution

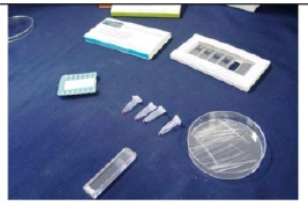
Prepare your macromolecular solution as pure as possible and use it after microfiltration. We recommend the use of a protein concentration of 5 to 10 mg/ml preferentially in water or alternatively in a buffered solution at buffer concentration smaller than 50mM. Initially, the sample should be free of any unnecessary additives in order to observe the effect of the precipitants in the kits.

Note:
We recommend testing two protein concentrations per protein (two capillaries in each box).

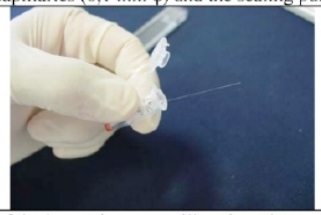
Counterdiffusion Protocols

STEP BY STEP

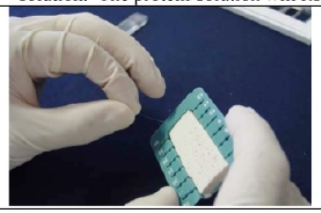
How to perform the screen

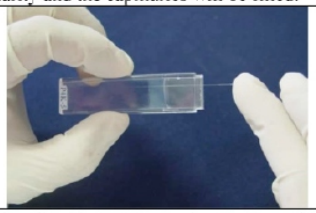


Select the kit you wish to use among the available GCB-Domino screening kits and have ready the material you will, i.e. the protein solutions, the capillaries (0,1 mm ϕ) and the sealing putty

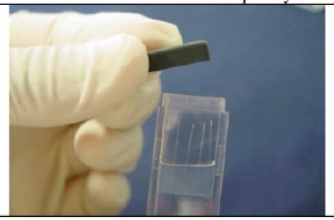


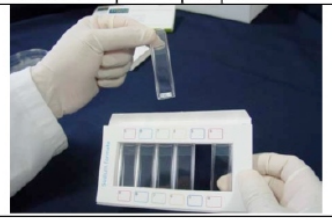
Take one GCB and cut the aluminium seal from top of the box. Dip one capillary into the protein solution. The protein solution will rise by capillarity and the capillaries will be filled.





Seal the upper end with the putty. Dip the filled capillary into the GCB-Domino. Just punch the unsealed end of the capillary across the gel located on top of the precipitant.





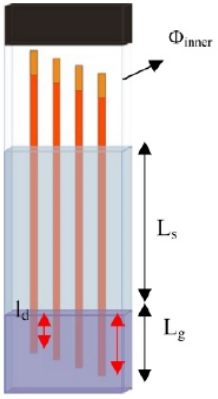
Repeat the sequence with other capillaries filled with your protein solution at different concentration or with selected additive like divalent metals or detergents. Close the GCB-Domino with its black tap. Repeat the procedure with the remaining five boxes. We suggest you to relocate the six boxes in the cardboard frame.

PROTOCOL 2: CD using empty GCB-Domino

Counterdiffusion in the GAME configuration.

The Gel Acupuncture Method (GAME) can be implemented in any container that can be further close avoiding evaporation.

The GCB-Domino (Triana S&T) is flat container that allows an easy implementation of the experiment, reduce the consumption of reactants and facilitate the observation.



As in any other crystallization experiment there are a number of variables. In counterdiffusion experiments it can be varied the

1. protein concentration
2. salt concentration
3. capillary inner diameter (Φ_{inner})
   - Φ_{inner} = 0.1 mm (for screening)
   - Φ_{inner} = 0.1 or 0.2 mm (for crystal improvement)
4. punctuation depth (l_d)
   - l_d = 5 mm
5. and value and ratio L_s:L_g

Set-up-tools

How to prepare the macromolecular solution

Prepare your macromolecular solution as pure as possible and use it after microfiltration. We recommend the use of a protein concentration of 5 to 10 mg/ml preferentially in water or alternatively in a buffered solution at buffer concentration smaller than 50mM. Initially, the sample should be free of any unnecessary additives in order to observe the effect of the precipitants in the kits.

Note:

We recommend testing two protein concentrations per protein (two capillaries in each box).

1. GEL LAYER

There are two way to prepare the agarose sol; in a water-heating bath or using a microwave.

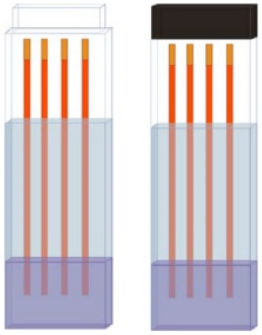
1.1. In both cases add the agarose powder to the appropriated volume (5 ml) of the buffer solution to a final agarose concentration of 1% (*w/v*).
1.2. Heat the mixture to boiling until the agarose solution becomes transparent.
1.3. Poor the agarose sol in the GCB and let it gel at room temperature.

Note: Agarose sol should contain any other additive present in your protein solution.

2. FILLING THE CAPILLARIES

2.1. Introduce one end of the capillary into the protein solution. The solution will flow up by capillary force.
2.2. Once it reaches almost the top of the capillary, remove it from the solution (you will see that the solution remains inside the capillary).
2.3. Seal the upper end of the capillary with a small amount of wax.
2.4. Puncture the capillary into the gel layer, typically 5 mm.
Repeat the procedure with the next capillary.

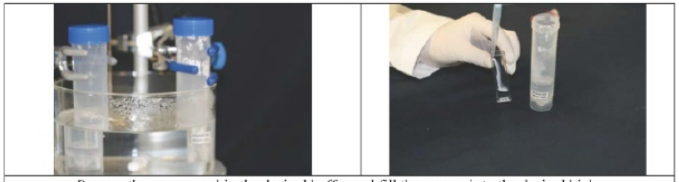
3. PRECIPITANT LAYER



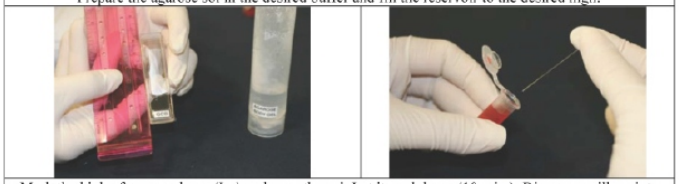
3. Pour the precipitant agent solution.
   - For screening we recommend the **Counterdiffusion Screening Solutions (CSS)®**

Close the box and sealed with parafilm.

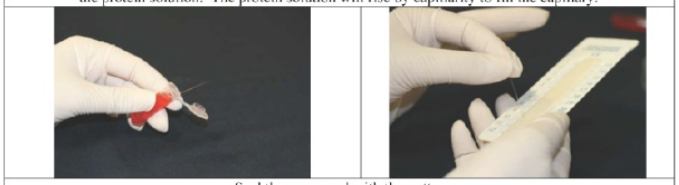
STEP BY STEP



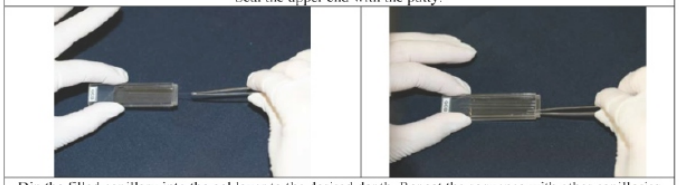
Prepare the agarose sol in the desired buffer and fill the reservoir to the desired high.



Mark the high of agarose layer (Lg) and pour the sol. Let it cool down (10 min.). Dip one capillary into the protein solution. The protein solution will rise by capillarity to fill the capillary.



Seal the upper end with the putty.



Dip the filled capillary into the gel layer to the desired depth. Repeat the sequence with other capillaries filled with your protein solution. Here you can use a different concentration.

Add the precipitant solution to the desired high (Ls). Close the reservoir to avoid evaporation.

Trace Fluorescent Labeling for Protein Crystallization Screening

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Hands-on Practical Outline.

I. Introduction to covalent modification of proteins.
   A. Several groups are commonly available as targets.
      1. Amines – side chain and N-terminal.
      2. Sulfhydryls – less commonly available.
      3. Carboxyls
   B. Easiest is Amines – use pH control to select type.
      1. pH ~7.5, N-terminal amines
      2. pH ~>8.5, side chain amines.
      3. I prefer side chain, less chance of systematic effects.

II. Solutions & Materials needed.
   A. Reaction buffer – 0.05M NaBorate, pH 8.75 used here. Must be free of primary and secondary amines (tertiary OK)!
   B. Reactive probe solution – carboxyrhodamine-SE used (5 mg in 1 mL DMF).
   C. Centrifugal desalting column – 0.5 mL (Pierce). Note – max speed is 1500xG.
   D. Crystallization buffer solution – whatever your protein is equilibrated in. I'm using 0.05 M NaHepes, 0.1 M NaCl, pH 7.5.

III. Procedure
   A. Equilibrate desalting column in reaction buffer (2-3 passes).
   B. Prepare protein solution – demo uses 0.8 mLs at 15 mg/mL as the stock solution.
   C. Pass 80 uL of protein solution thru desalting column.
   D. Add 0.5 uL of reactive probe solution.
   E. Interlude – dismantle microscope and show parts.
   F. Re-equilibrate column in crystallization buffer (2-3 passes).
   G. Pass derivatized protein solution thru desalting column.
   H. Add protein back to stock solution.
   I. Bring volume up to 1.0 mLs.
   J. Use microscope to verify fluorescence.
      1. Solution should be slightly colored at most.
      2. Should see more color in the desalting column – the unbound probe.
      3. From experience – this results in ~0.25% of the protein being labeled.
   K. Use protein as normal in setting up crystallization plates.

When viewing the plates – the crystals may show some slight color to those with more discerning eyes (not mine). Crystals are obvious as they give a much brighter emission than the solution or amorphous precipitate. For leads, look for precipitate that has 'bright spots', that cannot be obviously determined to be crystals.