Video Article

Intracellular Refolding Assay

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Abstract

This protocol describes a method to measure the enzymatic activity of molecular chaperones in a cell-based system and the possible effects of compounds with inhibitory/stimulating activity. Molecular chaperones are proteins involved in regulation of protein folding[1] and have a crucial role in promoting cell survival upon stress insults like heat shock[2], nutrient starvation and exposure to chemicals/poisons[3]. For this reason chaperones are found to be involved in events like tumor development, chemioresistance of cancer cells[4] as well as neurodegeneration[5]. Design of small molecules able to inhibit or stimulate the activity of these enzymes is therefore one of the most studied strategies for cancer therapy[7] and neurodegenerative disorders[9]. The assay here described offers the possibility to measure the refolding activity of a particular molecular chaperone and to study the effect of compounds on its activity. In this method the gene of the molecular chaperone investigated is transfected together with an expression vector encoding for the firefly luciferase gene. It has been already described that denaturated firefly luciferase can be refolded by molecular chaperones[10,11]. As normalizing transfection control, a vector encoding for the renilla luciferase gene is transfected. All transfections described in this protocol are performed with X-treme Gene [11] (Roche) in HEK-293 cells. In the first step, protein synthesis is inhibited by treating the cells with cycloheximide. Thereafter protein unfolding is induced by heat shock at 45°C for 30 minutes. Upon recovery at 37°C, proteins are re-folded into their active conformation and the activity of the firefly luciferase is used as read-out: the more light will be produced, the more protein will have re-gained the original conformation. Non-heat shocked cells are set as reference (100% of refolded luciferase).

Video Link

The video component of this article can be found at https://www.jove.com/video/3540/

Protocol

1. Seeding the cells

1. Before starting, warm up the culture medium, the PBS 1X and the trypsin in a water bath at 37˚C
2. Take the cells out of the incubator and aspirate the medium.
3. Gently apply 5 mL of PBS 1X on the cells to wash them.
4. Aspirate the PBS 1X and apply 1 mL of trypsin containing 0,025% EDTA.
5. Gently rotate the plate to have an even distribution of the trypsin.
6. Place the cells in the incubator at 37˚C for 5-10 minutes (depending on the cell type). From time to time check if cells are detached by shaking the plate.
7. Resuspend the cells in 10-20 mL of medium and collect them in a 50 mL falcon tube.
8. Count the cells using a Beckman counter. The number of cells should be enough to ensure a confluence of approximately 60-70%.
9. Plate the cells and allow them to seed for 6-8 hours.

2. Transfection

1. Prior to transfection, warm up an aliquot of serum-free medium in a water bath at 37˚C.
2. Bring the X-treme Gene 9 to room temperature.
3. Pipet the serum free medium in 2 eppendorf tubes (1 tube= 1 transfection).
4. Pipet the X-treme Gene 9 in the serum free medium paying attention that the tip does not touch the plastic wall of the eppendorf and incubate for 5 minutes. In the meantime prepare two DNA mixes: in one tube mix together the plasmids for renilla, firefly and an empty vector control, in another tube renilla, firefly and the molecular chaperone.
5. Add the DNA mix to each eppendorf containing serum free medium + X-treme Gene 9, vortex and incubate at room temperature for 20 minutes.
6. Add dropwise each mix onto each plate and incubate for 24 hours at 37°C and 5% CO_2

3. Splitting the cells

1. 24 hours after transfection, trypsinize the cells as described in 1.4 – 1.6 and dilute them to a final confluency of 60-70% in a 6 -well plate.
2. Prepare one plate for the non-shocked control and as many plates as the recovery time points that have to be analyzed, as showed in **Fig. 2**.

4. Heat shock

1. For heat shock, pre-warm serum containing medium in a water bath at 37˚C.
2. Prepare 'heat shock buffer' by diluting in serum containing medium cycloheximide to a final concentration of 20 µg/ml and MOPS to 20 mM.
3. Now take out the plates from the incubator and aspirate the medium.
4. Add to each well 1 mL of 'Heat shock buffer', and incubate for 30 minutes at 37˚C and 5% of CO_2 (include also the reference plate in this treatment). This will stop *de novo* protein synthesis.
5. In the meantime switch on a water bath and set the temperature at 45˚C.
6. After incubation, seal the lid of the plates with Parafilm previously cut in stripes. The reference plate (100% of refolfed firefly) is left in the incubator.
7. Incubate the plates at 45˚C for 30 minutes**.**
8. Remove the plates from the water bath, remove the parafilm and put the plates back to the incubator to allow recovery.
9. At each time point harvest the cells and wash them in PBS 1X by centrifuging at 2000Xg for 1 minute at 4˚C.

5. Cell lysis and luciferase assay

1. For an efficient cell lysis, snap freeze the cell pellet in liquid nitrogen.
2. Add 200 µl of passive lysis buffer diluted 1:5 in double distilled water in a glass tube.
3. Add 30 µl of the cell lysate in a 96 well plate. Each sample is pipetted in triplicate.
4. Only the 'non shocked' reference samples will be used to read the renilla luciferase activity, since this step is used to check equal transfection efficiency.
5. Prepare the solutions for the assay. For the luciferin solution dilute luciferin to a final concentration of 0.2 mM in Gly-Gly buffer. For the reaction buffer dilute DTT and ATP to 1mM final concentration in Gly-Gly buffer. For coelenterazine reaction buffer, dilute coelenterazine to a final concentration of 0.2 µM in coelenterazine buffer. Keep luciferin solution and coelenterazine reaction buffer light protected.
6. Now place the 96-well plate in the luminometer and start the program 'Wallac 1420 Workstation'.
7. Introduce the Pump1 into the falcon tube containing the coelenterazine solution. Close the lid so no light can come in contact with the tube.
8. Choose from the menu the option 'Dispenser maintenance' and tick Pump1.
9. Select the option 'Fill'.
10. To edit the plate layout choose the option 'Explore protocol and results' on the menu. Select the protocol and double click on it. Now select the wells to be read.
11. Go back to 'Wallac Manager' and click 'Start'. The reaction between the renilla luciferase and its substrate coelenterazine emits light with a peak wavelength of 482 nm.
12. After the reading, go on 'Dispenser maintenance' and select 'Empty' and Pump1 to release all the residual solution back to the tube.
13. Now move the tube to a falcon tube containing water and tick 'Flush'.
14. After the flushing step, empty the tube by selecting 'Empty'.
15. Place the tube from Pump1 in the Reaction buffer falcon tube and the one from Pump2 in the luciferin falcon tube .
16. Change the plate layout.
17. Select 'Fill' for Pump1 and Pump2 from the 'Dispenser Maintenance' menu.
18. Select the protocol and start the reading. The reaction between the Firefly luciferase and its substrate luciferin emits light with a peak wavelength of 560 nm.
19. At the end of the reading repeat the procedure Empty-Flush-Empty for Pump1 and Pump2.
20. Results are now available in the 'Wallac 1420 Explorer'.
21. Each experiment is associated to an assay number, username, begin and end date.
22. Results can now be exported as an Excel file.
23. For calculations, cells are divided into three groups: 1. non-shocked reference samples (set as 100% of refolded firefly luciferase); 2. cells without molecular chaperone and heat shocked; 3. cells with molecular chaperone and heat shocked.

6. Representative Results

Protein refolding is directly related to the time of recovery therefore to test if the system is working properly, an assay has to be performed collecting the cells at different time points. A direct correlation time/percentage of refolding indicates that the experiment has been properly performed and therefore represents a limiting factor. However it should be always considered that refolding after heat shock is a saturating event and therefore a proper time course analysis should be performed before starting any experiment.

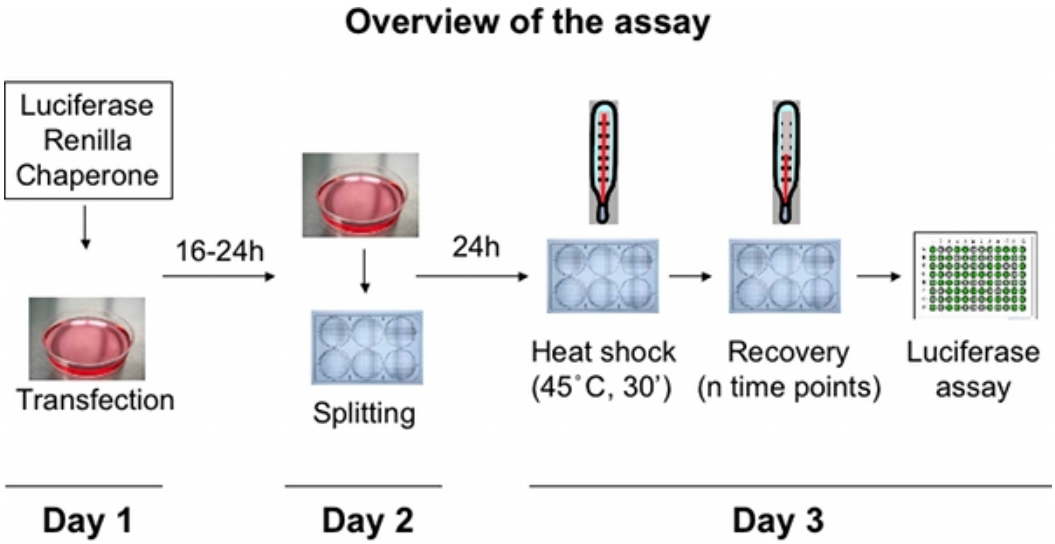

Figure 1. Overview of the experiment. In vivo refolding assay requires 3 to 4 days to be completed. On day 1 cells are seeded and transfected. The following day cells are splitted into a smaller format and on day 3 they are heat shocked. At this point is possible to perform the luciferase assay right after harvesting the cells or to save the cell pellet at -80°C and perform the assay in another moment

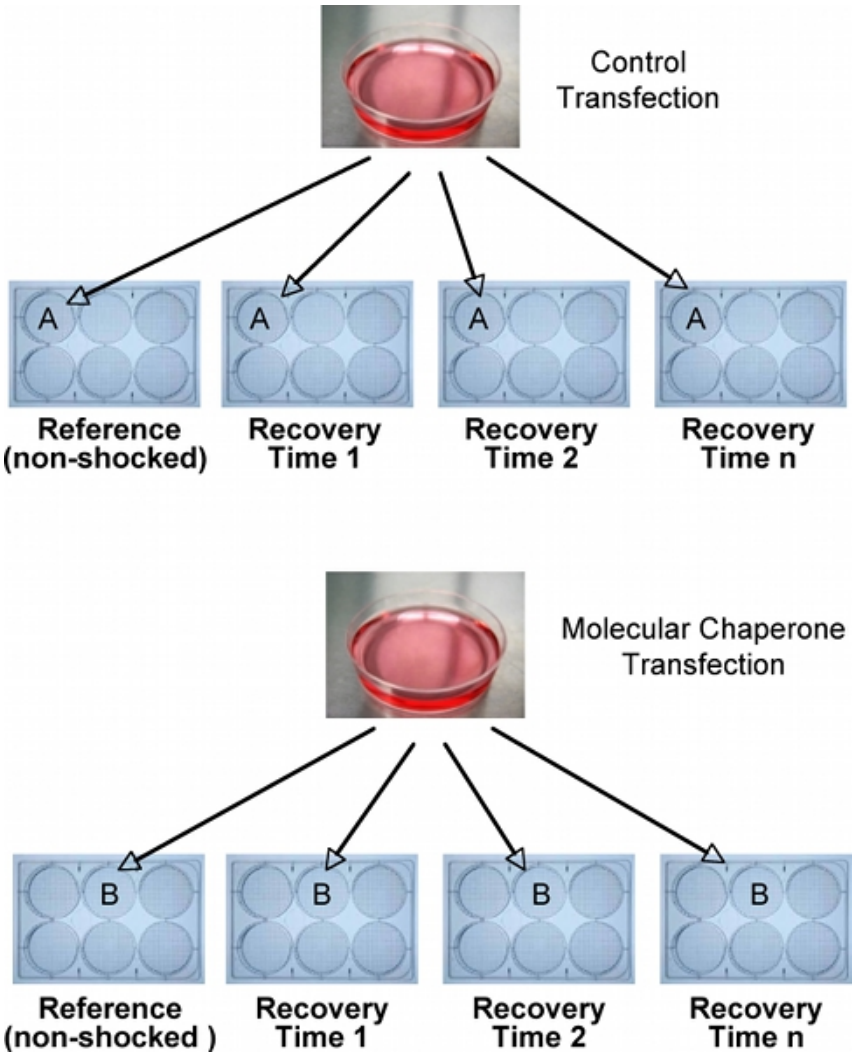

Figure 2. Cell splitting. On day 2 cells are split into a 6-well plate. Each transfection will be diluted in a well in order to have all the transfection on the same plate. Each plate will correspond to a particular recovery time plus a non-shocked reference plate.

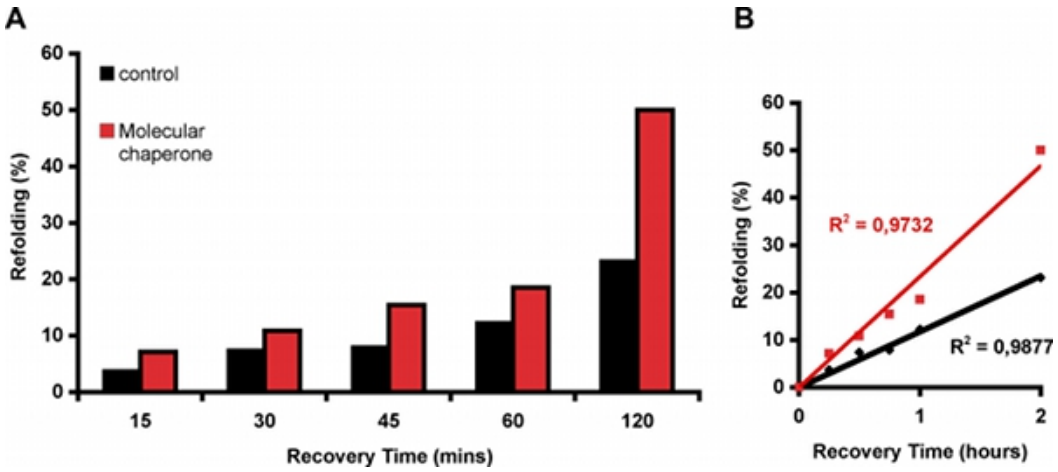

Figure 3. Representative good result. A. Percentage of refolding directly measured by firefly luciferase activity. Each bar chart represents the percentage of refolding in absence (black bars) and in presence (red bars) of a molecular chaperone at different recovery time points. B. Correlation between time and refolding in absence (black line) and in presence (red line) of chaperone. The r squared values indicate good correlation.

In **Fig.3A** is shown a representative results where cells were collected 15, 30, 45, 60 and 120 minutes after heat shock in absence (black bars) and in presence (red bars) of molecular chaperone. Percentage of refolded luciferase increases with prolonged recovery time and with the transfection of the molecular chaperone. The correlation between refolding and time for the control (**Fig. 3B**, black line) and the molecular chaperone-transfected cells (**Fig. 3B**, red line) is shown.

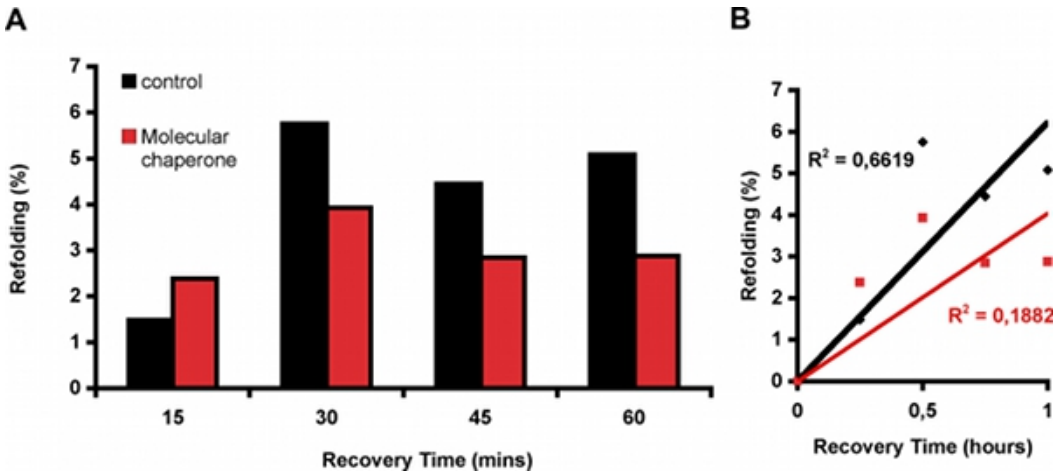

Figure 4. Representative bad result. A. Percentage of refolding directly measured by luciferase activity. Each bar chart represents the percentage of refolding in absence (black bars) and in presence (red bars) of a molecular chaperone at different recovery time points. B. Correlation between time and refolding in absence and in presence (red line) of chaperone. The r squared values indicate bad correlation.

Figure 4A. shows a representative result of an experiment not properly performed. Both the control (black bars) and the molecular chaperone-transfected cells (red bars) did not show an increase in protein refolding with the time. In addition, transfection of the molecular chaperone did not result in an increase of refolding. This poor correlation is confirmed in **Fig. 4B** showing an r squared value of 0.6619 and 0.1882 respectively for the control (black line) and the molecular chaperone-transfected cells (red line).

Discussion

In this work a protocol to measure intracellular refolding activity of molecular chaperones is presented. The whole assay can be performed in 3 to 4 days as shown by the overview in **Fig. 1**.

The robustness and linearity of the light signal produced by the firefly and the renilla luciferase represent a solid base for the reproducibility of the protocol.

The critical step of the assay is the choice of an efficient transfection reagent to ensure the overexpression of the molecular chaperone and the firefly luciferase. Reporter genes could be delivered also with other methods like adenoviral infection[12] or electroporation. Another possibility is the use of a transgenic cell line where the expression of the chaperone is chemically inducible (e.g. a tetracycline-responsive promoter)[13], while the gene of the firefly luciferase is stably transfected. This wide range of options makes thus possible the application of the assay to several cell lines, including primary cells.

Moreover, if a cell line is particularly sensitive to cycloheximide treatment, firefly luciferase protein translation could be arrested by the use a repressible/inducible system.

Since refolding activity can vary among the molecular chaperones, a titration of the amount of chaperone to be transfected should be always done. Once established in a particular cell line, the technique can be used to investigate how small molecules and/or gentic manipulations can affect chaperone activity. The assay can be performed also in a smaller format, like a 6- or even a 12-well plate.

One of the most attractive applications is the possibility to test entire libraries of compounds that can influence chaperone activity. In this particular case, cell lines stably transfected with the reporter gene as well as the chaperone are preferentially used, in order to minimize variations due to transfection efficiency.

This assay can be also used to investigate the role of co-activators or co-repressor of the molecular chaperones in protein refolding or how they influence refolding upon different stress stimuli (heat, oxidative stress, unfolded protein response).

Even if from a cell-based experiment is possible to have a more physiological readout of the effect of compounds and/or proteins on the activity of a particular chaperone, it is not possible to quantify the chaperone activity with the assay here presented. In this case would be more suitable a cell-free assay like *in vitro* refolding assay of firefly luciferase or β-galactosidase.

Disclosures

The authors have nothing to disclose.

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