IN VIVO REFOLDING ASSAY (IN VIVO CHAPERONE ACTIVITY)

The basis of this assay is to monitor refolding of heat denatured luciferase *in vivo* as a measure of chaperone activity. This assay has been performed using O23 cells (hamster) that have been transfected with a luciferase expression construct. Heat shocking the cells at 45˚C causes unfolding of luciferase and subsequent aggregation leading to loss of activity. Endogenous levels of molecular chaperones are not sufficient to fully refold and recover active luciferase. However, introducing excess amounts of molecular chaperones (by transfection) protects luciferase from unfolding and also enhances refolding after the heat denaturation. Therefore, since the ability to regain luciferase activity depends on the transfection of (introduction of exogenous) molecular chaperones, any activity recovered beyond basal level will be a reflection of the chaperone activity of the protein introduced to the system. The entire assay takes about a week (4-5days).

Materials:

Cell culture
O23 cells
1X PBS
DMEM/10% FBS
1X Trypsin

Transfection
Lipofectamin (Invitrogen) or Polyfect (Qiagen)
pCytLuc (B2.28 : luciferase expression construct)
molecular chaperone construct (e.g. pCMV-Hsp70)

Heat Shock

50X MOPS
MOPS 20.93g
q.s. to 100ml with DME/10% FBS
pH to 7.0, filter and store at -20°C

125X Cycloheximide
100mg cycloheximide
q.s. to 20ml with dH_2O
filter

MOPS/cycloheximide media
50X MOPS 1ml
125X Cycloheximide 400 l
q.s. to 50ml with DMEM/10%FBS

Luciferase assay
Dual-Luciferase Reporter Assay System (Promega: TM040)
- Passive Lysis Buffer
- Luciferase Assay Substrate (luciferin)
- Luciferase Assay Buffer

Procedure:

Day 1

Splitting cells

- *Make sure you write out how many different conditions you want to test so that you know how many 60mm plates you need for transfection.*

1. Split cells 60mm TC plate according the number of transfection conditions (e.g. A, B, C….). *Split 1:10 dilution using O23 cells.*
   Also include Negative control : pCytLuc alone
   Positive control: pCytLuc and pCMV-Hsp70

Day 2

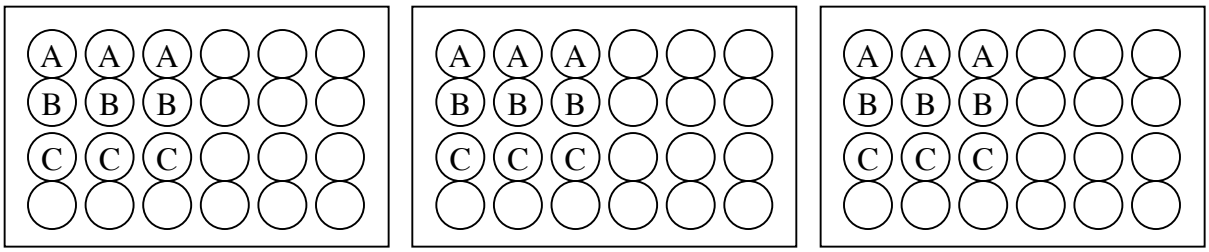
Transfection

1. Co-transfect cells with pCytLuc (B2.28) and molecular chaperone expression constructs at desired ratio. *Don't forget the negative and positive controls!*

Day 3

Splitting cells

1. Prepare three 24-well plates: Control, Heat Shock, and Heat Shock – Recovery.
2. For each transfection (sample) split the transfected cells as shown below.
   Split each transfection in three wells for each condition. (Triplicates)



Control (C) Heat Shock (HS) Heat Shock–Recovery(HR)

Day 4 (This assay is done using Dual-Luciferase Reporter Assay System : Promega)

Heat Shocking cells and cell lysate preparation

1. Prepare MOPS/cycloheximide media.
2. Remove media and add 500μl of MOPS/cycloheximide media to each well.
3. Incubate at 37˚C for 30min.
4. Heat a water bath to 45˚C.
5. Wrap the HS and HR plates with paraffin and immerse in 45˚C water bath for 30min-1hr. (Control plate (C) stays in 37˚C incubator.)
6. Unwrap and incubate the HR at 37˚C for 3hrs.
7. Remove media from C and HS plate and wash with 1xPBS (500μl –1ml per well).

STOP: once you remove the 1xPBS, you can store the cells at –70˚C until you are ready to analyze.

8. Remove 1xPBS and add 100μl of 1x PLB (Passive Lysis buffer).
   STOP: you can store this sample at –70˚C
9. Shake at RT for 30min. [Prepare Luciferase assay reagent II]
10. Pipet up and down to break-up the clumps.
11. Transfer 20μl of each cell lysate into white opaque luminometer microtiter plate.

Measuring luciferase activity

1. [Prepare Luciferase Assay Reagent II]
   Resuspend a bottle of lyophilized Luciferase Assay Substrate (luciferin) in Luciferase Assay Buffer and Label "LARII" (stable for one year at –80˚C)
   *Need 100μl per assay, plus about 2ml extra for the tube volume for luminometer.

2. [Read samples in Read samples in Molecular Devices "LMax" luminometer]
   Prime P injector (7 injections each) with LARII.
   Inject 100 μl LARII per well with P-injector.
   Read with a 2 sec premeasurement delay, 10 sec integration to detect firefly luciferase.
   Print out data.

Wash injectors:

Place the tube into a 50ml Falcon tube containing ddH_2O. Select **Wash Injectors…** from the **Control** menu. Accept the default settings (30 injections) and click **OK**.
Repeat this wash step with 70%EtOH (30 injections). Replace injector tube into the empty Falcon tubes and perform a final "Wash" that will just dry the injector tubing.

References:

Nollen, E.A. et al. *Proc. Natl. Acad. Sci. USA* **98**, 12038-12043 (2001)
Michels, A.A. et al. *Eur. J. Biochem*. **234**, 382-389 (1995)
Dual-Luciferase Reporter Assay System Technical Manual *Promega*
Dual-Luciferase Reporter Assay System by Sandy Westerheide

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