

Epidermal growth factor receptor (EGFR) recycling assay

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INTRODUCTION

This assay allows to have a quantitative assessment of the recycling rate of EGFR, by measuring the amount of intact ligand that is retrieved in the medium upon time. Data should be plotted as fraction of intact EGF in the medium/initial amount at zero time point (Y-axes), versus time (X-axes). In HeLa cells, after 1 h the curve reaches a plateau (around 40% recycling), meaning that all ligand that was initially internalized, has been extinguished (either recycled or degraded).

MATERIALS

Binding buffer

- DMEM
- 0.1% BSA
- 20 mM Hepes

Mild acid/salt wash buffer (pH 4.5)

- 0.2 M Na acetate pH 4.5
- 0.5 M NaCl

Acid wash buffer (pH 2.5)

- 0.5M NaCl
- 0.5M acid acetic

TCA precipitation

- 150 μ l of lysate or medium
- 125 μ l TCA 20%
- 225 μ l PBS/BSA 1%

METHODS

1. Cells are plated into 24 well in order to have 90 percent of confluence the day after (in the case of HeLa, 100.000 cells). Cells are plated in triplicates for each time point, plus one well for the unspecific binding.
2. The day after, cells are serum-starved for at least 4 hours in binding buffer and incubated for 15 minutes at 37°C with 20 ng/ml of 125 I-EGF: 5 ng/ml of 125 I-EGF (Perkin Elmer) + 15 ng/ml of cold EGF (Peprotech).
3. Put cells on ice and wash twice with cold PBS and incubate with mild acid/salt wash buffer (pH 4.5) for 5 min.
4. Wash 2 times with cold binding buffer. These cells (further referred to as " 125 I-EGF-loaded cells") contain a large pool of intracellular 125 I-EGF and a minimal (less than 5%) surface pool of 125 I-EGF.
5. The 125 I-EGF-loaded cells are further incubated in binding medium with 4 μ g/ml of unlabeled EGF at 37 °C for a chase time (0', 20', 40', 60'), and the amount of degraded and intact 125 I-EGF in the

medium as well as surface and intracellular labeled ligand is determined. Briefly, after each time points:

- a. Collect the medium, half to count directly (free total), half to perform TCA precipitation to distinguish between intact/recycled EGF (pellet) and degraded EGF (supernatant)*
 - b. Wash the cells twice with cold PBS and incubate with acid wash buffer pH 2.5 for 5 min at 4°, to determine the amount of surface-bound ¹²⁵I-EGF. Let dry the cells very well (at least 30 minutes at room temperature) before adding the lysis solution (c)
 - c. Lyse cells in 1 mM NaOH and leave an additional 30 min at RT in agitation. Then, scrape well by pipetting. Half of the lysate is used to count directly (internalized total), half to perform TCA precipitation to distinguish between intact EGF (pellet) and degraded EGF (supernatant)*
 - *The amount of degraded or intact ligand radioactivity in the medium/lysate is determined by precipitating with 5% trichloroacetic acid (TCA, final concentrations), and counting supernatant and pellet (cut the eppendorf's bottom).
6. Vortex well and incubate at 4° on
 7. Spin at 14000 rpm for 5 min
 8. Count 350 µl of supernatant (degraded ¹²⁵I-EGF)
 9. Eliminate the rest and count the eppendorf's bottom (intact ¹²⁵I-EGF)

BIBLIOGRAPHY

Sigismund S., Argenzio E., Tosoni D., Cavallaro E., Polo S. and Di Fiore P.P. (2008) Clathrin-mediated internalisation is essential for sustained EGFR signalling but dispensable for degradation. *Dev.Cell.* 15(2): 209-19.