

Thermo Scientific SCF-Skp2 E3 Ligase: p27 (RING) Degradation Redistribution[®] Assay

The Redistribution technology monitors the cellular translocation of GFP-tagged proteins in response to drug compounds or other stimuli and allows easy acquisition of multiple readouts from the same cell in a single assay run. In addition to the primary readout, high content assays provide supplementary information about cell morphology, compound fluorescence, and cellular toxicity.

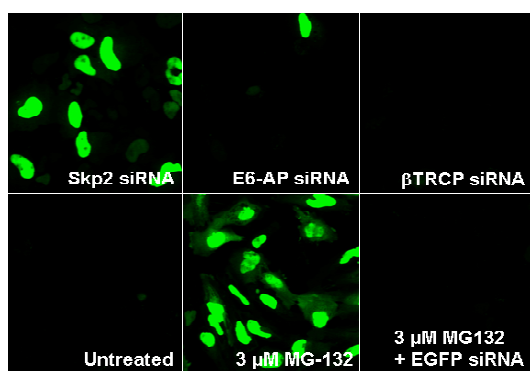


Figure 1. Inhibition of proteasomal degradation of p27Kip1(T187D)-EGFP. Cells were transfected with siRNAs or treated with the proteasome inhibitor MG132. Knockdown of Skp2 E3 ligase or treatment with MG-132 results in stabilization of p27(T187D)-EGFP, while knockdown of other E3 ligases results in no (β TRCP) or low (E6-AP) accumulation. EGFP siRNA is the positive control for efficiency of siRNA transfection.

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Conjugation of ubiquitin (a 76 aa protein) to a protein substrate during protein degradation by the proteasome follows a three-step mechanism. First, the ubiquitin activating enzyme E1 activates ubiquitin to generate a high-energy thiol-ester intermediate in a reaction dependent on ATP. The activated ubiquitin is then transferred to a member of the E2 ubiquitin conjugating enzymes, that finally transfers the ubiquitin to a substrate protein that is specifically bound by an E3 ligase enzyme [1]. One such E3 ligase is the SCF-Skp2 complex that belongs to the RING finger class of E3 ubiquitin ligases. SCF consists of three primary subunits (Skp1, Cullin and Rbx/Roc1) that are responsible for binding to E2 enzyme and to so-called F-box proteins that recruit protein substrates and thereby bridge the reaction between the E2 ubiquitin conjugating enzyme and the substrate. The SCF-Skp2 E3 ligase is mainly involved

in promoting cell cycle progression, most notably through targeted degradation of p27Kip1 phosphorylated at Thr187 in S-phase [2, 3]. Furthermore, Skp2 has been found to promote and be upregulated in a number of human cancers.

Features

- Designed to assay compounds for their ability to modulate activation of SCF-Skp2 ubiquitin E3 ligase activity
- Coupled to EGFP for easy monitoring of the cellular translocation event
- Robust cell-based assay for use in high content analysis and fluorescence microscope applications

Highlights:

- **Biologically relevant data**
Compounds tested in a cellular environment
- **Validated**
Functionally tested cells provided with an optimized assay protocol
- **Easy to use**
Just plate cells, add compounds, and image

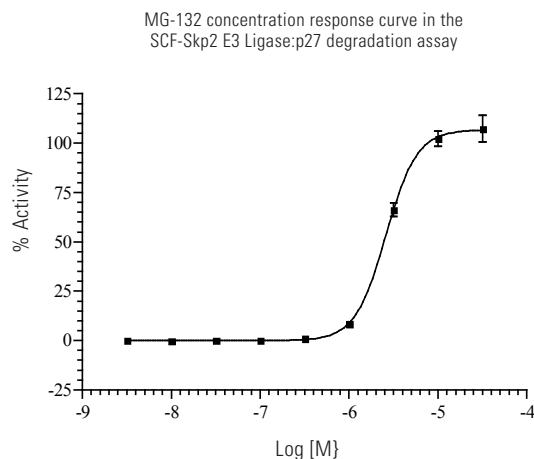


Figure 2. MG-132 concentration response in the SCF-Skp2 E3 Ligase:p27 degradation assay. The EC_{50} is $\sim 3 \mu\text{M}$. Concentration response was measured in 9 point half log dilution series ($n = 8$). Cells were treated with test compound for 24 hr. Cells were then fixed and increase in fluorescence intensity was measured using the Cellomics ArrayScan V^{TI} Reader and the MolecularTranslocationV2 BioApplication. % activity was calculated relative to the positive ($5 \mu\text{M}$ MG-132) and negative control (0.25% DMSO).

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Assay Details

Recombinant HeLa cells stably expressing human p27 containing a T187D mutation (p27Kip1(T187D)) fused to the N-terminus of enhanced green fluorescent protein (EGFP). In this assay, a constitutively degraded mutant of p27Kip1, p27Kip1(T187D) is fused to GFP and used as a reporter for SCF-Skp2 ubiquitin E3 ligase activity. Untreated cells will appear dark without green fluorescence while knockdown of Skp2 by siRNA transfection or inhibition of the proteasome by proteasome inhibitors, such as MG-132, leads to stabilization of p27Kip1(T187D)-GFP and thereby an increase in green fluorescence. MG-132 is used as reference compound and compounds are assayed for their ability to inhibit degradation of p27Kip1. Such compounds may be general proteasome inhibitors, direct inhibitors of the SCF-Skp2 ubiquitin E3 ligase activity, or potentially inhibitors of other E3 ligases relevant for p27Kip1 degradation. General proteasome inhibitors may be identified by running test compounds in the Redistribution Proteasome Assay. The SCF-Skp2 E3 Ligase: p27 degradation assay is validated with an average $Z' = 0.82 \pm 0.08$, suitable for both screening and profiling applications.

Imaging

The SCF-Skp2 E3 Ligase:p27 degradation Redistribution assay can be imaged on most HCS platforms and fluorescence microscopes. The filters should be set for Hoechst (350/461 nm) and GFP/FITC (488/509 nm) (wavelength for excitation and emission maxima). Consult the instrument manual for the correct filter settings. The translocation can typically be analyzed on images taken with a 10x objective or higher magnification. The primary output in the assay is the accumulation of p27Kip1(T187D)-EGFP. The data analysis should therefore report an output relating to GFP fluorescence intensity.

Imaging on Thermo Scientific Cellomics ArrayScan V^{TI}

This assay has been validated on the Cellomics Arrayscan V^{TI} using a 10x objective (0.63X coupler), XF100 filter sets for Hoechst and FITC, and the MolecularTranslocationV2 BioApplication. The output parameter used was MEAN_CircAvgInten. The minimally acceptable number of cells used for image analysis in each well was set to 400 cells. Other BioApplications that can be used for this assay include Redistribution V3 BioApplication, CompartmentalAnalysisV2, NucTransV2, and ColocalizationV3.

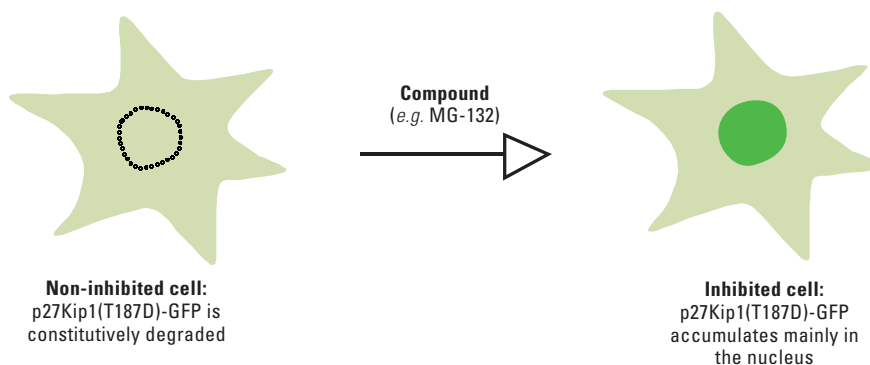


Figure 3. Illustration of the SCF-Skp2 E3 Ligase:p27 degradation translocation event.

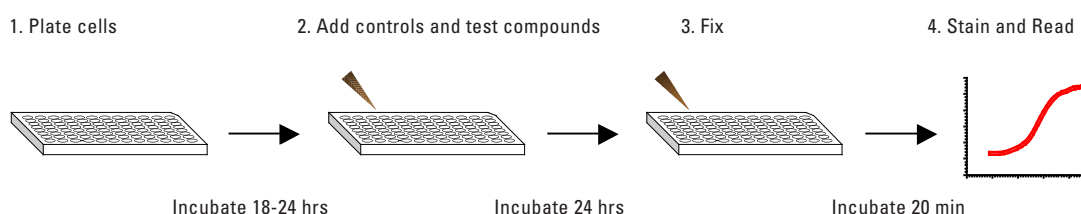


Figure 4. The SCF-Skp2 E3 Ligase:p27 degradation Redistribution assay is very easy and fast to perform.

Ordering Information

PRODUCT #	DESCRIPTION	CELL LINE	PROFILING	SCREENING	CRYOREDI
052_01	SCF-Skp2 E3 Ligase: p27 degradation Redistribution Assay	HeLa	•	•	

The Redistribution Assays are available in 3 product formats, Profiling, Screening and CryoRedi, for different volume and level of convenience needs. The Redistribution Assays can also be accessed through the Thermo Scientific Managed Services.

Related Thermo Scientific Products

PRODUCT #	DESCRIPTION	CELL LINE	PROFILING	SCREENING	CRYOREDI
021_02	Proteasome Redistribution Assay	U2OS	•	•	•
055_01	E6-AP E3 Ligase:p53 degradation Redistribution Assay	HeLa	•	•	
8407701	Cellomics Poly-Ubiquitin Detection HCS Reagent Kit	Antibody- and dye-based reagent kit			
8407801	Cellomics LC3B and Poly-Ubiquitin Detection HCS Reagent Kit	Antibody- and dye-based reagent kit			
8408001	Cellomics Multiparameter Cell Death Detection HCS Reagent Kit	Antibody- and dye-based reagent kit			
CX03004-INS	Cellomics ONE BioApplication Suite	High content data acquisition and analysis software			
CX03102A/B	Cellomics ArrayScan V ^{TI}	Flexible, high throughput, high content reader			
N01-3001	CellWoRx	Economical high content reader			

References

1. Glickman, M.H. et al. *Physiol. Rev.* 82: 373-428, 2002.
2. Carrano, A.C et al. *Nat. Cell Biol.* 1: 193-199, 1999.
3. Bloom-J et al. *Sem. in Cancer Biol.* 13: 41-47, 2003.

