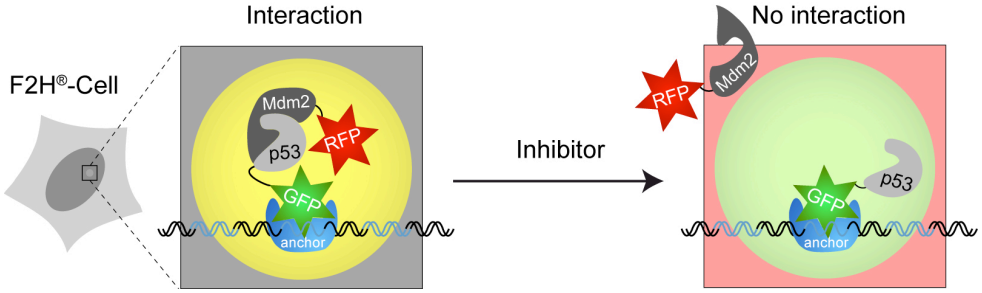
F2H®-p53/Mdm2 Kit for Live-Cell Analyses of Interaction between p53 and Mdm2 Proteins

A microscopy-based protein interaction assay for quantitative evaluation of p53/Mdm2-targeting treatments (e.g. screening for inhibitors).

Only for research applications, not for diagnostic or therapeutic use.

Introduction

The Fluorescent Two-Hybrid (F2H®) p53/Mdm2 Kit enables intracellular analysis of the interaction between the tumor suppressor p53 and its negative regulator Mdm2. In this assay, GFP-tagged human p53 (1-81 aa) is anchored at a specific location in the nucleus of F2H®-Cells, forming a bright fluorescent spot in the green channel. Interaction with the RFP-tagged human Mdm2 (7-134 aa) can be easily evaluated by conventional fluorescence microscopy as enrichment of red fluorescence at the location of the green spot. Compounds' ability to disrupt the p53/Mdm2 interaction is determined based on the disappearance of the red spots from the anchored green spots.



Content

Code	Reagent	Quantity
f2h-bhk	**F2H®-Cells** (F2H®-BHK Cell Line) genetically engineered BHK cells stably expressing components of the PPI-platform	**1 vial,** 5 X 10^6 frozen cells in FCS-free cryopreservation reagent PAA CryoMaxx
f2h-ppm2	**Platform-p53-Mdm2 (red cap)** transfection mixture containing plasmids pTagGFP2-p53(1-81aa) and pTagRFP-Mdm2(7-134aa), as well as anchoring components	**1 vial, 100 µL, 1 mg/mL,** for 125 transfections in 24-well format or 500 transfections in 96-well format
f2h-cp	**Control-p53 (yellow cap)** transfection mixture containing plasmids pTagGFP2-p53(1-81aa) and pTagRFP, as well as anchoring components (no interaction, low control)	**1 vial, 30 µL, 1 mg/mL,** for 37 transfections in 24-well format or 150 transfections in 96-well format

NOTE: Both Platform-p53-Mdm2 as well as Control-p53 mixtures are ready for transient transfection into F2H®-BHK cells with a DNA-transfection reagent of choice. The plasmid-to-anchor ratios within each mixture are validated for the best assay performance.

Stability and Storage

Shipped on dry ice. Upon receipt store frozen F2H®-Cells in -80°C (short term, days) or in liquid nitrogen (long term, months). Store Platform-p53-Mdm2 and Control-p53 at +4°C (short term) or in -20°C (long term).

All kit components are stable at least six months from the date of receipt if stored and handled correctly. We recommend to expand F2H®-BHK cells and to generate back-up aliquots (liquid nitrogen stocks).

Further Reagents Required

- Transfection reagent (e.g. Lipofectamine® 2000), complete growth medium (see protocol below), trypsin, DPBS.
- Test compounds and the reference compound Nutlin-3 (e.g. from Cayman Chemical).

Assay Principle

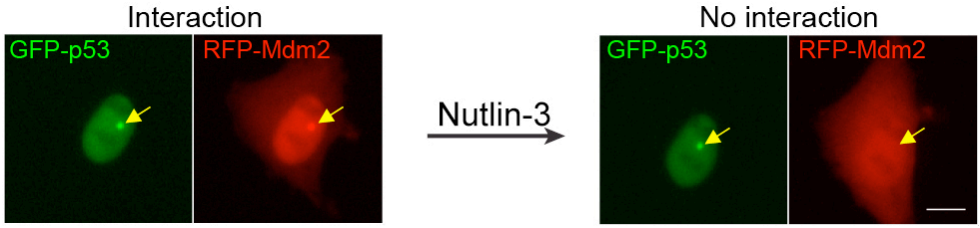
• F2H®-Cells express components of the protein-protein interaction platform which recruits GFP-tagged proteins to a specific location in the nucleus;

• GFP-tagged human p53 and RFP-tagged human Mdm2 (both supplied pre-mixed in Platform-p53-Mdm2 reagent) are co-transfected into F2H®-Cells;

• GFP-tagged p53 is anchored at the PPI platform, forming a bright green spot
- co-localizing **green** and **red** spot in the nucleus → interaction,
- only **green** spot (red is disperse) → no interaction.

Results: Analysis of Interactions

For visual inspection of the interactions we recommend using a conventional fluorescence microscope equipped with 20X and/or 40X objectives and standard filter sets for detection of GFP and RFP.



F2H®-Cells were transfected with Platform-p53-Mdm2 and subjected to live-cell imaging during Nutlin-3 treatment (5 µM). Before Nutlin-3 addition, both green and red spots co-localize (left image, interaction), whereas after 3 h incubation with Nutlin-3 the red spot is dispersed, while the green one remains intact (right image, no interaction). Scale bar, 10 µm.

Interactions can be analyzed manually or, alternatively, the samples can be imaged and subjected to quantitative image analysis with a software of choice

Related Products

Use our Nano-Traps® (e.g. p53-Trap®, GFP-Trap®, RFP-Trap®) for biochemical analyses of protein-protein interactions from cell lysates.

Support

Please contact *support@chromotek.com* or call +49 89 78797310.

Limited Use Label License

By use of these Products, you accept the terms and conditions of the applicable Limited Use Label License.

F2H®-p53/Mdm2 Kit: Protocol

Only for research applications, not for diagnostic or therapeutic use.

Part I: Culture F2H®-Cells

> Use aseptic technique for sterile handling of cell cultures when working with F2H®-Cells.

> F2H®-Cells are genetically modified Baby Hamster Kidney fibroblasts (BHK) and can be cultured according to standard protocols for maintenance of BHK cells.

Thaw F2H®-Cells

> Prepare complete growth medium (not provided) prior to thawing procedure:

Complete growth medium
• Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/L glucose, 110 mg/L sodium pyruvate and L-gluthamine • 10% Fetal bovine serum (FCS) • 50 µg/mL Gentamycin

> Remove frozen cells from storage and thaw quickly in +37°C water bath.

> **Immediately** upon thawing transfer thawed cells (~1.5 mL) into a Falcon tube containing 10 mL of complete growth medium. Mix gently and centrifuge at ~80 *g* for 3 min.

> Aspirate supernatant without disturbing the pellet.

> Gently resuspend pelleted cells in 1 mL of complete growth medium and transfer into a 100-mm cell culture dish, containing 10 mL of complete growth medium.

Note: To culture F2H®-Cells, 100-mm polystyrene cell culture dishes with standard tissue culture/gas plasma treated surface (Ref. 353003, Corning, USA) can be used.

Subcultivate F2H®-Cells

> Culture cells in a humidified +37°C, 5% CO_2 incubator. Check daily if cells are confluent. When confluent, subculture (split) by trypsinization as outlined below.

> Briefly wash cells with Dulbecco's Phosphate Buffered Saline (DPBS, 1X) without Ca & Mg supplemented with 0.5 mM EDTA.

> Aspirate and add 0.5 mL Trypsin/EDTA onto cells for ~3 min at 37°C.

Note: For trypsinization, 0.05% Trypsin / 0.02% EDTA in DPBS or HBSS, e.g. 1XTrypsin-EDTA Solution (T3924, Sigma, USA) can be used.

> When cells are loose, add 10 mL complete growth medium to the plate, resuspend gently. Use light microscopy to check that the cells are well resuspended. Plate trypsinized cells 1:3 – 1:30 (see table below):

Subcultivation ratio for F2H®-Cells (starting with confluent cultures)
• Plate cells 1:25-1:30 if to be kept longer in culture (at this splitting ratio cells should be subcultivated at least twice a week, maximum 25 passages recommended). • Plate cells 1:5 if to be transfected upon splitting (e.g. by reverse transfection, recommended for 96-well plates). • Plate cells 1:10 if to be transfected the next day (recommended for coverslips).

Tipp 1: Do not let the cells overgrow! Do not plate them too thin either (max. splitting ratio 1:30).

*Tipp 2: **Cell density is critical** for transfection efficiency! If transfecting BHKs for the first time, try several densities, e.g. 1:3, 1:6, 1:12. For reverse transfection, pre-splitting cells the day before (1:3 – 1:5) increases transfection efficiency.*

Part II: F2H®-Assay

On Day 1 F2H®-Cells are transfected.

On Day 2 the cells are incubated with test and reference compounds and interactions are analyzed.

Day 1:

Transfect F2H®-Cells

> Experimental design: As a general guideline, we recommend including the following conditions in your experiment: (1) high control, (2) reference compound, (3, 4) at least two concentrations of your test compound and (5) low control. This adds up to 5 wells in total, one condition per well:

Well	Condition	Transfection (Day 1)	Treatment (Day 2)
1	High control	Platform-p53-Mdm2	Untreated
2	Reference comp.	Platform-p53-Mdm2	5 µM Nutlin-3, 3 h
3	Test comp. conc. 1	Platform-p53-Mdm2	X* µM test comp, Y** h
4	Test comp. conc. 2	Platform-p53-Mdm2	10X* µM test comp, Y** h
5	Low control	Control-p53	Untreated

* X= 0.1 to 100 µM, ** Y = 3 - 8 h (more details in the section Day2)

> Transfect 4 wells of F2H®-Cells with **Platform-p53-Mdm2 (red cap)** and one well of F2H®-Cells with **Control-p53 (yellow cap)**. Use a reagent appropriate for DNA-transfection of BHK cells according to manufacturer's instructions. We recommend Lipofectamine® 2000 (Thermo Fisher Scientific., USA).

Example: Transfection in 24-well plate format.

Seed F2H®-Cells on coverslips one day in advance. In this experiment, 5 wells with cells are required. Cells should be ~25% confluent at the time of transfection. To transfect one well:

- *Pre-mix in one 1.5 mL tube:*
  - *50 µL DMEM (without FCS & Gentamycin) and*
  - *0.8 µL DNA (**Platform-p53-Mdm2** or **Control-p53**, each contains 1 µg/µL DNA).*
- *Pre-mix in another 1.5 mL:*
  - *50 µL DMEM (without FCS & Gentamycin) and*
  - *1.6 µL Lipofectamine® 2000.*
- *Combine the content of the tubes, mix gently.*
- *Let stand for 5-20 min at room temperature.*
- *Add dropwise into the well containing cells and media. Place the plate in the incubator.*

Note: To scale the transfections down to 96-well or up to 12-well formats, check the manufacturer's instructions for the transfection reagent you use.

Day 2:

Incubate with Compounds

> Check the transfection efficiency 16-24 h after transfection and treat the cells with the reference and test compounds.

> Reference compound: We recommend applying 5 µM Nutlin-3 for 3 h.

> Test compound: Working concentration and the best incubation time depend on the test compound and should often be determined empirically.

In general, the concentration can range from 0.1, 1, 10 up to 100 µM depending on the properties (IC_{50}, toxicity, solubility, etc.) of the compound. As a starting point, we recommend testing two concentrations which differ 10 times, e.g. 5 and 50 µM. For dose response analysis, several dilutions of the test compound can be evaluated in one experiment (one dilution per well).

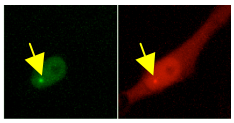
Incubation can be carried on for 3-8 h. For small molecule compounds, 3-4 h incubation is usually sufficient to observe a response; for peptidic inhibitors we recommend 8 h incubation.

When the working concentration of the test compound is determined, F2H® assay can be used for analysis of compounds' kinetics by subjecting cells to live imaging during incubation with the compound (e.g. image every 30 min from 0 up to 8 h).

Detect Interactions

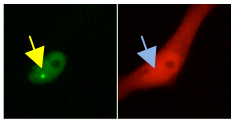
> Analyze interactions in living cells during compound treatment, or fix the cells after incubation before imaging. For better image quality, we recommend fixing cells with 4% formaldehyde in PBS (10 min at RT) and staining the nuclei, e.g. with DAPI.

Interaction:



> Use a 20X or 40X objective and standard filter sets for detection of GFP and RFP.
• Excitation/emission maxima for TagGFP2 are 483/506 nm.
• Excitation/emission maxima for TagRFP are 555/584 nm.
www.evrogen.com/products/TagFPs.shtml

No Interaction:



> Examine nuclei of co-transfected cells in green channel to find GFP-tagged p53 anchored at PPI-platforms → identify one, rarely two bright **green** spots per nucleus.

> Switch to the red channel and check for accumulations of the red fluorescence of RFP-tagged Mdm2 at the locations corresponding to the green spots:
• **Red** spot co-localizes with a **green** spot → **interaction**,
• No clearly distinguishable red spot standing out from the rest of the nucleus and nucleoli (the cell is co-tranfected and has a **green** spot) → **no interaction**.

When analyzing per visual inspection, you should evaluate at least 50 co-transfected cells (carrying a signal in green, a signal in red and a green spot). For automated analysis we recommend evaluating at least 100 co-transfected cells with a green spot.

*> **High control:** Co-localizing green and red spots should be detectable in ~90-95% of cells transfected with the Platform-p53-Mdm2 mixture.*

*> **Reference compound:** Co-localizing green and red spots should be reduced down to ~20-30 % in cells transfected with the Platform-p53-Mdm2 mixture.*

*> **Low control:** Co-localizing green and red spots should be detectable only in very few cells (~1-2%) transfected with the Control-p53 mixture.*

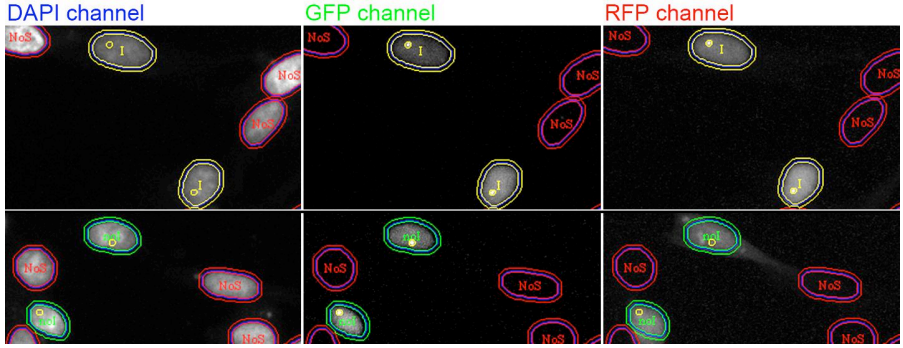
The table below shows an example of evaluation of interactions in one single experiment.

Normalized interactions, % = 100% X Interaction / (Interaction + NoInteraction)

Well	Condition	Evaluation, example		Result, example
		Cells with interaction	Cells with no interaction	Interactions normalized to number of analyzed cells, %
1	High control	45	5	100% X 45 / (45+5) = 90%
2	Reference comp.	10	40	100% X 10 / (10+40) = 20%
3	Test comp. conc. 1	?	?	?
4	Test comp. conc. 2	?	?	?
5	Low control	1	49	100% X 1 / (1+49) = 2%

Alternatively to manual analysis, the samples can be imaged and subjected to quantitative image analysis with a software of choice.

For automated cell-by-cell analysis, we recommend segmenting the nuclei (based on their size and shape in DAPI channel), identifying co-transfected cells (based on the intensities in green and red channels) and identifying spots in the nuclei in green channel. Only co-transfected cells carrying a green spot should be further evaluated for co-localization of red and green spots.



Above are exemplary screen-shots of interaction quantification with IN Cell Analyzer 1000 Workstation 3.5 (GE Healthcare). I - interaction, noI – no Interaction, NoS – no green spot.

Note: This protocol is just a general guideline; the optimal experimental design (test compound concentrations, incubation time, number of replicates) should be determined by the scientist according to the study requirements