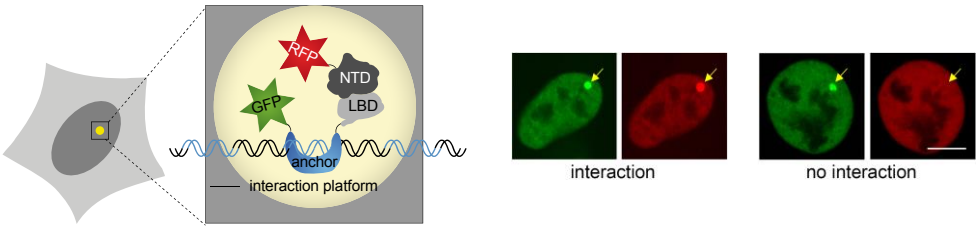
F2H®-Kit AR-N/C for Live-Cell Analyses of Interactions between N- and C-Termini of the Androgen Receptor

A microscopy-based assay for quantitative evaluation of the effects of chemical compounds on AR N/C interaction (e.g. screening for inhibitors)

Only for research applications, not for diagnostic or therapeutic use

Introduction

The Fluorescent Two-Hybrid (F2H®) Androgen Receptor Kits enable intracellular analyses of interactions between the N- and C-termini of human Androgen Receptor (AR). In this assays, GFP-tagged Ligand Binding Domain (LBD) of human AR (669-919 aa, *wt* or mut) 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 N-Terminal Domain (NTD) of human AR (1-558 aa) can be easily evaluated by conventional fluorescence microscopy as enrichment of red fluorescence at the location of the green spot.



Content

Code	Reagent	Quantity
f2h-bhk	**F2H®-Cells** (F2H®-BHK Cell Line) genetically engineered BHK cells stably expressing components of the protein-protein interaction platform (PPI Platform)	**1 vial** 5×10^6 frozen cells in serum-free cryopreservation medium (Waymouth formulation)
f2h-ar-wt	**Platform-ARLBDwt-ARNTD** DNA mix containing plasmids pTagGFP2-LBD (669-919 aa, *wt*) and pTagRFP-NTD (1-558 aa), as well as anchoring sequences for the GFP-tagged protein	**1 vial, 100 µL, 1 µg/µL** for 125 transfections in 24-well format or 500 transfections in 96-well format
f2h-ca	**Positive Control** (yellow cap) DNA mix containing plasmids coding for interacting TagGFP2- and TagRFP-fusion proteins, as well as anchoring components	**1 vial, 30 µL, 1 µg/µL** for 37 transfections in 24-well format or 150 transfections in 96-well format

NOTE: Mutant AR variants can be analyzed with the Platform Reagents f2h-ar-w741l, f2h-ar-f876l, f2h-ar-t877a, f2h-ar-f876l/t877a, which can be used instead of the wt Platform Reagent f2h-ar-wt. Both Platform-ARLBDwt/mut-ARNTD, as well as Positive Control mixes 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 DNA mixes f2h-ar-*wt*/mut and f2h-ca at +4°C (short term, days) or in -20°C (long term, months).

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 agonists (e.g. DHT)

Assay Principle

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

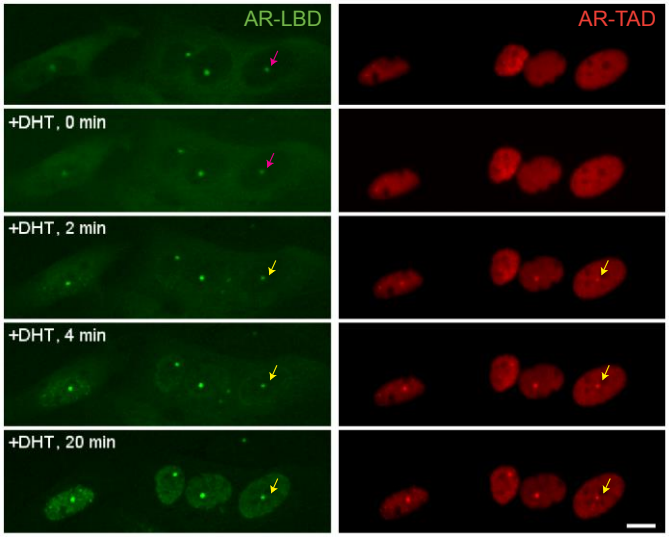
• GFP-tagged LBD (*wt* or mut) of human AR and RFP-tagged NTD of human AR (both supplied pre-mixed in f2h-ar-*wt*/mut reagents) are co-transfected into F2H®-Cells

• GFP-tagged LBD (*wt* or mut) 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 20× and/or 40× objectives and standard filter sets for detection of GFP and RFP.



F2H®-Cells were transfected with Platform-LBDwt-NTD and subjected to live-cell imaging during DHT treatment (10 nM). Before DHT addition, green spots are present, but there are no red spots (upper row, no interaction). Within 20 min incubation with 10 nM DHT red spots appear at the positions corresponding to the green spots (bottom row, 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® (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.

F2H®-Kit AR-N/C: Protocol

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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 thawn 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 transfected cells are incubated with test and reference compounds and interactions are analyzed.

Day 1:

Transfect F2H®-Cells

> Use a reagent appropriate for DNA-transfection of BHK cells according to manufacturer's instructions. We recommend Lipofectamine® 2000 (Thermo Fisher Scientific., USA). All DNA mixes in the Kit are supplied at concentration 1 µg/µL.

> To analyze N/C interactions of the *wt* AR, transfect F2H®-Cells with the DNA mix **f2h-ar-wt**.

> To analyze N/C interactions of the AR carrying a specific mutation (for example, T877A), transfect F2H®-Cells with the DNA mix **f2h-ar-(mut)** (in this case**, f2h-ar-t877a**).

> To have a reference for visualization of protein-protein interactions with F2H®, transfect F2H®-Cells with the Positive Control DNA **f2h-ca**.

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

Day 2:

Incubate with Compounds

> Test compounds: Working concentration and incubation time depend on the test compound. For a start, we recommend testing a range of concentrations from 1 to 50 µM.

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

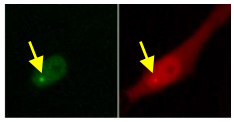
> Induce interactions: We recommend applying 0.25 nM DHT or higher for at least 2 h.

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.

> Use a 20× or 40× 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

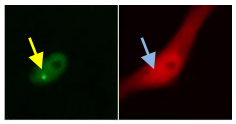
Interaction:



> Examine nuclei of co-transfected cells in green channel to find GFP-tagged AR-LBD 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 AR-NTD at the locations corresponding to the green spots:
• **Red** spot co-localizes with a **green** spot → **interaction**,
• A cell is co-transfected and has a **green** spot, but no clear red spot standing out from the rest of the nucleus and nucleoli can be distinguished → **no interaction**.

No Interaction:



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

*> **Positive Control:** Co-localizing green and red spots should be detectable in ~90-95% of cells transfected with the **f2h-ca** mix.*