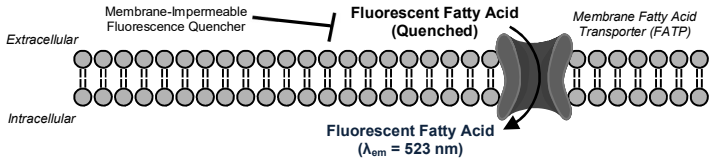
Fatty Acid Uptake Assay Kit (Cell-Based)

(Catalog # BN00661; 100 Reactions; Store at -20°C)

I. Introduction:

Fatty acids are essential dietary nutrients consisting of a carboxylic acid moiety linked to a long aliphatic hydrocarbon chain. They are the fundamental building blocks of more complex lipids such as those that form cellular membranes. In the blood, long-chain fatty acids travel bound to serum albumin and are transported into cells by members of the SLC27 family of transmembrane transporter proteins. Uptake of long-chain free fatty acids into adipocytes is essential for regulating circulating lipid levels and cellular lipid metabolism. Abnormalities in adipocyte fatty acid uptake and lipid metabolism are major contributing factors to obesity, diabetes/metabolic syndrome, cardiovascular disorders and nonalcoholic fatty liver disease (NAFLD). As diet and exercise consistently fail to achieve any lasting results in combating obesity, pharmacological targeting of fatty acid uptake is being investigated as a potential treatment strategy. Assay Genie's Fatty Acid Uptake Assay Kit enables rapid determination of real-time fatty acid uptake kinetics in live cells expressing fatty acid transporter proteins. The assay uses a highly fluorescent long-chain fatty acid analogue (Ex/Em = 488/523 nm) that acts as a substrate for transmembrane fatty acid transporters (FATPs) and accumulates in intracellular lipid droplets. A proprietary non-toxic membrane-impermeable quenching agent eliminates any fluorescence arising from the extracellular space, ensuring specific measurement of intracellular fatty acid accumulation without requiring any tedious wash steps. The assay is highly sensitive, has a simple "mix-and-read" protocol and is high-throughput adaptable for screening of fatty acid uptake-modulating test compounds or treatment conditions. The kit contains a complete set of reagents sufficient for performing 100 reactions in a 96-well plate format.



II. Applications:

- Characterization of fatty acid uptake kinetics in live cells.
- Screening and characterization of drugs and test compounds for modulation of fatty acid uptake.

III. Sample Type:

- Adherent cells expressing transmembrane fatty acid transporters (*e.g.* differentiated 3T3-L1 or 3T3-F442A adipocytes)

IV. Kit Contents:

Components	BN00661	Cap Code	Part Number
Uptake Assay Buffer	25 ml	NM	BN00661-1
Extracellular Quenching Solution (100X)	200 µl	Amber	BN00661-2
Fluorescent Fatty Acid Probe (200X)	100 µl	Red	BN00661-3

V. User Supplied Reagents and Equipment:

- Cell line for testing: cells that express membrane fatty acid transporter proteins (differentiated 3T3-L1 adipocytes or heterologous cells stably transfected with desired FATP(s))
- Serum-free/phenol red-free cell culture medium and 5% CO_2 cell culture incubator
- Multiwell fluorescence microplate reader (capable of bottom read)
- Black-walled 96-well tissue-culture plates with clear flat bottom wells

VI. Storage Conditions and Reagent Preparation:

Store kit at -20°C and protect from light. Briefly centrifuge all small vials prior to opening. Open all of the reagents under sterile conditions (*e.g.* a cell culture hood) only. Read entire protocol before performing the assay procedure.

- **Uptake Assay Buffer:** Allow to thaw to room temperature and open under sterile conditions. Store at 4°C.
- **Extracellular Quenching Solution (100X):** Aliquot the stock solution as desired and store aliquots at -20°C, **protected from light**. Stable for at least 4 freeze/thaw cycles.
- **Fluorescent Fatty Acid Probe (200X):** Aliquot the stock solution as desired and store aliquots at -20°C, **protected from light**. Avoid repeated freeze/thaw cycles.

VII. Fatty Acid Uptake Assay Protocol:

The procedure described below employs differentiated 3T3-L1 adipocytes as a model cell line for measuring fatty acid uptake. Undifferentiated 3T3-L1 cells (ATCC CL-173) display a fibroblast-like morphology but become rounded and accumulate lipid droplets several days after the initiation of differentiation. Primary adipocytes derived from tissues or other cell lines known to import long-chain fatty acids (such as Caco-2 or HepG2 cells) may also be used if desired.

1. 3T3-L1 Adipocyte Differentiation and Seeding:

a. Grow 3T3-L1 fibroblasts in preadipocyte growth medium (DMEM with 10% BCS) until cells are maximally confluent (>95%). At two days post-confluence, replace preadipocyte medium with differentiation medium (DMEM/F12 with 10% FBS) containing a cocktail of induction agents. Incubate cells for 3 days at 37°C in a humidified cell culture incubator with a 5% CO_2 atmosphere, then replace differentiation medium with maintenance medium (DMEM/F12 with 10% FBS, supplemented with 1.5 µg/ml insulin). Change maintenance medium every 2-3 days, until at least 90% of the cells exhibit an adipocyte-like morphology, with intracellular lipid droplets visible by brightfield microscopy (typically 7-10 days after initiation of differentiation). Once 3T3-L1 adipocytes are fully differentiated, the insulin-supplemented medium should be replaced with standard DMEM/F12 with 10% FBS.

b. Trypsinize 3T3-L1 adipocytes the night prior to assay and seed approximately 7-8 x 10^4 cells/well in an black-walled 96-well tissue culture plate (with flat, clear-bottom wells) using 200 µl culture media (DMEM/F12 with 10% FBS) per well. Grow cells overnight in a 5% CO_2 atmosphere 37°C incubator (adherent cell monolayer should be ≈80-90% confluent for optimal assay).

Notes:

- Differentiated 3T3-L1 adipocytes are easily ruptured in response to mechanical shearing during trypsinization and seeding. For best results, handle cells gently and avoid excessive mechanical agitation or intense, vigorous pipetting force.
- If desired, 3T3-L1 adipocytes may be seeded in the 96-well plate on the day of the fatty acid uptake assay rather than the night before. Seed 7-8 x 10^4 cells per well in 200 µl culture media and incubate at 37°C in 5% CO_2 atmosphere for 5 hours. If seeding cells on the day of the assay, we recommend using plates coated with an adhesion-promoting polymer such as poly-D-lysine.

2. Serum Starvation and Uptake Assay Reaction Preparation:

a. Warm Uptake Assay Buffer to 37°C. Remove plate from the incubator. Prepare background control (no-cells) well(s) by adding 90 µl of serum-free, phenol red-free medium to blank well(s). For sample wells (with cells), gently aspirate growth medium from wells and replace with 90 µl of serum-free, phenol red-free medium. Return cells to CO_2 incubator and incubate at 37°C for 1 hour.

Note: To minimize the loss of the cells during medium exchange, we recommend centrifuging the plate in a 96-well plate-compatible centrifuge at 800 x *g* for 3 min **with the brake function turned off** prior to aspiration of growth medium.

b. Dissolve fatty acid uptake-modulating test compounds in proper solvent(s) to produce stock solutions. For each test compound, prepare a 10X working solution of each desired test concentration by diluting stock solutions in serum-free, phenol red-free medium. To determine IC_{50} / EC_{50} values for test compounds, 10X working solutions should be prepared in a range of concentrations in order to generate a multi-point dose-response curve. *If an organic solvent is used to dissolve test compounds, the concentration of organic solvent should be the same for all test compound dilutions.*

c. Following serum starvation, remove cells from incubator and treat cells by adding 10 µl of either 10X test compound working solution to designated wells. For vehicle control and blank wells, add 10 µl of serum-free, phenol red-free medium (with the same final concentration of organic solvent used to solubilize test ligands, if applicable). Return plate to 5% CO_2 atmosphere incubator and incubate treated cells at 37°C for 30 min (or desired time based upon your specific treatment conditions and protocol).

d. Prepare a 2X solution of quenched Uptake Reaction Mix by adding 200 µl of the 100X Extracellular Quenching Solution stock and 100 µl of the 200X Fluorescent Fatty Acid Probe to 9.7 ml pre-warmed Uptake Assay Buffer. This preparation yields 10 ml of 2X Uptake Reaction Mix, which is sufficient for 100 reaction wells, but can be scaled depending upon the number of reactions to be performed. Keep the 2X Uptake Reaction Mix protected from light and warmed at 37°C until use.

3. Measurement: Remove plate from cell culture incubator and transfer to microplate reader. Add 100 µl of prewarmed 2X Uptake Reaction Mix (**see step 2d**) to all wells, including blank wells (for a final volume of 200 µl per well). Immediately begin measuring the fluorescence (Ex/Em = 488/523 nm) of all of the wells in kinetic mode at 37°C for 60 min **using the 'bottom read' function.**

Note: While we recommend reading in kinetic mode, the assay may also be performed in endpoint mode. Following addition of Uptake Reaction Mix, incubate the plate at 37°C for 60 min (protected from light), then measure endpoint fluorescence in bottom-read mode.

4. Calculation: For each test compound or treatment condition, quantify the relative stimulation/inhibition of fatty acid uptake versus vehicle control using the equation below, where $\boldsymbol{RFU_{vehicle}}$ is the fluorescence intensity of the vehicle control condition, $\boldsymbol{RFU_{treatment}}$ is the fluorescence intensity of the treated well and $\boldsymbol{RFU_{blank}}$ is the fluorescence intensity of the background control (no cells) well:

$$\% \textbf{ Activity} = \left(\frac{RFU_{treatment} - RFU_{blank}}{RFU_{vehicle} - RFU_{blank}}\right) \times \mathbf{100}$$

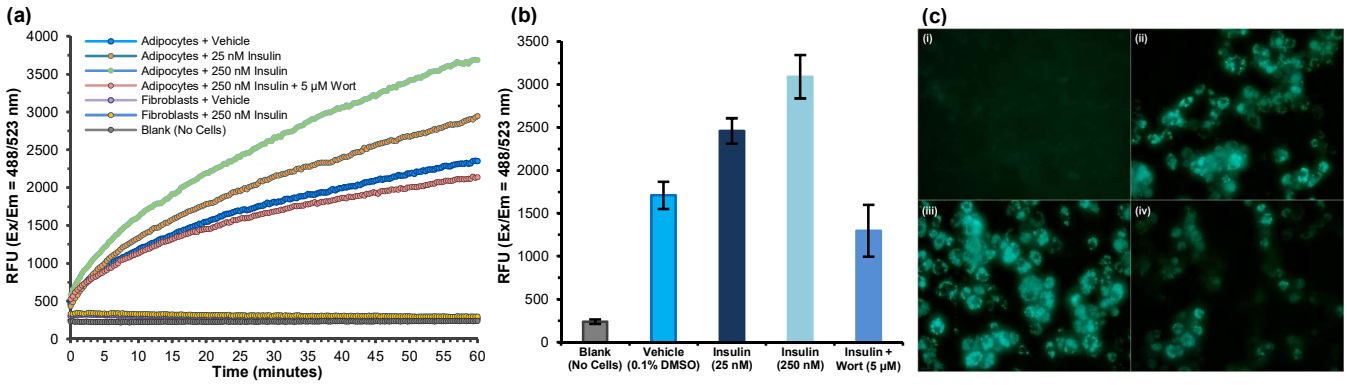


Figure: (a) Kinetics of fatty acid uptake in serum-starved 3T3-L1 fibroblasts and differentiated 3T3-L1 adipocytes treated with insulin (25 and 250 nM) or insulin plus wortmannin (250 nM + 5 µM), a PI3-Kinase inhibitor that blocks insulin signaling pathways. **(b)** Endpoint fluorescence in 3T3-L1 adipocytes in the presence of ascending doses of insulin (0, 25 and 250 nM), as well as a mixture of insulin and wortmannin (250 nM + 5 µM). Fluorescence was measured 60 minutes after the addition of Uptake Reaction Mix. Data are mean RFU ± SEM of 4 independent plates, with each data point representing the mean of quadruplicate wells. **(c)** Fluorescence imaging showing the extent of fluorescent fatty acid analogue uptake after 60 minutes. **(i)** undifferentiated 3T3-L1 fibroblasts, **(ii-iv)** differentiated 3T3-L1 adipocytes pretreated with **(ii)** vehicle (medium w/ 0.1% DMSO), **(iii)** 250 nM insulin or **(iv)** 250 nM insulin and 5 µM wortmannin. Fluorescence images were obtained with a Nikon TE2000 inverted microscope using a 20X Plan Fluor objective and a FITC filter cube.

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