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Cell-based RAF1 Functional Assay: RAF1/β-arrestin Interaction LinkLight™ U2OS Cells

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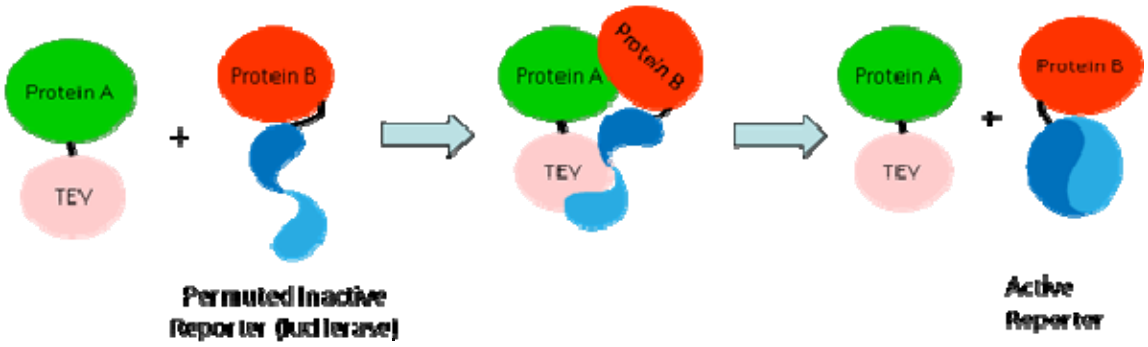
1 Product Description

Product Code: 5002-S
Product Description: **RAF1 and β-arrestin-2 LinkLight™ U2OS cells**
Human RAF1 (MAP3K, c-Raf) gene: GeneBank ID_NM_002880.3 (Consensus CDS: CCDS2612.1)
Human β-arrestin-2 gene: GeneBank ID_NM_004313.3 (Consensus CDS: CCDS11050.1)
TEV protease (Tobacco tech protease): Gene ID_1502321 (modified sequence, proprietary information)
Permuted Firefly Luciferase: (modified proprietary firefly luciferase sequence)
Host cells: U2OS (ATCC number HTB-96) Osteosarcoma; Bone sarcoma from the tibia of a human female

2 Technology Principle

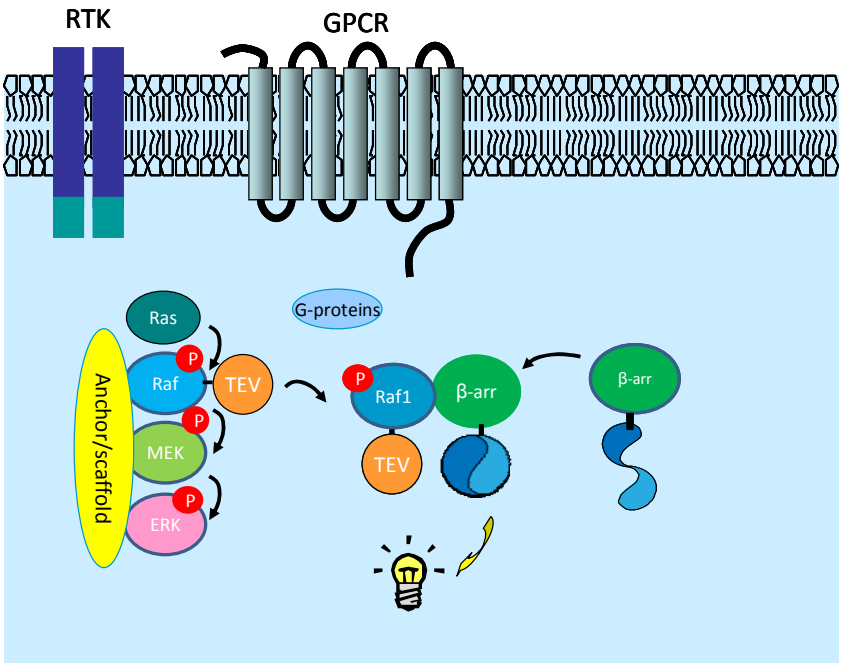
LinkLight™ technology[1-2] consists of two components: an inactive permuted luciferase (pLuc) containing a Tobacco Etch Virus (TEV) protease cleavage site fused to a protein B and the TEV protease fused to a protein A. Upon interaction between protein A and protein B, the inactive permuted luciferase is cleaved by the TEV protease, the cleaved luciferase fragments spontaneously refold, and active luciferase is reconstituted. For transient protein-protein interaction events, even the interaction partners are separated, the interaction signal is stably generated. Highly sensitive luminescent signals produced by the reconstituted, active luciferase can be sensitively and cost-effectively detected.



3 Potential Applications

Both GPCRs (G-protein coupled receptors) and RTKs (receptor tyrosine kinases) can activate RAF1. Upon on activation, RAF1 is released from the RAF/MEK/ERK signal complex. The liberated, phosphorylated Raf1 then binds to other cellular proteins. Depending on the binding partners, activated RAF1 translocate to various subcellular locations, and phosphorylate a subset of RAF1 substrates. The interaction of RAF1 and β-arrestin-2 is kinase activity dependent. The activated RAF1/ β-arrestin-2 complex remains in the cytoplasm.

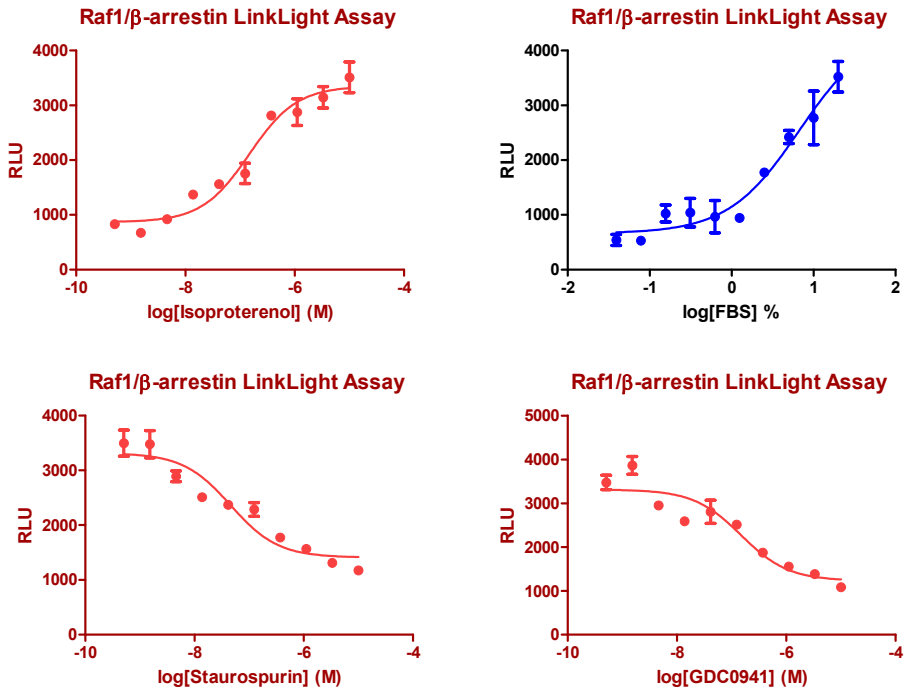
The RAF1/β-arrestin-2 LinkLight™ U2OS cells can be used to assess the RAF1 kinase activity. Molecules modulate RAF1 and β-arrestin-2 functional interaction trough cell surface receptors could be identified. Potentially, allosteric modulators, RAF1 ATP-non-competitive inhibitors and RAF1 upstream kinase inhibitors could also be identified by the RAF1 and β-arrestin-2 LinkLight™ assays.



Protein-protein interactions are dynamically regulated by reversible post-translational modifications (PTM) such as phosphorylation and dephosphorylation. The dynamic balance between phosphorylation and dephosphorylation mediates cell physiology. However, the transient protein-protein interactions that are especially sought for therapeutic intervention are not easy to be captured or characterized in the dynamic cellular environment. The irreversible feature of LinkLight™ assays can be very useful for assessing dynamic, time and physiological sensitive protein interaction events.

Signal adaptors such as beta-arrestins[3-4] are multi-functional proteins that lack intrinsic enzymatic activity but link protein-binding partners together and facilitate the formation of signaling complexes. The interactions mediate a remarkable range of cellular activities. Signal adaptor proteins may be considered as general switch proteins whose function depends on the client protein.

U2OS cells are known to contain endogenous RTKs such as HGFR, PDGFR and FGFR. Fetal bovine sera (FBS) contain many growth factors to activate RTKs. Fetal bovine serum (FBS) treatments of Raf1/β-arrestin LinkLight cells produced dose-dependent luciferase signals. Pan-kinase inhibitor staurosporine and PI3K/AKT pathway inhibitor GDC0941 can block RAF1/β-arresin-2 interaction signals, whereas RAF1 downstream kinase ERK inhibitor CI-1040 dose not block the signals (data not shown). U2OS cells contain endogenous ADRB2 receptor.



ADRB2 agonist Isoproterenol treatment generated dose-dependent β-arrestin-mediated RAF1 activation signals.

By exogenous expression of your interested GPCRs or RTKs in the RAF1/β-arrestin-2 LinkLight™ U2OS cells, molecules modulate receptor activities could also be assessed through the RAF1 and β-arrestin-2 interaction.

4 Materials Provided & Storage Conditions.

One cryovial containing ~2x10e6 cells each in 1 mL's of freezing media is shipped on dry ice. Upon receipt, cells should be transferred to liquid nitrogen for storage beyond 24 hours. If cells will be thawed and used within 24 hours, they can be stored temporarily at -80°C.

5 Materials & Equipment required (not provided)

• Cell culture plates/flasks, pipettes and pipette tips
• 384-well or 96-well microplates (Tissue culture treated, sterile, white-walled with solid white or clear bottom, example: BD Falcon Cat.# 353286)
• Luminescence plate reader (EnVision, EnSpire equipped with ultra sensitive luminescent detector, TopCount, or other ultra sensitive luminescent readers)

• LinkLight™ Detection Reagent: ONE-Glo™ , Bight-Glo, Steady-Glo (Promega Cat. No. E6110, E61120, E61130, E2650, E2550) or NeoLite (Perkin Elmer)
• Test compounds
• Growth medium McCoy's 5A (Invitrogen, Cat. #16600) and supplements for cells culture

6 Cell Culture Procedures

6.1. Rapidly thaw the vial of cells by placing at 37°C in a water bath with gentle agitation for 1–2 minutes. Do not submerge vial in water.
6.2. Decontaminate the vial by wiping with 70% ethanol before opening in a Class II biological safety cabinet.
6.3. Transfer the vial contents drop-wise into 10 mL of culture medium in a sterile 15-mL conical tube.
6.4. Centrifuge cells at 200 × *g* for 5 minutes.
5.5. Aspirate supernatant and resuspend the cell pellet in 1 mL fresh McCoy's 5A culture media.
6.6. Count the cells and adjust cells density with culture media according assay procedure.
6.7 ERK2 and β-arrestin-2 LinkLight™ U2OS cell Culture Media:

- McCoy's 5A (Invitrogen, Cat. #16600)
- 10% FBS (Invitrogen/BRL, Cat. #10082-147)
- 1X Pen/Strep (Invitrogen/BRL, Cat. #10378-016)
- G418 (Geneticin) 400 µg/ml (Invitrogen/Life Technology, Cat. #10131-027)
- Hygromycin B 200 µg/ml (Invitrogen/ Life Technology, Cat. #10687-010)

6.8 Change the culture media every 3~4 days until 90% confluent. Split the cells by washing one time with PBS (no Ca^{2+}, Mg^{2+}) and adding 0.05% Typsin-EDTA (GIBCO # 25300) and allow the cells to incubate in room temperature until cells detach, add new culture media and transfer viable cells (1: 6 split) to a new culture vessel and place in a 37ºC incubator at 5% CO2.

7 Assay Procedure

The following outlines the procedure for performing the LinkLight™ assay in 384-well plate format (BD Falcon 384-well plate Cat.# 353286).
7.1 Seed 10,000 to 15,000 cells per well with 40 µL culture media without G418 and hygromycin B, culture cells in a humidified 37°C, 5% CO2 incubator for over 16 hours.
7.2 Gently replace with 20 µL McCoy's 5A media without FBS and antibiotics.
7.2 Add 5 µL ligand/compound to each well, incubate 90~120 minutes in a humidified 37°C, 5% CO2 incubator.
7.3 After incubation, take the plate out and equalize to room temperature for 10 to 15 min.
7.3 Add 25 µL detection reagent ONE-Glo™ reagent (Promega, Cat. No. E6110, E61120, E61130) to each well, read luminescent light on a luminescence plate reader. Alternatively, you can dump off the media, add 15 µL detection reagent, and record the luminescence.

8 Guidelines for Use

This LinkLight™ Cell Line is engineered for expression of TEV tag fused to the target protein, human Raf1 and β-arrestin-2 with a permuted luciferase. Please refer to the

Detection Kit product insert for information on how to prepare the reagents for use. Cells should be cultured according to standard procedures for tissue culture. DMSO concentration should be kept below 1%. The Raf1and β-arrestin-2 LinkLight cells can be frozen with 20%FBS with 10% DMSO according to standard cell freezing procedures.

9 Tips for Optimal Performance

- Cells density and viability influence S/B ratio.
- Serial dilutions of compound stock are recommended to maintain DMSO at < 1%.
- Use serum-free media for the LinkLight™ assay.
- Ligand/compound incubation time can be adjusted within 60 to 120 minuets.
- Use clear bottom plate to observe cell growth condition, if you have doubt of cell health condition.
- Detection reagents should be prepared just prior to use and are light sensitive.
- Optimal signal is generated within 2 to 10 minutes incubation in detection reagents.
- Adjust luminescence plate reader to appropriate sensitivity mode.
- High signal can be obtained by aspirating out media and adding 15 µL LinkLight™ detection reagent, be careful not to suck the cells out.

10 Limited Use License Agreement

The cells (collectively Materials) purchased from BioInvenu are expressly restricted in their use. BioInvenu has developed LinkLight assay (Assay) that employs genetically modified cells and vectors (collectively, the “Cells”). By purchasing and using the Materials, the Purchaser agrees to comply with the following terms and conditions of this label license and recognizes and agrees to such restrictions:

10.1. Purchaser is permitted only for use in the Assay and in connection with Reagents purchased from BioInvenu Corporation or its authorized distributor.

10.2. The Materials are not transferable and will be used only at the site for which they were purchased. Transfer to another site owned by Purchaser will be permitted only upon written request by Purchaser followed by subsequent written approval by BioInvenu.

10.3. The Reagents are developed on the patented LinkLight technology and contain the proprietary and valuable know-how developed by BioInvenu. Purchaser will not analyze or reverse engineer the Materials nor have them analyzed on Purchaser’s behalf.

10.4. In performing the Assay, Purchaser will use only Reagents supplied by BioInvenu or an authorized BioInvenu distributor for the Materials.

10.5. Purchaser will not use the Cells with any other reagents or substrates, other than the Reagents that are provided by or purchased from BioInvenu or an authorized BioInvenu distributor, in connection with the Materials.

10.6. Purchaser will not transfer the Cells to a third Party.

10.7. Purchaser will not use the Cells for customer services.

10.8 . The LinkLight cells are used for Researches only.

If the purchaser has any further questions regarding the rights conferred with purchase of the Materials, please contact:

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References:

1 Eishingdrelo H, et al. (2011). A cell-based protein-protein interaction method using a permuted luciferase reporter. Current. Chem. Genomics 5:122-128.
2 Eishingdrelo H, et al. (2014) ERK and β-arrestin interaction: a converging-point of signaling pathways for multiple types of cell-surface receptors. J. Biomolecular Screen (DOI: 10.1177/1087057114557233).
3 J. J. Kovacs, M. R. Hara1, C. L. Davenport, J. Kim, and R. J. Lefkowitz. (2009) Arrestin Development: Emerging Roles for β-arrestins in Developmental Signaling Pathways. Dev Cell. 17: 443–458.
4 E. G. Strungs, L. M. Luttrell. (2014) Arrestin-Dependent Activation of ERK and Src Family Kinases. Handbook of Experimental Pharmacology Volume 219: 225-257.