Description

The Dcaf11-driven p21 ubiquitination assay kit is a sensitive AlphaLisa® high-throughput screening (HTS) assay kit, designed to measure Dcaf11 (DDB1- and Cul4-associated factor 11) E3 ligase activity in a homogeneous 384 reaction format. The assay kit comes with enough biotinylated ubiquitin, ATP, GST-tagged p21, assay buffer, detection buffer, purified UBE1 (E1), UbcH5b (E2), and Rbx1/Cul4B/Dcaf11/Ddb1 complex (E3) for 384 reactions. The assay detects mono-ubiquitination and poly-ubiquitination of p21.

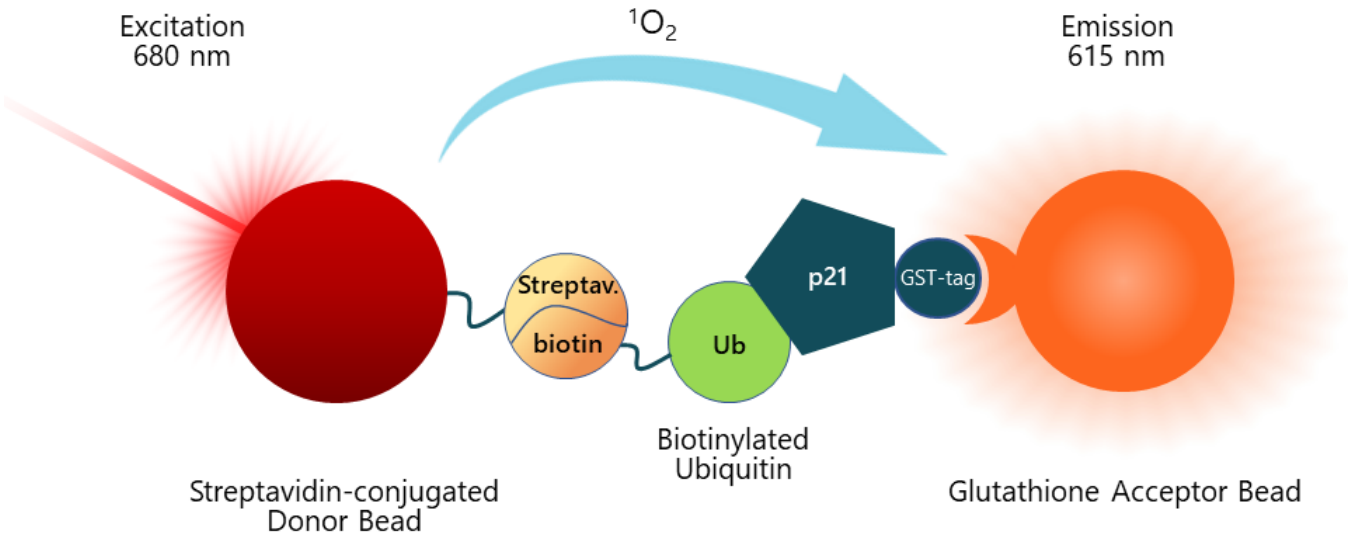

Figure 1. Dcaf11-driven p21 ubiquitination assay kit schematic.

First, E1 and E2 enzymes are incubated with the E3 complex and with the target protein p21 (GST-tag) in the presence of biotin-conjugated ubiquitin and ATP. Ubiquitination of p21 occurs in a multistep ubiquitin transfer from E1 to E2 to E3, and E3-mediated conjugation of ubiquitin to p21. Next, glutathione-acceptor beads are added, followed by streptavidin-conjugated donor beads, and Alpha-counts are measured. The increase in Alpha-counts is proportional to the mono- or poly-ubiquitination of the GST-tagged p21.

Background

Covalent conjugation to ubiquitin (Ub) is one of the major post-translational modifications regulating protein stability, function, and localization. Ubiquitination is the concerted action of three enzymes: a Ub-activating enzyme (E1), a Ub-conjugating enzyme (E2), and a Ub ligase (E3). The specificity and efficiency of ubiquitination are largely determined by the E3 enzyme, which directs the last step of the Ub-conjugating cascade by binding to both an E2~Ub conjugate and a substrate protein. This step ensures the transfer of Ub from E2~Ub to the substrate, leading to its mono- or poly-ubiquitination.

DDB1 and CUL4-associated Factor 11 (Dcaf11, also known as WD Repeat Domain 23, WDR23) is a protein that associates with Cul4A or Cul4B to form a complex with E3 ligase activity. Dcaf11 E3 ligase can ubiquitinate a variety of substrates, including p21 (cyclin-dependent kinase inhibitor 1 or CDK-interacting protein 1). P21 is involved in cell cycle regulation by inhibiting CDK (cyclin-dependent kinase) and PCNA (proliferating cell nuclear antigen). It also mediates p53 response to DNA damage, and its levels have been linked to lymph node metastasis and overall survival rate to cancer. Knocking down any component of the Dcaf11 complex attenuates the ubiquitination level of p21, inhibiting osteosarcoma cell proliferation. The use of drugs targeting the Dcaf complex may be a viable cancer therapy approach.

Applications

- Screen molecules that inhibit Dcaf11 Ub ligase activity in drug discovery high throughput screening (HTS) applications.
- Determine compound IC_{50} values.
- Analyze Dcaf11-driven ubiquitination.

Supplied Materials

Catalog #	Name	Amount	Storage
80301	UBE1 (UBA1), Flag-Tag Recombinant*	25 µg	-80°C
80314	UbcH5b, His-Tag (Human) Recombinant*	50 µg	-80°C
101495	Rbx/Cul4B/Dcaf11/Ddb1 Complex*	5 µg	-80°C
101584	p21, GST-Tag*	5 µg	-80°C
	Biotin-Ubiquitin	400 µl	-80°C
	ATP (10 mM)	400 µl	-80°C
	U2 Assay Buffer	2 x 10 ml	-80°C
	4x U2 Detection Buffer	2 x 2 ml	-20°C

**The initial concentration of enzyme is lot-specific and will be indicated on the tube containing the protein.*

Materials Required but Not Supplied

Name	Catalog #
AlphaLISA® glutathione acceptor beads	Perkin Elmer #AL109C
AlphaScreen® Streptavidin-conjugated donor beads	Perkin Elmer #6760002S
Optiplate-384	Perkin Elmer #6007290
AlphaScreen® microplate reader	
DNAse free water	

Storage Conditions

This assay kit will perform optimally for up to **6 months** from date of receipt when the materials are stored as directed. **Avoid multiple freeze/thaw cycles!**

Safety

This product is for research purposes only and not for human or therapeutic use. This product should be considered hazardous and is harmful by inhalation, in contact with skin, eyes, clothing, and if swallowed. If contact occurs, wash thoroughly.

Contraindications

- The Dcaf11-driven p21 Ubiquitination Assay Kit is compatible with up to 1% final DMSO concentration. We recommend preparing the inhibitor in no higher than 4% DMSO solution in buffer and using 2.5 µl per well.
- Green and blue dyes that absorb light in the AlphaScreen® signal emission range (λ=520-620 nm), such as Trypan Blue, interfere with the assay.
- Avoid the presence of potent singlet oxygen quenchers such as sodium azide (NaN_3) or metal ions (Fe^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+} and Ni^{2+}).
- The presence of the culture medium RPMI 1640 at >1% leads to signal reduction due to the presence of excess biotin and iron in this medium. MEM, which lacks these components, does not affect AlphaScreen® assays.

Assay Protocol

- All samples and controls should be tested in duplicate.
- The assay should include "Blank", "Positive Control", "Negative Control" and "Test inhibitor" conditions.
- We recommend preincubating inhibitors with p21 and/or Dcaf11 before initiating the reaction.
- If the assay plate is going to be used more than once, prepare enough of each protein and aliquot the remaining undiluted proteins into single-use aliquots depending on how many times the assay plate will be used. Store the protein aliquots at -80°C and store aliquots of U2 Assay Buffer and ATP at -20°C. Refer to #2 (dilutions) and to #4 (preparing the master mix) to calculate how much of each protein is needed.

Step 1:

1) Thaw **U2 Assay Buffer**, **ATP, Biotin-Ubiquitin**, **UBE1**, **UbcH5b**, **Dcaf11 complex,** and **p21** on ice. Briefly spin the tubes to recover their full content.

   UBE1, UbcH5b, Dcaf11 complex, p21, Biotin-Ubiquitin, and U2 Assay Buffer are sensitive to freeze/thaw cycles. Avoid multiple freeze-thaw cycles.

2) Dilute proteins, as follows, and keep on ice:

   a) Dilute UBE1 in U2 Assay Buffer to 19 ng/µl (160 nM – the final concentration in the reaction will be 40 nM) (2.5 µl/well),

   b) Dilute UbcH5b in U2 Assay Buffer to 90 ng/µl (5 µM – the final concentration in the reaction will be 500 nM) (1 µl/well),

   c) Dilute Dcaf11 complex in U2 Assay Buffer to 9.3 ng/µl (30 nM – the final concentration in the reaction will be 3 nM) (1 µl/well),

   d) Dilute p21 in U2 Assay Buffer to 1.34 ng/µl (30 nM – the final concentration in the reaction will be 3 nM) (1 µl/well).

   Note: The concentration of each protein is lot-specific and is indicated on the tube. Verify the initial concentration and dilute accordingly. Do not freeze and re-use diluted proteins.

3) Prepare the Test Inhibitor (2.5 µl/well): for a titration prepare serial dilutions at concentrations 4-fold higher than the desired final concentrations. The final volume of the reaction is 10 µl.

   a) If the Test Inhibitor is water-soluble, prepare serial dilutions in the U2 Assay Buffer, 4-fold more concentrated than the desired final concentrations. For the positive and negative controls, use U2 Assay Buffer (Diluent Solution).

   b) If the Test inhibitor is soluble in DMSO, prepare the test inhibitor at 100-fold the highest desired concentration in DMSO, then dilute the inhibitor 25-fold in U2 Assay Buffer to prepare the highest concentration of the 4-fold intermediate dilutions. The concentration of DMSO is now 4%.

      Prepare serial dilutions of the Test Inhibitor at 4-fold the desired final concentrations using 4% DMSO in U2 Assay Buffer to keep the concentration of DMSO constant.

      For positive and negative controls, prepare 4% DMSO in U2 Assay Buffer (vol/vol) so that all wells contain the same amount of DMSO (Diluent Solution).

      The final concentration of DMSO should not exceed 1%.

4) Make a Master Mix (5 µl/well, except "Blank" wells): N wells × (1 µl Biotin-Ubiquitin + 1 µl ATP (10 mM) + 1 µl diluted UbcH5b + 1 µl diluted Dcaf11 complex + 1 µl p21).

5) Add 5 µl of Master Mix to the "Negative Control", "Positive Control" and "Test Inhibitor" wells.

6) Make a Dcaf-Deficient Master Mix for the "Blank" wells (7.5 µl/ well): N wells x (1 µl of Biotin-Ubiquitin +1 µl ATP (10 mM) + 1 µl of diluted UbcH5b + 2.5 µl of Diluent Solution + 2 µl of U2 Assay Buffer).

7) Add 7.5 µl of Dcaf-Deficient Master Mix to the "Blank" wells.

8) Add 2.5 µl of the test inhibitor serial dilution to each well designated "Test Inhibitor".

9) Add 2.5 µl of the Diluent Solution to the "Positive Control" and "Negative Control" wells.

10) Add 2.5 µl of U2 Assay Buffer to the wells designated "Negative Control".

11) Initiate the reaction by adding 2.5 µl of UBE1 to the wells labeled "Positive Control", "Test Inhibitor", and "Blank".

12) Cover the plate with a plate lid and incubate the reaction at Room Temperature (RT) for one hour with gentle agitation.

	Test Inhibitor	Negative Control	Positive Control	Blank
Master Mix	5 µl	5 µl	5 µl	-
Dcaf11-Deficient Master Mix	-	-	-	7.5 µl
Test Inhibitor	2.5 µl	–	–	-
Diluent solution	–	2.5 µl	2.5 µl	-
U2 Assay Buffer	–	2.5 µl	–	-
Diluted UBE1 (160 nM)	2.5 µl	–	2.5 µl	2.5 µl
Total	**10 µl**	**10 µl**	**10 µl**	**10 µl**

Note: Protect your samples from direct exposure to light for steps 2 and 3!

Step 2:

1. Dilute **4x U2 Detection Buffer** 4-fold with DNAse-free water to make **1x U2 Detection Buffer**. Prepare only the amount required for the assay.

2. Dilute AlphaLISA® Glutathione acceptor beads (PerkinElmer #AL109C) 250-fold in 1x U2 Detection Buffer. Mix well.

3. Add 10 µl of acceptor bead mix to each reaction well.

4. Agitate on a rotator platform for 30 minutes at RT.

Step 3:

1. Dilute Streptavidin-conjugated donor beads (PE #6760002S) 125-fold with 1x Detection Buffer 3D.

2. Add 10 µl per well.

3. Agitate on a rotator platform for 15-30 minutes* at RT.

   ** The Signal-to-Noise ratio depends greatly on the performance of the beads from PerkinElmer. Duration of incubation may be extended for some lots of the beads, if necessary.*

4. Read Alpha-counts on an AlphaScreen® microplate reader.

5. The "Blank" control might be important to determine the background A-screen counts in the assay. The blank value should be subtracted from all other values.

Example Results

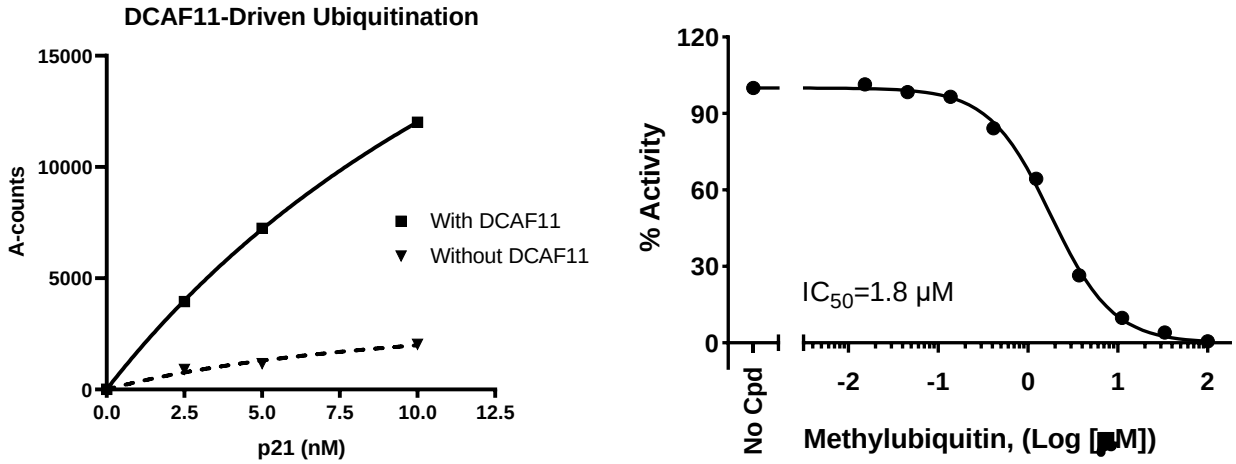

Figure 1: Dcaf11-driven p21 ubiquitination.
Left: Dcaf11-driven ubiquitination of p21 was measured with increasing amounts of p21 in the presence and absence of Dcaf11 complex. Right: Dcaf11-dependent ubiquitination of p21 was measured in the presence of increasing concentrations of methylubiquitin (R&D Systems #U-501). Results are expressed as percent Activity (in which positive control activity in the absence of inhibitor is set at 100%).

Data shown is representative. For lot-specific information, please contact BPS Bioscience, Inc. at support@bpsbioscience.com.

Troubleshooting Guide
Visit bpsbioscience.com/assay-kits-faq for detailed troubleshooting instructions. For all further questions, please email support@bpsbioscience.com

Related Products

Products	*Catalog #*	*Size*
Cereblon Ubiquitination Homogeneous Assay Kit	79881	384 reactions
UBC5b TR-FRET Assay Kit	79896	384 reactions
UBE1, GST-Tag	100402	100 µg
Cereblon/DDB1/Cul4A/Rbx1 Complex	100329	10 µg
VHL/CUL2/ELOB/ELOC/RBX1 Complex	100373	10 µg