

Product Information

A549 RFP-STAT3 Lung Carcinoma Cell Line with RFP-tagged STAT3

Catalog Number **CLL1140**

Storage Temperature $-196\text{ }^{\circ}\text{C}$ (liquid nitrogen)

TECHNICAL BULLETIN

Product Description

This product is a human A549 cell line in which the genomic STAT3 gene has been endogenously tagged with a Red Fluorescent Protein (RFP) gene using CompoZr[®] zinc finger nuclease (ZFN) technology. The cell line shows redistribution of STAT3 to the nucleus upon activation with a ligand such as IL-6, making it useful for high content screening of compounds that modulate STAT3 activity.

CompoZr[®] technology is a fast and reliable way to manipulate the genome in a targeted fashion. ZFNs are synthetic proteins engineered to bind DNA at a sequence-specific location and create a double strand break (www.compozrzn.com). The cell's natural machinery repairs the break in one of two ways: non-homologous end joining or homologous recombination. The homologous recombination pathway was used to insert a transgene into a desired target location – behind the start codon of the STAT3 locus. A donor construct containing a fluorescent reporter gene (RFP) flanked by sequences homologous to the cut site was nucleofected into the A549 cells along with ZFNs designed to cut near the genomic target site (see Figures 1a and 1b). Integration resulted in endogenous expression of fluorescent fusion protein RFP-STAT3. Knock-in cells were sorted to single cells by flow cytometry and then grown up into clonal populations. Testing of these clones was used to select a single RFP-STAT3 clone as a stable cell line (see Figures 2a and 2b). Junction PCR showed at least one allele is tagged (see Figure 3a) and Southern analysis showed there were no off-target insertions of the RFP (see Figure 3b).

Signal transducer and activator of transcription 3 (STAT3) belongs to the STAT protein family (STAT1-7), which are signaling intermediates that mediate the action of many cytokines and growth factors.¹ STAT3 is an oncogene that is constitutively active in many

different cancers including prostate, breast, lung, head and neck, colon, liver, and pancreas as well as in multiple myeloma and large granular lymphocytic leukemia.² Numerous studies have repeatedly demonstrated that inhibiting STAT3 results in decreased tumor growth and improved animal survival by inducing tumor cell apoptosis, inhibiting angiogenesis and enhancing antitumor immune-mediated cytotoxicity. It has also been demonstrated recently that a STAT3-mediated mechanism drives self-renewal and tumorigenesis of CD24⁺ hepatic tumor-initiating cells.³ Thus, STAT3 has been identified as a promising drug discovery target for many cancers.

To date, a few non-peptidic small-molecules have been reported to inhibit STAT3 by direct binding to its SH2 domain. Most studies used virtual screening to identify candidate compounds with an increased likelihood of binding to the STAT3 SH2 domain. The result of these studies was the identification of a specific STAT3 inhibitor called Stattic (STAT three inhibitory compound). Stattic selectively inhibits activation, dimerization, and nuclear translocation of STAT3 and induces apoptosis in STAT3-dependent cancer cell lines.⁴

The screening of potential inhibitors for STAT3 is based on their ability to compete with a high-affinity phosphopeptide targeted to the SH2 domain of STAT3.⁴ This *in vitro* screening yields many false leads because it doesn't take into account that many small molecules are not permeable to cell membranes in cell-based assays or in animal models. Therefore, a secondary screen for visualizing the inhibition of IL-6-driven STAT3 nuclear localization is utilized to identify the best compounds.^{4,5} Using immunofluorescence to detect native protein localization⁴ is expensive and fixation can cause reproducibility concerns in these assays. Alternatively, overexpressing a fluorescently tagged STAT3 creates aberrant physiological conditions⁵ and may create artifactual results.

With ZFN-mediated gene tagging in knock-in cell lines, STAT3's native gene regulation is conserved resulting in normal protein expression levels and preservation of protein function. Following confirmation of IL-6-driven STAT3 nuclear translocation, we successfully tagged the endogenous STAT3 locus with either GFP or RFP in SKOV3 and A549 cell lines, respectively. Next, we confirmed fluorescently tagged STAT3 was predominantly in the cytoplasm of uninduced cells and nuclear translocation could be detected within 15 minutes of induction with 100 ng/mL IL-6. Within 30-40 minutes of IL-6 treatment RFP-STAT3 was primarily localized in the nucleus of the A549 cells (see Figure 2a and Figure 4, Panels A, B). However, after preincubation of the cells with 20 μ M Stattic for one hour, the IL-6-induced nuclear translocation of STAT3 was inhibited (see Figure 4, Panels C, D).

RFP and TagRFP are all synonymous for the fluorescent reporter gene in this document. The RFP used in this cell line originated from Evrogen, referred to as TagRFP:

<http://evrogen.com/products/TagFPs.shtml>

For further information on our CompoZr modified cell lines go to the website:
www.wheribiobegins.com/biocells

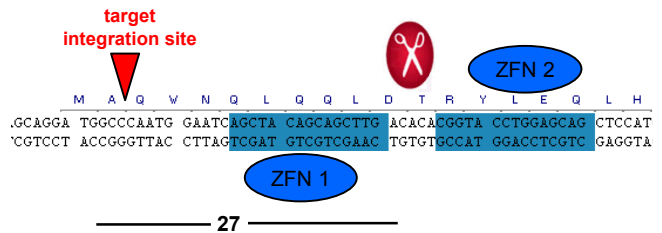
Component

A549 knock-in cell line having the STAT3 gene 1 vial
tagged at the N-terminus with RFP
Catalog No. CLL1140

One vial of modified A549 cells contains $\sim 2 \times 10^6$ cells
in Cell Freezing Medium-DMSO 1 \times , Catalog No.
C6164.

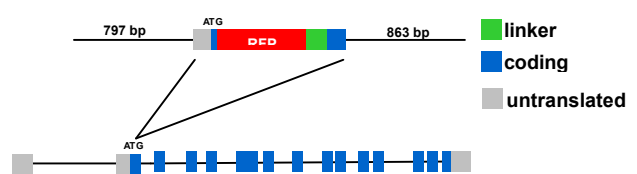
Design of tag sequence integration at the STAT3 gene locus

Figure 1a.



Schematic of the genomic sequence at the target region for integration of the fluorescent tag RFP. DNA of STAT3, showing the start codon, CompoZr ZFN binding sites (blue boxes), the ZFN cut site (scissors), and the tag sequence integration site (red arrow).

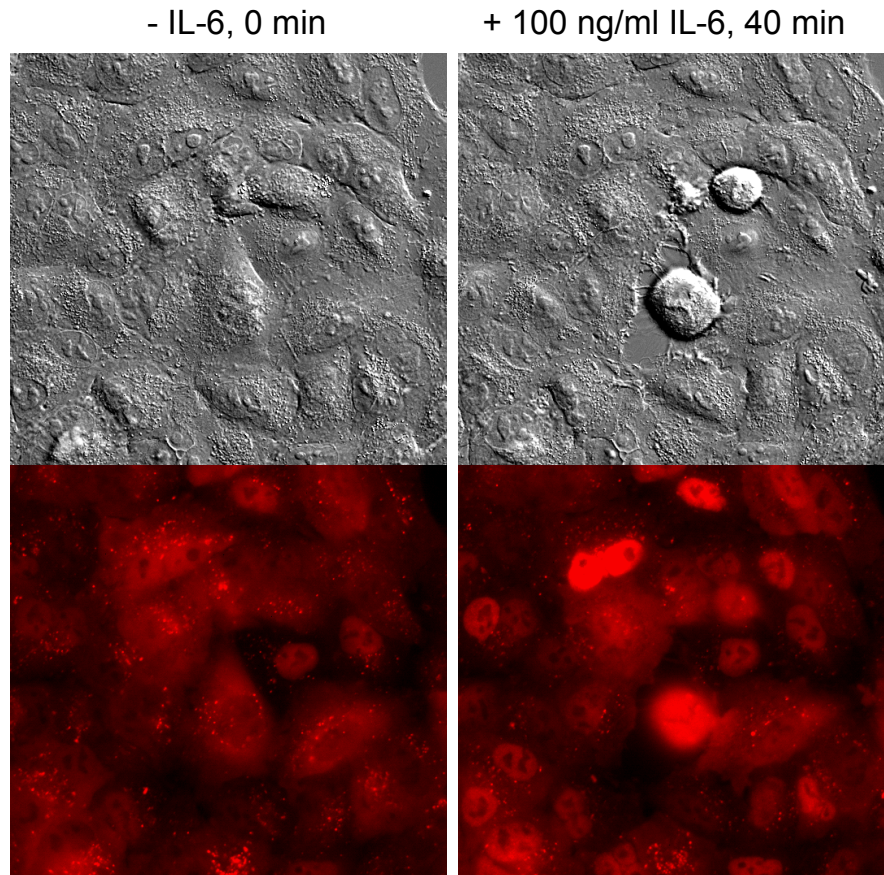
Figure 1b.



Schematic of the STAT3 locus and the donor with the locus showing the coding regions (blue) and untranslated regions (gray). The Donor (top) has the homology arms of indicated length and the RFP sequence (red) fused to the beginning of the STAT3 coding sequence (an N-terminal fusion).

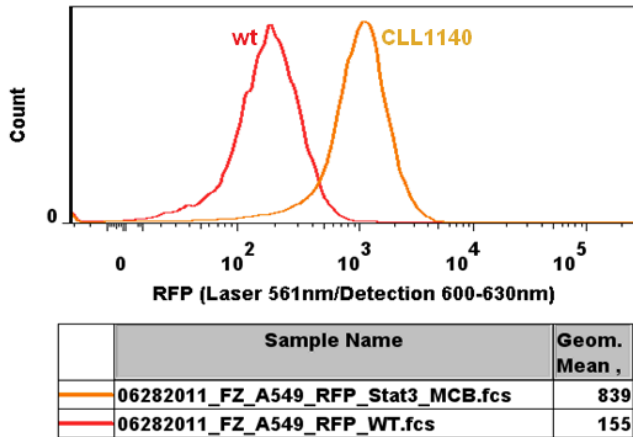
Localization and expression levels of endogenously tagged STAT3 (RFP-STAT3) in A549 cells

Figure 2a.



Differential interference contrast (DIC) and fluorescence microscopy images of an isolated cell clone expressing endogenous STAT3 protein tagged with RFP before and 40 minutes after addition of 100 ng/mL IL-6 (ex 530–560/em 590–650, 40×/1.3 oil/1 second exposure in HBSS+2% FBS). Endogenous STAT3 expression levels are low and near autofluorescence levels (see Figure 2b) and following IL-6 stimulation nuclear translocation of RFP-STAT3 fusion protein is distinctly detectable.

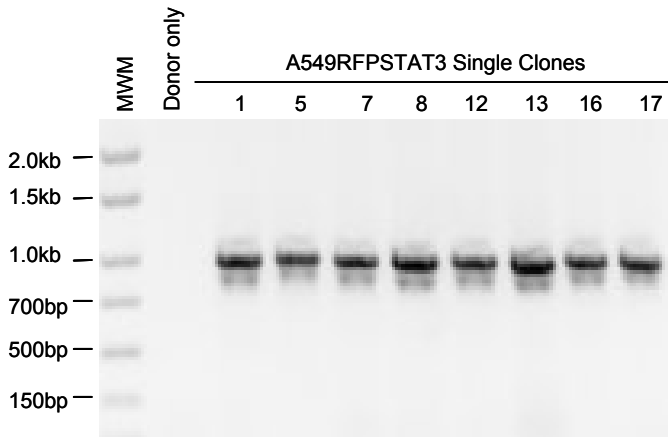
Figure 2b.



Fluorescence analysis of the RFP-STAT3 clone compared to wild type A549 (autofluorescence) using BD FACS Aria III.

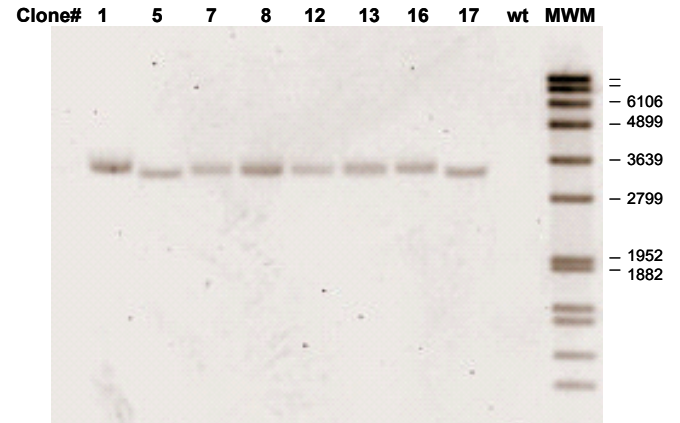
Molecular analysis to identify targeted integration in A549 RFP-STAT3 clones

Figure 3a.



Junction PCR using RFP forward and STAT3 reverse primers produced a 926 bp characteristic fragment for targeted integration for all clones tested. No PCR product can be detected in the negative control that contained donor only.

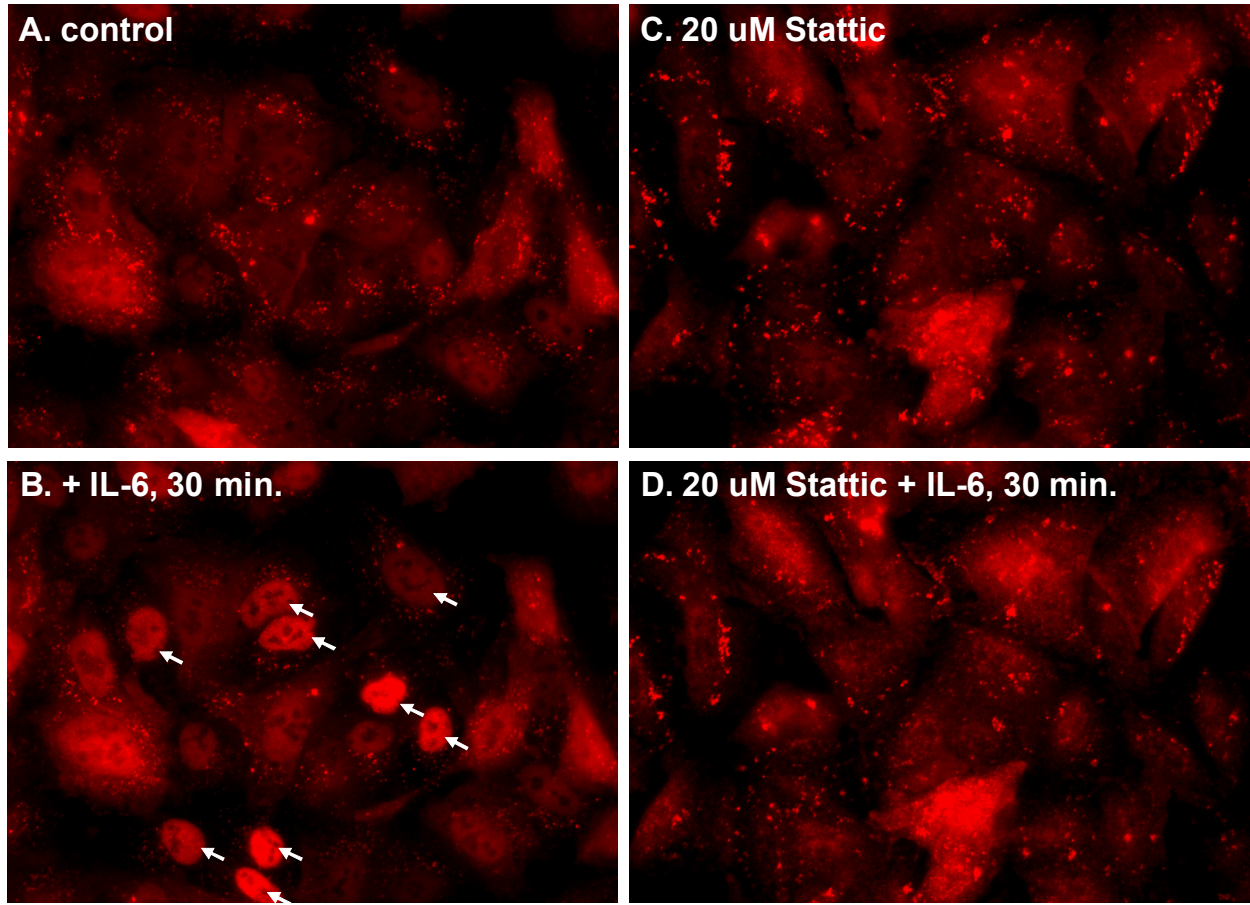
Figure 3b.



Southern Blot Hybridization was performed using DIG-labeled RFP probe for all single clones isolated. Only the expected DNA-hybridization band at 3271 bp can be detected across all clones. No random DNA bands were observed. A549 wild type served as a negative control for RFP-STAT3 integration. Clone #13 was selected based on cell morphology, molecular analyses and imaging/translocation analyses

Inhibition of translocation of RFP-STAT3 fusion protein from cytosol to nucleus in A549 cells by Stattic

Figure 4.



Fluorescence microscopy analysis indicated that RFP-STAT3 protein was translocated from cytoplasm (A) to nucleus (B, indicated by arrows) when cells were cultured in medium containing 100 ng/ml IL-6 for 30 minutes. If cells were preincubated with 20 μ M Stattic for 1 hour (C,D), the IL-6 treatment did not induce RFP-STAT3 translocation (D).

Cell Line Description

Organism: *Homo sapiens* (human)

Tissue: Carcinoma; Lung

Age: 58 years

Gender: Male

Ethnicity: Caucasian

Morphology: Epithelial

Growth properties: Adherent

DNA profile

Short Tandem Repeat (STR) analysis:

Amelogenin: X, Y

CSF1PO: 10, 12

D13S317: 11

D16S539: 11,12

D5S818: 11

D7S820: 8, 11

TH01: 8,9,3

TPOX: 8, 11

vWA: 14

Parental Cell Line: ATCC® Catalog No. CCL-185™

Note: Please see CCL-185 product datasheet from ATCC for additional information about the origin of these cell lines. Cytogenetic information is based on initial seed stock at Sigma Life Science. Cytogenetic instability has been reported in the literature for some cell lines.

Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Biosafety Level: 1

This cell line is not known to harbor an agent known to cause disease in healthy adult humans. Handle as a potentially biohazardous material under at least Biosafety Level 1 containment. The parental cell line, A549, was obtained from ATCC. All animal products used in the preparation of the knockout line and maintenance of both, parental and knockout clone, have been screened negative by 9CFR for adventitious viral agents. Cell lines derived from primate lymphoid tissue may fall under the regulations of 29 CFR 1910.1030 Bloodborne Pathogens. Appropriate safety procedures are recommended to be used when handling all cell lines, especially those derived from human or other primate material. Detailed discussions of laboratory safety procedures have been published.⁶⁻⁸

Storage/Stability

Upon receiving a shipment of frozen cells it is important the end user gives the shipment attention without delay. To ensure the highest level of viability, thaw the vial and initiate the culture as soon as possible upon receipt. If upon arrival, continued storage of the frozen culture is necessary, it should be stored in liquid nitrogen vapor phase and not at -70 °C. Storage at -70 °C will result in loss of viability.

Precaution: It is recommended that protective gloves and clothing always be used, and a full face mask always be worn when handling frozen vials. It is **important to note that some vials leak when submersed in liquid nitrogen** and will slowly fill with liquid nitrogen. Upon thawing, the conversion of the liquid nitrogen back to the gas phase may result in the rapid expansion of the vessel, potentially blowing off its cap with dangerous force creating flying debris.

At the time a cell line is ordered, end users should also consider the culture conditions for the new cell line and make sure the appropriate medium will be available when the cells arrive.

Complete Medium Preparation Instructions

Complete Medium: To make the complete growth medium, add fetal bovine serum, Catalog No. F2442, to a final concentration of 10% and L-Glutamine, Catalog No. G7513, at a final concentration of 2 mM in RPMI-1640 Medium, Catalog No. R0883. This medium is formulated for use with a 5% CO₂ in air atmosphere.

Procedure

Thawing of Frozen Cells.

1. Thaw the vial by gentle agitation in a 37 °C water bath. To reduce the possibility of contamination, keep the O-ring and cap out of the water. Thawing should be rapid (~2 minutes).
2. Remove the vial from the water bath as soon as the contents are thawed, and decontaminate by dipping in or spraying with 70% ethanol. All of the operations from this point on should be carried out under strict aseptic conditions.
3. Transfer the vial contents to a centrifuge tube containing 9.0 mL of Complete Medium and spin at ~125 × g for 5–7 minutes.
4. Resuspend cell pellet with the Complete Medium and dispense into a 25 cm² or a 75 cm² culture flask. It is important to avoid excessive alkalinity of the medium during recovery of the cells. It is suggested, prior to the addition of the vial contents, the culture vessel containing the Complete Medium be placed into the incubator for at least 15 minutes to allow the medium to reach its normal pH (7.0–7.6) and temperature (37 °C).
5. Incubate the culture at 37 °C in a suitable incubator. A 5% CO₂ in air atmosphere is recommended for the Complete Medium.

Sub-culturing Procedure

Volumes used in this procedure are for a 75 cm² flask; proportionally reduce or increase volume of dissociation medium for culture vessels of other sizes.

1. Remove and discard culture medium.
2. Briefly rinse the cell layer with Trypsin-EDTA solution (Catalog No. T3924)
3. Add 2.0–3.0 mL of Trypsin-EDTA solution to flask and incubate at 37 °C for 7 minutes to detach the cells.
4. Add 6.0–8.0 mL of Complete Medium and aspirate cells by gentle pipetting.
5. Add appropriate aliquots of the cell suspension into new culture vessels.
Subcultivation Ratio: 1:3 to 1:20
6. Incubate cultures at 37 °C.

Note: More information on enzymatic dissociation and subculturing of cell lines is available in the literature.⁸

References

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Additional product and technical information can be obtained from the catalog references and the Sigma Life Science Website
www.wherebiobegins.com/biocells

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