3050 Spruce Street, St. Louis, MO 63103 USA
Tel: (800) 521-8956 (314) 771-5765 Fax: (800) 325-5052 (314) 771-5757
email: techservice@sial.com sigma-aldrich.com

Product Information

A549 GFP-SMAD4
Lung Carcinoma Cell Line with GFP-tagged SMAD4

Catalog Number **CLL1167**
Storage Temperature –196 °C (liquid nitrogen)

TECHNICAL BULLETIN

Product Description
This product is a human A549 cell line in which the genomic SMAD4 gene has been endogenously tagged with a Green Fluorescent Protein (GFP) gene using CompoZr® zinc finger nuclease (ZFN) technology. The cell line shows redistribution of SMAD4 from the cytoplasm to the nucleus upon activation with a ligand such as transforming growth factor beta (TGF-β), making it useful for high content screening to identify compounds that modulate SMAD2/3 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.compozrzfn.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 for GFP into a desired target location – in front of the stop codon of the SMAD4 locus – to result in the target protein with its C-terminus fused to the N-terminus of GFP. A donor construct containing a fluorescent reporter gene (GFP) flanked by sequences homologous to the regions on either side of the genomic target site was nucleofected into the A549 cells along with ZFNs designed to cut near the genomic target site (Figures 1a and 1b). Integration resulted in endogenous expression of fluorescent fusion protein SMAD4-GFP.

Knockin cells were sorted into single cells by flow cytometry and then expanded into clonal populations. Testing of these clones was used to select a single SMAD4-GFP clone as a stable cell line (Figures 2 and 3). Junction PCR showed at least one allele is tagged (Figure 4a) and Southern analysis showed there were no off-target insertions of the GFP (Figure 4b).

The SMAD proteins are homologs of both the *Drosophila* protein mothers against decapentaplegic (MAD) and the *C. elegans* protein SMA. The name is a combination of the two.[1] The SMAD proteins are divided into three distinct classes based on their structure and function in signaling by TGF-β family members.

Receptor-regulated SMADs (R-SMADs) are phosphorylated on two serine residues at the C-terminus and thus, activated in a ligand-specific manner. SMAD2 and SMAD3 mediate signaling by TGF-β and activin; whereas, SMAD1, SMAD5, and presumably SMAD8 are known to be involved in BMP signaling.

Upon phosphorylation by type I receptors, R-SMADs form heteromeric complexes with SMAD4. SMAD4, also referred to as Co-SMAD, serves as a common partner for all R-SMADs. The heterocomplexes translocate to the nucleus where they modulate the transcription of TGF-β target genes that include cell cycle regulators such as the CDK inhibitors, p15 and p21, and the proto-oncogene c-myc.[2-3]

TGF-β-mediated SMAD signaling has been shown to be required for the antiproliferative activity of TGF-β, and components in this signaling pathway are frequently inactivated by mutation or silenced in several human cancers.[4] This indicates that wild-type SMAD4 activity in the TGF-β signal pathway may be critical for maintaining an environment that inhibits tumorigenesis. SMAD4 in the basal state is found mostly as a homooligomer, most likely a trimer.[2-3] SMAD4 is recognized as a key tumor suppressor as it is defective in a number of cancers.[4]

To date, a few non-peptidic small-molecules (e.g., SB431542) have been reported to inhibit SMAD2/3/4 by direct inhibition of the activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7 (the TGF-β type I receptors).[5] SB431542 also suppresses the TGF-β-induced proliferation of human osteosarcoma cells.[6] However, there are no effective modulators that directly interfere with R-SMAD/SMAD4 heterocomplex formation. A small molecule that selectively affects formation and/or nuclear translocation of this heterocomplex may have potential as a human antitumor agent.

Biochemical screening based on such a complicated event as R-SMAD/SMAD4 complex formation is extremely difficult due to issues associated with *in vitro* modeling. If accomplished, such screening is likely to yield many false-positive leads because it does not take into account the low permeability of many small molecules across the cell membrane. Therefore, a cell-based high-content screen for visualizing inhibition of TGF-β-driven endogenous SMAD4 nuclear localization will provide a key tool to identify efficacious compounds compared to other methods which are either expensive and/or subject to artifacts or reproducibility concerns.[7,8]

Using ZFN-mediated tagging of the endogenous gene locus in A549 cells, SMAD4's native gene regulation is conserved resulting in normal protein expression levels and preservation of protein function. This was confirmed by demonstrating fluorescently tagged SMAD4 was predominantly in the cytoplasm of uninduced A549 cells (Figure 2C) and nuclear translocation could be detected within 10–15 minutes after application of 100 ng/mL of TGF-β. After 20 minutes of TGF-β treatment, SMAD4-GFP was primarily localized in the nucleus of the cells (Figure 2D). Notably, preincubation of the cells for 1 hour with 20 μM SB431542 (inhibitor of activin receptor-like kinase ALK5) inhibited the TGF-β-induced nuclear translocation of SMAD4 (data not shown). If SB431542 was added after TGF-β-induced nuclear accumulation of SMAD4-GFP, it triggered fast and robust nuclear export of SMAD4-GFP within 40–50 minutes; whereas, the usual nuclear retention time for activated SMAD2/3/4 complexes is 6–9 hours. Thus, SMAD4-GFP in the A549 cell line behaves as endogenous SMAD4 and its dynamics reveal the key step of the SMAD2/3/4 pathway - nuclear translocation of the heterocomplexes.

GFP and TagGFP are all synonymous for the fluorescent reporter gene in this document. The GFP used in this cell line originated from Evrogen, referred to as TagGFP:
http://evrogen.com/products/TagFPs.shtml

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

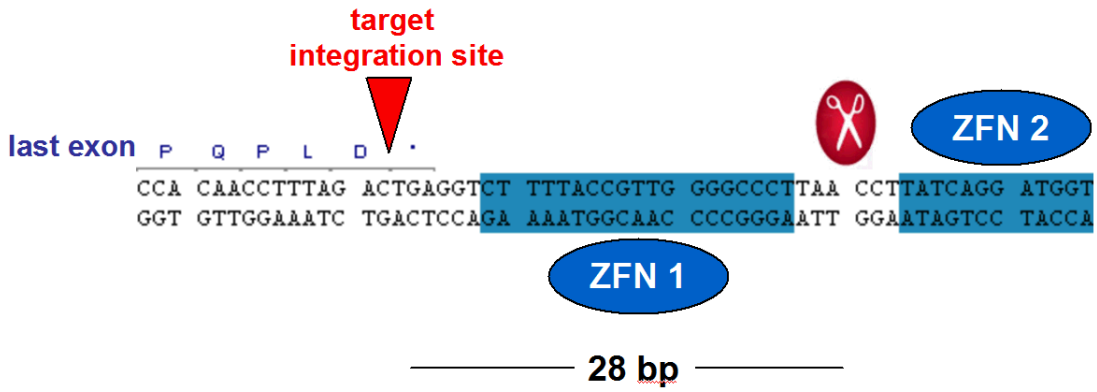
Component

A549 knockin cell line having the SMAD4 gene tagged at the C-terminus with GFP Catalog No. CLL1167	1 vial

One vial of modified A549 cells contains ~2×10^6 cells in Cell Freezing Medium-DMSO 1×, Catalog No. C6164.

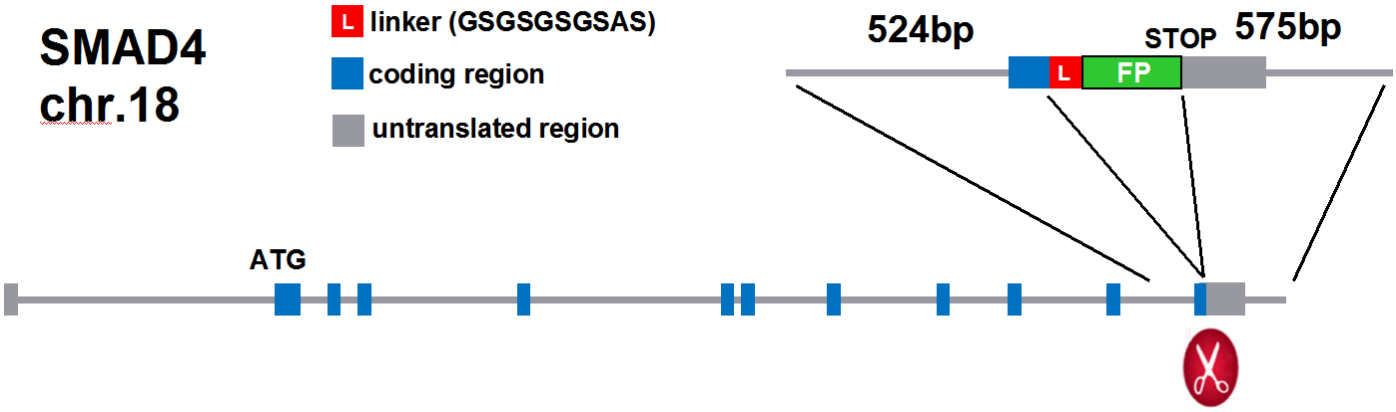
Design of tag sequence integration at the SMAD4 gene locus

Figure 1a.



Schematic of the genomic sequence at the target region for integration of the fluorescent tag GFP. DNA of SMAD4, showing the end of the last exon, CompoZr ZFN binding sites (blue boxes), the ZFN cut site (scissors), and the tag sequence integration site (red arrow).

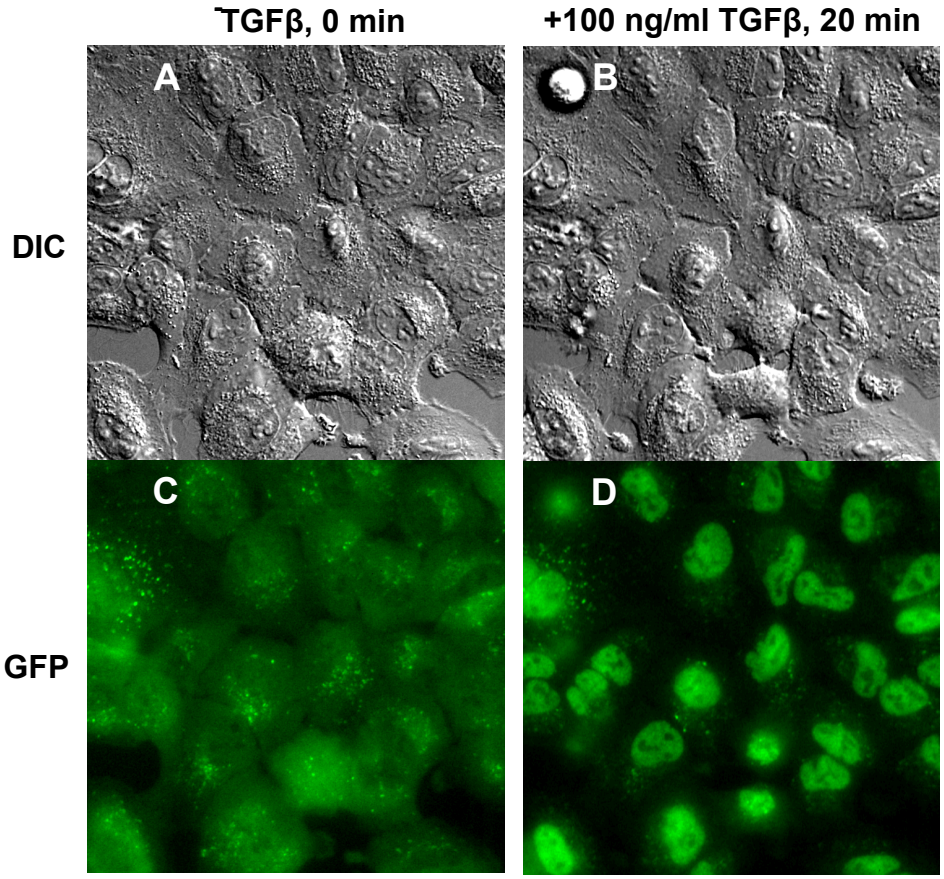
Figure 1b.



Schematic of the SMAD4 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 GFP sequence (green) fused to the end of the SMAD4 coding sequence (an C-terminal fusion).

Localization of endogenously tagged SMAD4 (SMAD4-GFP) in A549 cells

Figure 2.

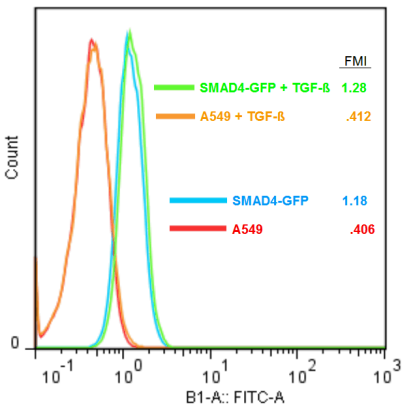


Differential interference contrast (DIC) and fluorescence microscopy images of an isolated single cell clone expressing the SMAD4 gene (Mothers Against Decapentaplegic homolog 4) endogenously tagged with GFP (A549 lung carcinoma) at the C-terminus. The images were taken either before (A, C) or 20 minutes after addition of 100 ng/mL of TGF-β (B, D). The cells were imaged live in Hanks balanced salt solution (Catalog No. H8264) supplemented with 2% fetal bovine serum (Catalog No. F2442) using a GFP filter set (ex 450–490/em 550) and 40x/1.3 oil objective. Endogenous SMAD4 expression levels are low and near autofluorescence levels (see Figure 2b). TGF-β-triggered nuclear translocation of the SMAD4-GFP fusion protein is distinctly detectable.

Expression levels of endogenously tagged SMAD4 (SMAD4-GFP) in A549 cells

Figure 3.

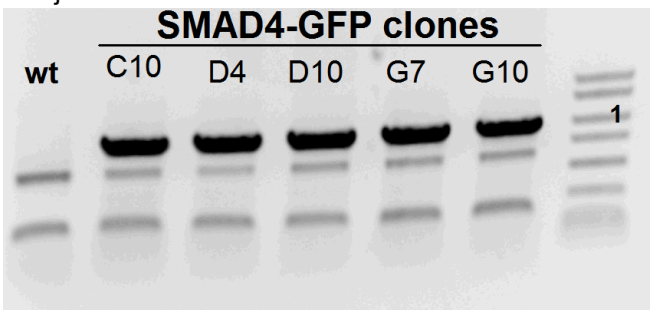
Fluorescence analysis of the SMAD4-GFP clone compared to the wild type A549 (autofluorescence) with or without 30 minute treatment of 100 ng/mL of TGF-β using MACSQuant® from Miltenyi Biotech.
FMI = Fluorescent Mean Intensity



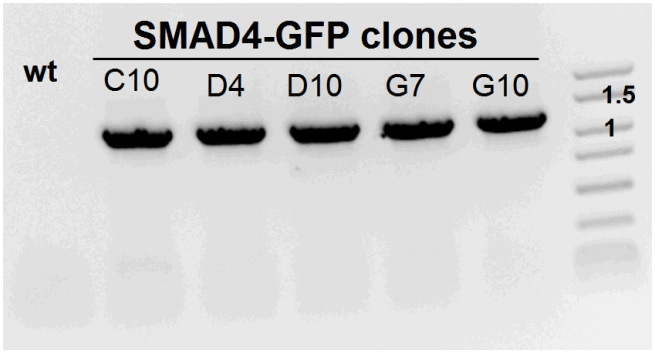
Molecular analysis to identify targeted integration in A549 SMAD4-GFP clone

Figure 4a.

Left junction PCR

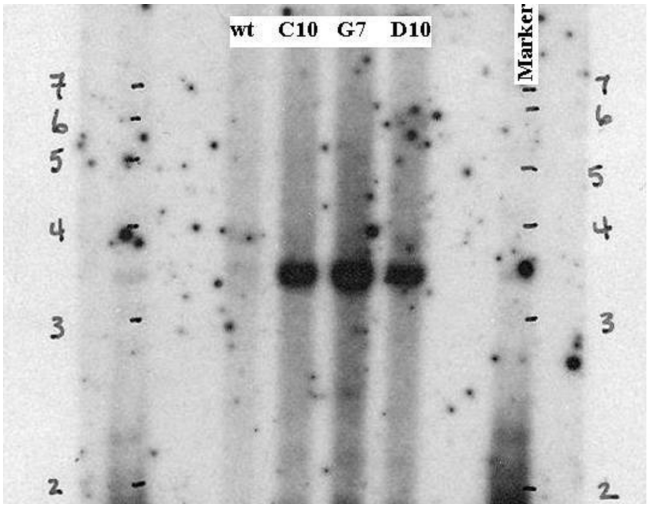


Right junction PCR



Junction PCR was performed on genomic DNA isolated from the wild type (wt) and SMAD-GFP clones. The 0.9 kb expected fragment for left junction and 1.2 kb for right junction confirm the targeted integration of GFP in front of the stop codon of SMAD4 locus for all clones tested. No PCR product can be detected in the wild type control. Junction PCR products were confirmed by sequencing.

Figure 4b.



Southern blotting shows no random integration from the plasmid donor initially used to create the clones. Genomic DNA from single cell clones C10, G7, D10, and from A549 wild type (wt - served as a negative control) were digested with *Nco* I and *Spe* I restriction endonucleases. Proper targeted insertion of the GFP into the SMAD4 locus should produce a hybridized band of ~3.3 kb in size. Radioactive-labeled GFP probe was used. Clone C10 was chosen as the final product based on cell morphology, molecular analyses, and imaging/translocation analyses.

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

The STR profile of this cell line matches that of its 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.[9-11]

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
To make the complete growth medium, add L-Glutamine, Catalog No. G7513, at a final concentration of 2 mM, and fetal bovine serum, Catalog No. F2442, to a final concentration of 10% in the base medium, RPMI-1640 Medium, Catalog No. R0883. This medium is formulated for use with a 5% CO_2 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 cm^2 or a 75 cm^2 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% CO_2 in air atmosphere is recommended for the Complete Medium.

Sub-culturing Procedure
Volumes used in this procedure are for a 75 cm^2 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.
   Sub-cultivation 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.[11]

References

1. http://en.wikipedia.org/wiki/Smad4
2. Matsuzaki, K. Smad phosphoisoform signaling specificity: the right place at the right time. Carcinogenesis, **32**(11), 1578-88 (2011).
3. Massagué, J. et al., Smad transcription factors. Genes Dev., **19**(23), 2783-810 (2005).
4. He, S.M. et al., Reduced expression of SMAD4 in gliomas correlates with progression and survival of patients. J. Exp. Clin. Cancer Res., **30**, 70 (2011).
5. Inman, G.J. et al., SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. Mol. Pharmacol., 62(1), 65-74 (2002).
6. Matsuyama, S. et al., SB-431542 and Gleevec inhibit transforming growth factor-beta-induced proliferation of human osteosarcoma cells. Cancer Res., 63(22), 7791-7798 (2003).
7. Pierreux, C.E. et al., Transforming growth factor beta-independent shuttling of Smad4 between the cytoplasm and nucleus. Mol. Cell Biol., **20**(23), 9041-54 (2000).
8. Nicolás, F.J. et al., Analysis of Smad nucleo-cytoplasmic shuttling in living cells. J. Cell Sci., **15**;117(Pt 18), 4113-25 (2004).
9. Centers for Disease Control, Biosafety in Microbiological and Biomedical Laboratories Human Health Service Publication No. (CDC) 21-1112. U.S. Dept. of Health and Human Services; 5th Edition (2009) U.S. Government Printing Office Washington D.C. The entire text is available online at www.cdc.gov/biosafety/publications/index.htm
10. Fleming, D.O. & Hunt, D.L., Biological Safety: Principles And Practices, 4th Edition, ASM Press, Washington, DC (2006).
11. Freshney, R.I., Culture of Animal Cells, a manual of Basic Technique, 6th edition, published by John Wiley & Sons, Hoboken, NJ (2010).

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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