

## Product Information

### A549 GFP-CTNNB1 RFP-LMNB1 Lung Carcinoma Cell Line with 3XFLAG™-GFP- tagged CTNNB1 and RFP-tagged LMNB1

Catalog Number **CLL1149**

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

## TECHNICAL BULLETIN

### Product Description

This product is a human A549 cell line in which the genomic CTNNB1 ( $\beta$ -catenin) and LMNB1 (lamin B1) genes have been endogenously tagged with the 3XFLAG™ epitope linked to a Green Fluorescent Protein (GFP) and a Red Fluorescent Protein (RFP) gene, respectively, using CompoZr® zinc finger nuclease (ZFN) technology. The cell line shows 3XFLAG-GFP- $\beta$ -catenin fusion protein localized mostly in the adherens junctions but also in the cytosol (see Figure 1). It redistributes into the nucleus upon activation with LiCl or recombinant Wnt3a ligand similarly to the untagged  $\beta$ -catenin.<sup>1-3</sup> The RFP-lamin B1 fusion protein participates in the nuclear envelope formation and could serve as a nuclear marker. This makes the analysis of  $\beta$ -catenin redistribution possible without the addition of DNA binding dyes like Hoechst or DAPI. The cell line is useful for high content screening of compounds that regulate  $\beta$ -catenin to find novel anticancer drugs.

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](http://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 fluorescent protein transgenes into the desired target locations, in front of the start codons of the CTNNB1 and LMNB1 loci, resulting in the target proteins with their N-termi fused to the C-termi of the fluorescent proteins. Donor constructs containing each insert flanked by sequences homologous to the regions on either side of the genomic target site were nucleofected into A549 cells along with ZFNs designed to cut near the genomic target site (see Figure 2).

The double integration was done in two rounds. During the first round the cells were nucleofected with the CTNNB1 ZFNs and a donor construct containing a 3XFLAG epitope and GFP. Integration resulted in endogenous expression of fluorescent fusion protein 3XFLAG-GFP- $\beta$ -catenin. The cells were GFP-sorted to single cells by flow cytometry and expanded into clonal populations. Testing of the clones was performed to select a single 3XFLAG-GFP-CTNNB1 clone as a stable cell line.

This line was used as starting material for the second round of integration in which nucleofection was carried out with the LMNB1 ZFNs and corresponding RFP-containing donor. A single RFP-LMNB1 clone was isolated similar to the first round, resulting in generation of the double knockin reporter line (3XFLAG-GFP-CTNNB1/RFP-LMNB1) (see Figures 1, 3, and 4). Junction PCR showed at least one allele was tagged for each target (see Figure 5) and Southern analysis showed there were no off-target insertions of the reporter sequences (see Figure 6).

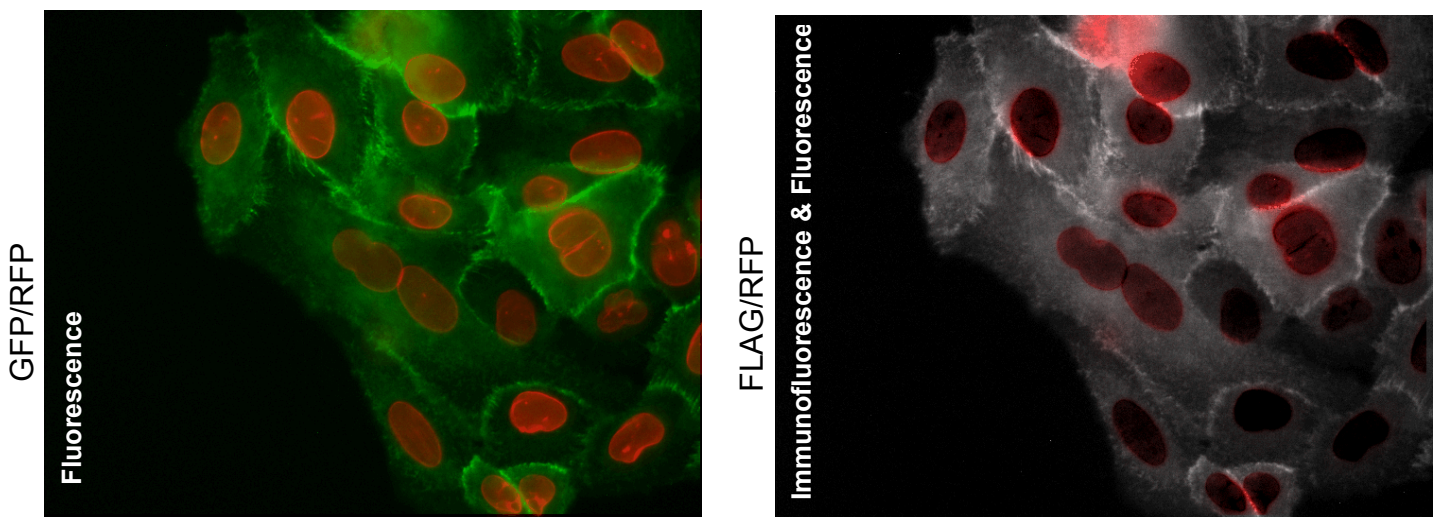
$\beta$ -catenin (CTNNB1) is a multifunctional protein involved in cell-cell adhesion and serves as a mediator of nuclear signaling in the Wnt pathway. Membrane-localized  $\beta$ -catenin is mostly bound to E-cadherin at adherens junctions and helps tether it to the actin cytoskeleton. Disruption of this process causes a dedifferentiation phenotype (called epithelial-to-mesenchymal transition) associated with the progression of epithelial-derived cancers.<sup>2,4-5</sup> Another well-known role of CTNNB1 is to enter the nucleus and act as a co-transcriptional expression modulator of the growing number of target genes. How  $\beta$ -catenin accumulates in the nucleus is not well understood and there are multiple potential regulatory checkpoints: nuclear retention, nuclear import, and nuclear export.<sup>1-3</sup>

One way to affect nuclear import is to affect the amount of free cytosolic  $\beta$ -catenin available for import. Glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ) targets  $\beta$ -catenin for degradation by the proteasome through phosphorylation of  $\beta$ -catenin at its N-terminus. It is hypothesized the inhibition of GSK3 $\beta$  allows  $\beta$ -catenin to accumulate in the cytosol and thus in the nucleus.<sup>2</sup> Lithium chloride and Wnt ligands that bind to a group of membrane Frizzled receptors cause the deactivation of GSK3 $\beta$  and thus indirectly allow CTNNB1 to accumulate in the nucleus.<sup>2</sup> Recent findings suggest the LiCl treatment also promotes the interaction with a protein binding partner, LEF-1, and this interaction increases  $\beta$ -catenin retention in the nucleus.<sup>3</sup>

Using ZFN-mediated tagging of the endogenous gene loci in A549 cells, regulation of the CTNNB1 native gene is conserved resulting in normal protein expression levels and preservation of protein function. This was confirmed by demonstrating fluorescently tagged  $\beta$ -catenin was predominantly in the membrane at cell-cell junctions and absent in the nucleus of unstimulated A549 cells (see Figure 1). Nuclear translocation/enrichment of  $\beta$ -catenin could be detected with Wnt3a or LiCl/leptomycin B treatments and the translocation/enrichment amount in the nucleus was the same with untagged and GFP-tagged  $\beta$ -catenin (see Figure 3).

### Figure 1.

CLL1149 cells have endogenously labeled CTNNB1 (3XFLAG-GFP) and LMNB1 (RFP)



Fluorescence and immunofluorescence microscopy images of an isolated cell clone expressing endogenous  $\beta$ -catenin (CTNNB1) tagged with 3XFLAG-GFP and endogenous lamin B1 (LMNB1) tagged with RFP. Cells were fixed with 3.7% formaldehyde and permeabilized with 0.1% Triton™ X-100. For FLAG® immunostaining: ANTI-FLAG® M2 (Catalog No. F1804) was diluted 1:500. The secondary was goat anti-mouse antibody conjugated to the fluorochrome ATTO 655 (Catalog No. 50283). Note GFP (left panel) and FLAG (right panel) localization is identical.

GFP and TagGFP2, and RFP and TagRFP are synonymous for the fluorescent reporter genes in this document. The GFP and RFP used in this cell line originated from Evrogen, referred to as TagGFP2 and TagRFP, respectively:  
<http://evrogen.com/products/TagFPs.shtml>

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

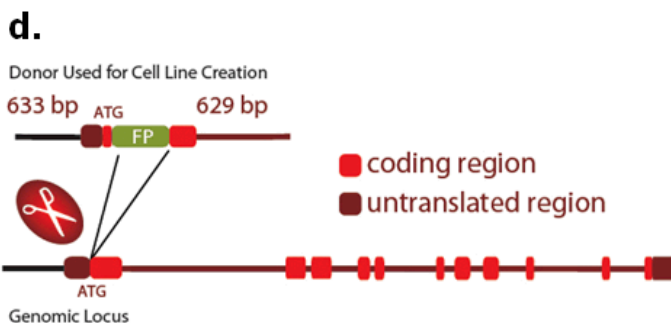
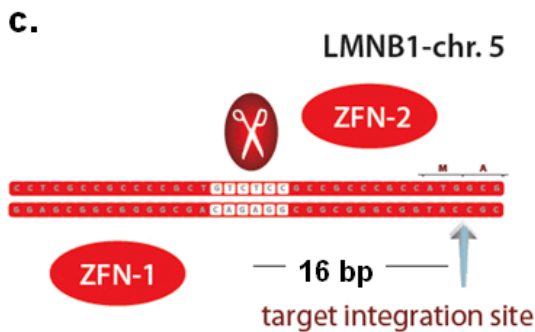
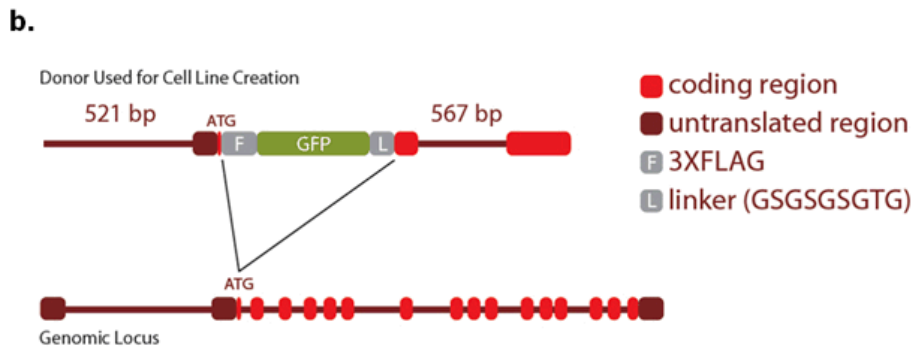
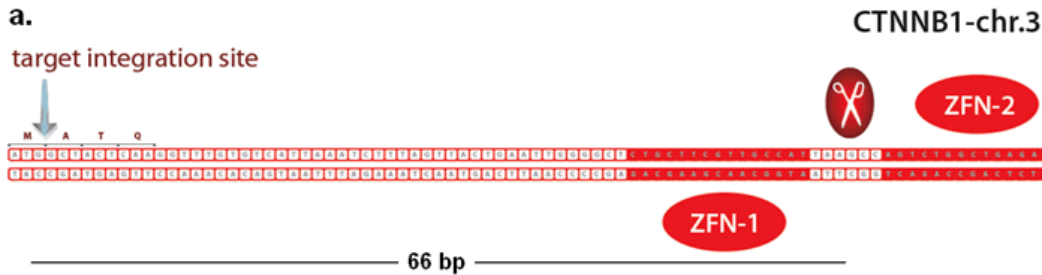
### Component

A549 knockin cell line having the CTNNB1 gene tagged at the N-terminus with 3XFLAG-GFP and the LMNB1 gene tagged at the N-terminus with RFP	1 vial
Catalog No. CLL1149	

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

**Figure 2**

The design of tag sequence integration at the CTNNB1 and LMNB1 gene loci

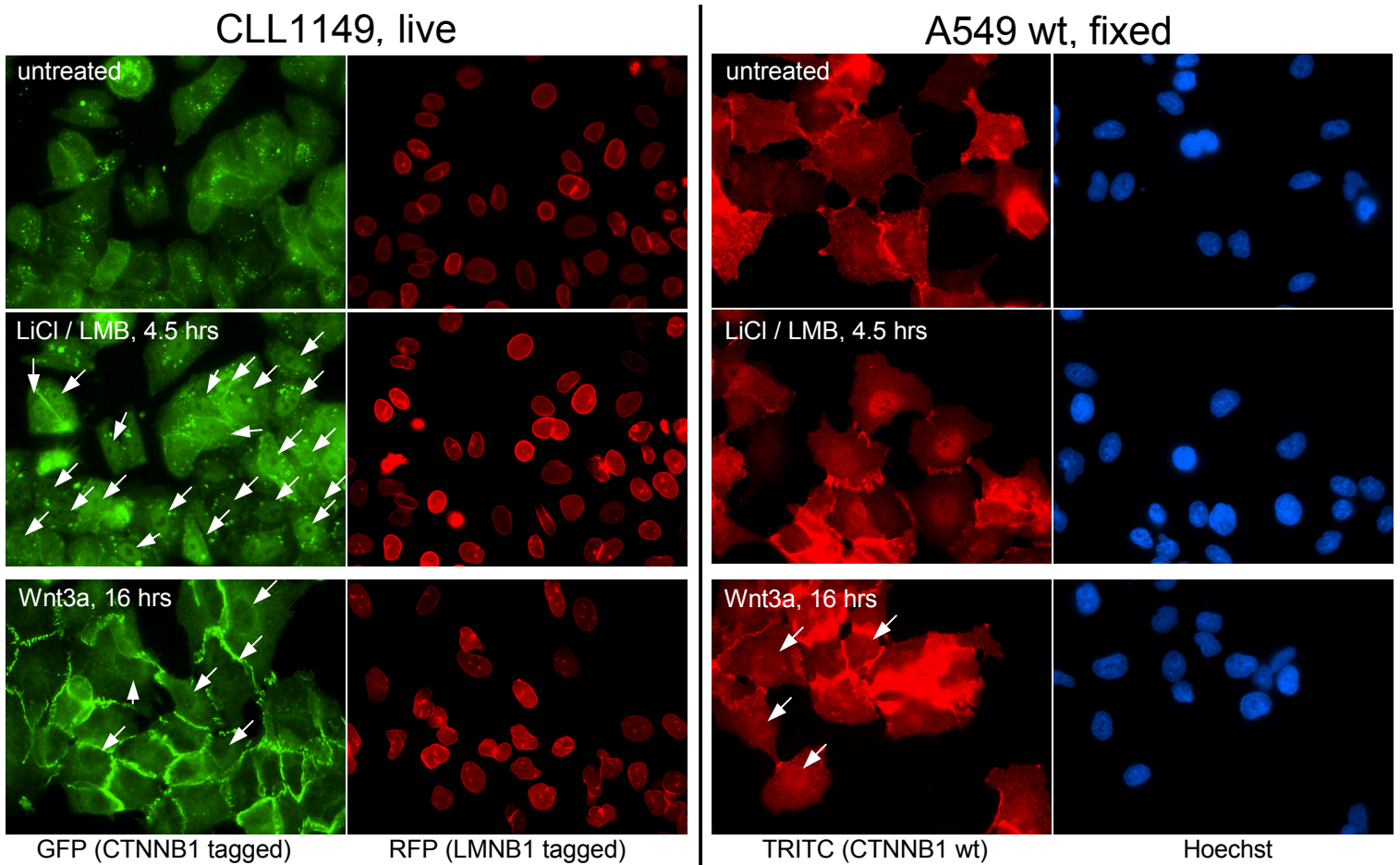


Schematic of the genomic sequence of CTNNB1 (a.) and LMNB1(c.) at the target region (the first translational codon) for integration of the fluorescent tag - FP DNA. ZFN binding sites (red boxes), the ZFN cut site (scissors), and the tag sequence integration site (blue arrow) are shown. Target locus names with the chromosome number are indicated at the top.

Schematics of the CTNNB1 (b.) and LMNB1 (d.) loci showing the coding region (red), untranslated region (brown), and the ZFN cut site (scissors). The Donors (top) have the homology arms of indicated length and the FP sequence (green) fused to the beginning of the target gene coding sequence (both inserts result in N-terminal fusions).

**Figure 3.**

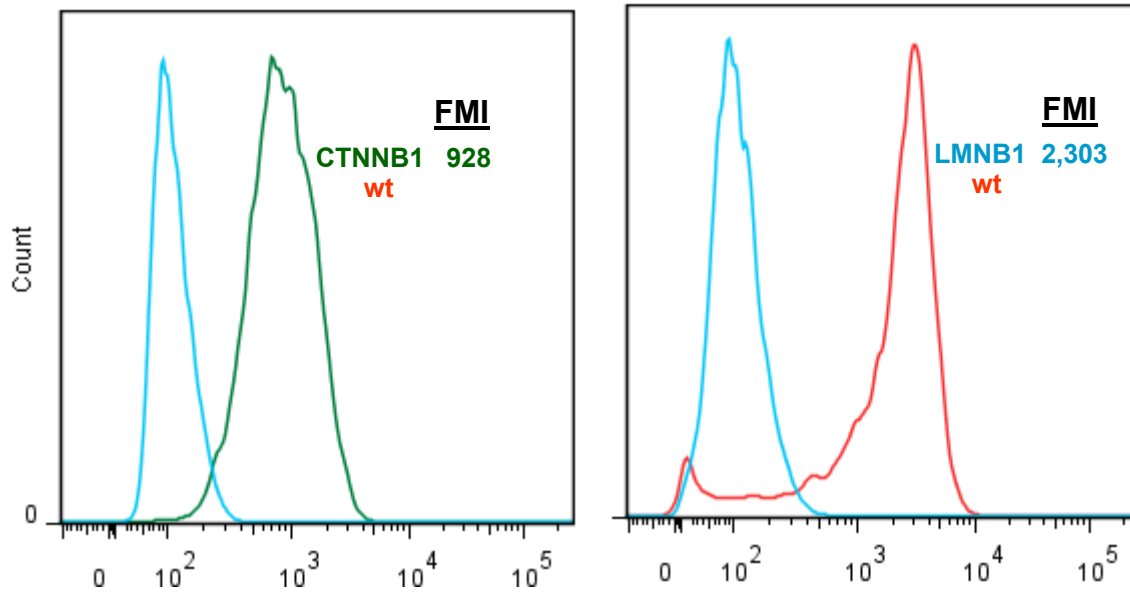
Localization and expression levels of endogenously tagged  $\beta$ -catenin (3XFLAG-GFP-CTNNB1) and wt  $\beta$ -catenin in A549 cells are the same



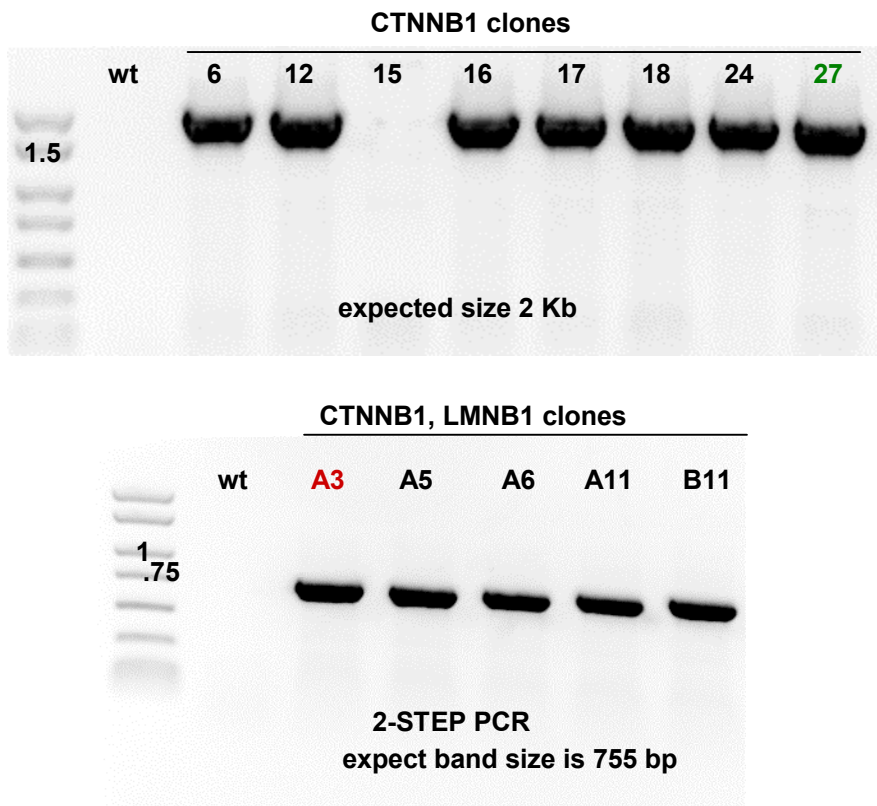
Fluorescence images of CLL1149, a clone expressing endogenous  $\beta$ -catenin (CTNNB1) tagged with 3XFLAG-GFP and endogenous lamin B1 (LMNB1) tagged with RFP (left column), and  $\beta$ -catenin immunofluorescence microscopy images of wild type A549 cells stained with Hoechst dye (right columns). Wild type cells were fixed as in Figure 1. The respective fluorophore (GFP, RFP, TRITC labeled secondary, or Hoechst) giving the signal is shown below the column. Cells were untreated (first row), treated with 40 mM LiCl with 20 ng/mL of leptomycin B for 4.5 hours (second row, the same cells as untreated for CLL1149), or treated with 200 ng/ml Wnt3a for 16 hours. The white arrows highlight the dark nucleolus, which is only observed when CTNNB1 is translocated into the nucleus. CLL1149 and wild type cells respond with a similar degree of nuclear translocation for both treatments.

**Figure 4.**

Expression levels of endogenously tagged  $\beta$ -catenin (CTNNB1-GFP) and lamin B1 (LMNB1-RFP) in A549 cells



Fluorescence analysis of the GFP-CTNNB1 RFP-LMNB1 clone compared to the wild type A549 (autofluorescence) using BD FACSAria™ III. FMI stands for Fluorescence Mean Intensity.

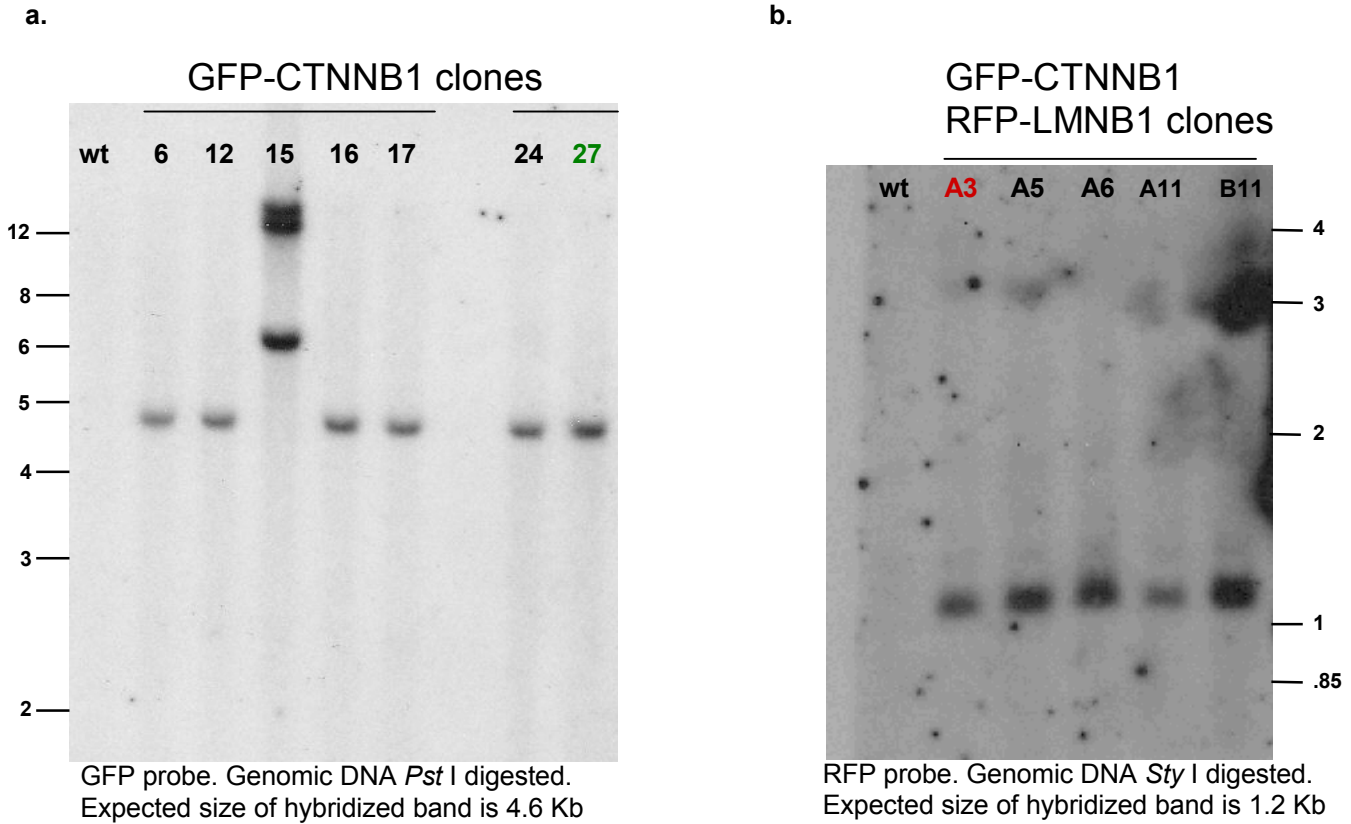
**Figure 5.**

Molecular analysis to identify targeted integration in A549 3XFLAG-GFP-CTNNB1 RFP-LMNB1 clones

3' Junction PCR using GFP forward and CTNNB1 reverse primers (top panel) and RFP forward and LMNB1 reverse primers (bottom panel) produce characteristic fragments for targeted integration (TI) of GFP and RFP, respectively. Clone 27 that had CTNNB1 tagged with 3XFLAG-GFP was used as the background cell line to tag LMNB1 with RFP. No PCR product can be detected in the wild type A549 cell line or clone 15. Clone 15 had GFP randomly integrated in the genome (see Figure 6a) and did not produce the TI band.

**Figure 6.**

Molecular analysis to identify targeted integration in A549 GFP-CTNNB1/RFP-LMNB1 clones



Southern blotting tests for possible random integration of the plasmid donor initially used to create the clones. Genomic DNA from single cell clones and from A549 wild type (wt - served as a negative control) were digested with *Pst* I and *Sty* I restriction endonuclease, respectively. **a.** Proper targeted insertion of the GFP into the CTNNB1 locus should produce a hybridized band of ~4.6 Kb in size. Radioactive-labeled GFP probe was used. Random integration of the donor was seen in Clone 15. Clone 27 was chosen and nucleofected with LMNB1 ZFNs and RFP donor. **b.** Proper targeted insertion of the RFP into the LMNB1 locus should produce a hybridized band of ~1.2 Kb in size. Radioactive-labeled RFP probe was used. Clone A3 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<sup>®</sup> Catalog No. CCL-185<sup>™</sup>.

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.<sup>6-8</sup>

**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<sub>2</sub> 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).
1. 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.
2. Transfer the vial contents to a centrifuge tube containing 9.0 mL of Complete Medium and spin at ~125 × g for 5–7 minutes.
3. Resuspend cell pellet with the Complete Medium and dispense into a 25 cm<sup>2</sup> or a 75 cm<sup>2</sup> 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<sub>2</sub> in air atmosphere is recommended for the Complete Medium.

### Sub-culturing Procedure

Volumes used in this procedure are for a 75 cm<sup>2</sup> 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.<sup>8</sup>



## 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](http://www.wherebiobegins.com/biocells)).

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