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Product Information

SKOV3 GFP-HER2 RFP-EGFR
Ovarian Adenocarcinoma Cell Line with
GFP-tagged HER2 and RFP-tagged EGFR

Catalog Number **CLL1143**
Storage Temperature –196 °C (liquid nitrogen)

TECHNICAL BULLETIN

Product Description
This product is a human SKOV3 cell line in which the genomic HER2 gene has been endogenously tagged with a Green Fluorescent Protein (GFP) gene and the genomic EGFR gene with a Red Fluorescent Protein (RFP) gene using CompoZr® zinc finger nuclease (ZFN) technology.

CompoZr® ZFN 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 into a desired target location – in front of the stop codon for both the HER2 and EGFR loci – to result in the target proteins being fused to the insert at their C-terminus. Donor constructs containing each insert (a fluorescent reporter gene, GFP or RFP, flanked by sequences homologous to the regions on either side of the genomic target site) were nucleofected into SKOV3 cells along with ZFNs designed to cut near the genomic target site (see Figures 1a-d).

The double integration was done in two rounds. During the first round, the cells were nucleofected with the HER2 ZFNs and corresponding GFP-containing donor that resulted in endogenous expression of the fluorescent fusion protein HER2-GFP. 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 HER2-GFP clone as a stable cell line (HER2-GFP cell line available as Catalog No. CLL1135).

This line was used as starting material for the second round of integration in which nucleofection was carried out with the EGFR ZFNs and corresponding RFP-containing donor. A single EGFR-RFP clone was isolated similar to the first round, resulting in generation of the double knockin reporter line (HER2-GFP/ EGFR-RFP) (see Figures 2a and 2b). Junction PCR showed at least one allele was tagged for each target (see Figure 3a) and Southern analysis showed there were no off-target insertions of the reporter sequences (see Figure 3b).

Receptor tyrosine kinases (RTKs), such as Human Epidermal Growth Factor Receptor 2 (HER2/neu or ErbB2) and epidermal growth factor receptor (EGFR or ErbB1), both members of the ErbB protein family, are cell surface growth factor receptors that dimerize and autophosphorylate upon ligand binding. They transduce signals across the cell membrane and thus regulate diverse cell functions involved in growth and proliferation.[1-3]

The HER2 gene is a proto-oncogene located at the long arm of human chromosome 17. Breast cancers are closely associated with an amplification of the HER2/neu gene or overexpression of its protein product.[4,5] Overexpression also occurs in other cancers such as ovarian cancer, stomach cancer, and biologically aggressive forms of uterine cancer.[4] HER2 is thought to be an orphan receptor, with none of the known EGF family of ligands able to activate it.[6] However, ErbB receptors dimerize on ligand binding, and HER2 is the preferential dimerization partner of other members of the ErbB family.[5]

The EGFR gene is located on chromosome 7p12.3-p12.1. Overexpression of EGFR has been found in various cancers. This has lead to the development of clinically approved therapeutics that target EGFR for colon, lung, breast, pancreatic, and head and neck cancers.[7]

ZFN-mediated gene tagging in knockin cell lines provides a basis for the development of various assays for compound screening. Unlike cell lines with overexpression of the gene driven by an exogenous promoter, target gene regulation and corresponding protein function are preserved in these cell lines. In contrast to biochemical assays or immunostaining, using a tagged protein under endogenous regulation allows detection of RTK activation in live cells.

In wild type cells, EGF-stimulated activation of EGFR leads to receptor internalization.[3] HER2 inhibits internalization of ligand-bound EGFR retaining the EGFR-HER2 heterodimeric complexes at the plasma membrane so that only EGFR homodimers are internalized.[8]

Having HER2 and EGFR tagged by different FPs allows tracking of the EGFR internalization process triggered by EGF while simultaneously observing HER2 retention at the plasma membrane (see Figure 4a). The internalization kinetics for EGFR can be quantified by image analysis software to count intracellular granules. The reporter response is abolished by a selective inhibitor of EGFR, Tyrphostin AG 1478 (see Figure 4b).

GFP and TagGFP2, RFP and TagRFP are all synonymous for the fluorescent reporter gene 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

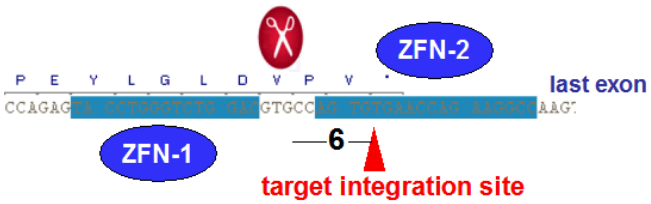
Component

SKOV3 knock-in cell line with HER2 gene C-terminally tagged with GFP and EGFR gene C-terminally tagged with RFP Catalog No. CLL1143	1 vial

One vial of modified SKOV3 cells contains ~2×10^6 cells in Cell Freezing Medium-DMSO 1×, Catalog No. C6164.

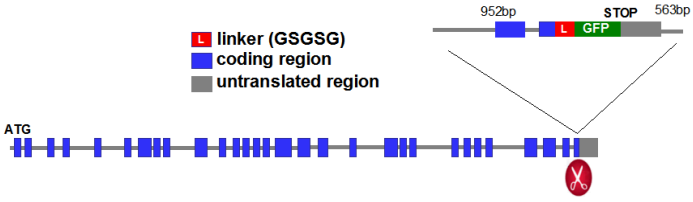
The design of tag sequence integration at the HER2 and EGFR loci

Figure 1a.



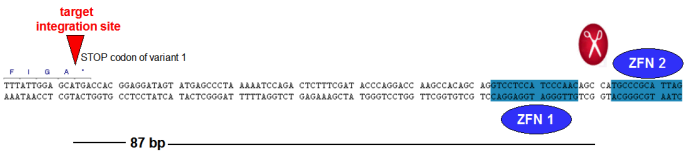
Schematic of genomic sequence of HER2's last exon at the target region for integration of the fluorescent tag GFP. CompoZr ZFN binding sites (blue boxes), ZFN cut site (scissors), and the tag sequence integration site (red arrow).

Figure 1b.



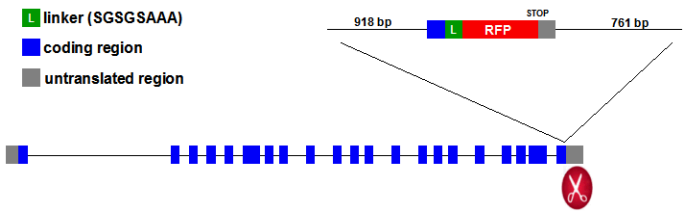
Schematic of HER2 locus showing the coding region (blue), untranslated region (gray), and the ZFN cut site (scissors). The donor (top) has the homology arms of indicated length and the GFP sequence (green) fused at the end of HER2 coding sequence (a C-terminal fusion).

Figure 1c.



Schematic of genomic sequence of EGFR's last exon at the target region for integration of the fluorescent tag RFP.

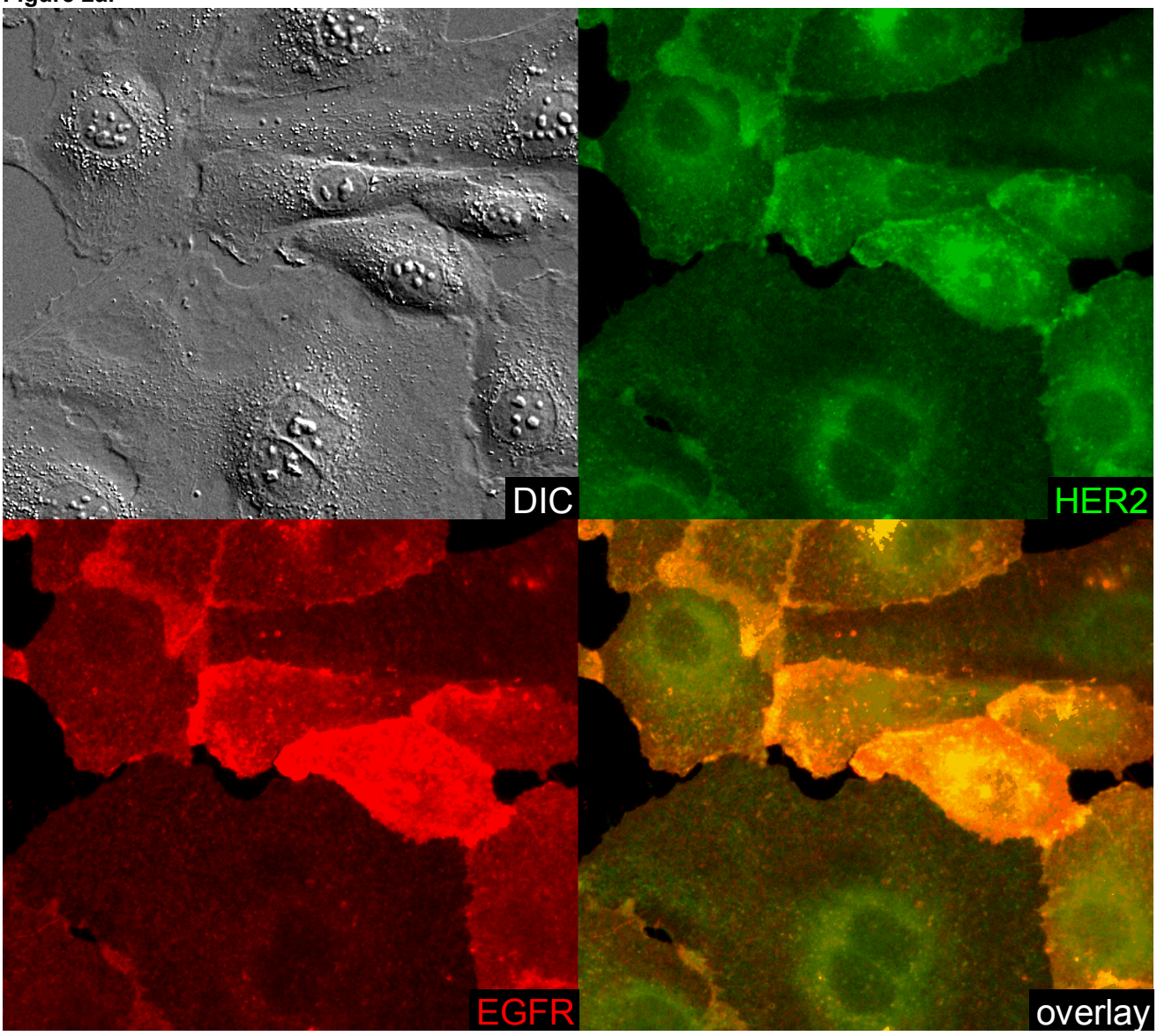
Figure 1d.



Schematic of EGFR locus showing the donor (top) has the homology arms of indicated length and the RFP sequence (red) fused at the end of EGFR coding sequence (a C-terminal fusion).

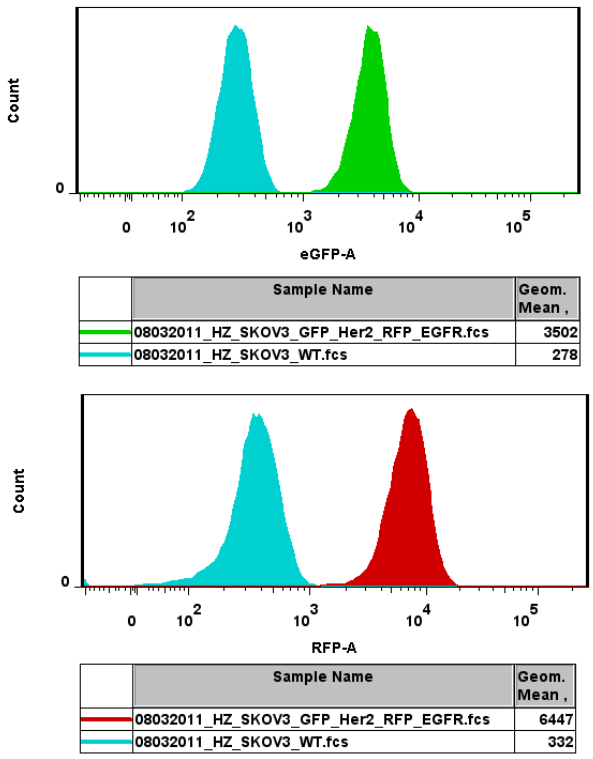
Localization & expression levels of endogenously-tagged HER2 and EGFR

Figure 2a.



Differential interference contrast (DIC) and fluorescence microscopy images of an isolated cell clone expressing endogenous HER2 protein tagged with GFP and endogenous EGFR protein tagged with RFP (GFP: ex 450–490/ em 500–550, RFP: ex 530–560/em 590–650, 40×/1.3 oil). The cells were imaged in Hanks balanced salt solution (Catalog No. H8264) supplemented with 2% fetal bovine serum (Catalog No. F2442). Endogenous expression levels allow for good fluorescence signal separation of the endogenously expressed HER2-GFP/EGFR-RFP fusion versus autofluorescence levels (see Figure 2b).

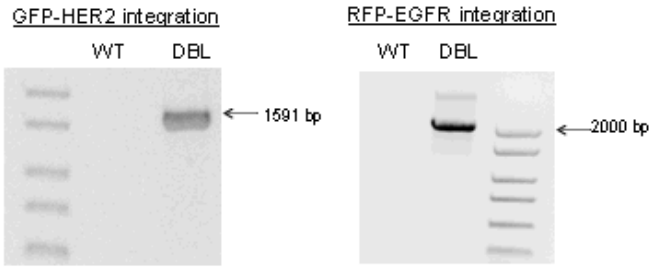
Figure 2b.



Fluorescence analysis of the GFP-HER2/RFP-EGFR double knock-in clone compared to wild type SKOV3 (autofluorescence) using MACSQuant®.

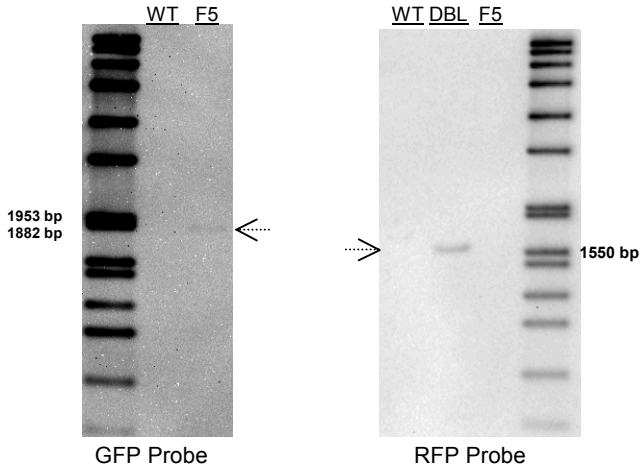
Molecular diagnostics of endogenously tagged HER2/EGFR clones

Figure 3a.



Junction PCR confirms the targeted integration (TI) of GFP at the site of the HER2 locus and RFP at the site of the EGFR locus in the double clone (DBL). PCR was performed on genomic DNAs isolated from the wild type (WT) and the double-tagged clone.

Figure 3b.



Southern blotting shows no random integration from the plasmid donors (either GFP-HER2 or RFP-EGFR) initially used to create the clones. Dig-labeled GFP probe was used for GFP-HER2 clone F5, and its genomic DNA was digested with *Kpn* I. Accurate targeted insertion of the GFP into the HER2 locus should produce a hybridized band of 1868 bp. Dig-labeled RFP probe was used for RFP-EGFR integration in the double knockin cell line, and its genomic DNA was digested with *Bam*H I and *Hin*d III. Accurate targeted insertion of RFP into the EGFR locus should produce a hybridized band of 1550 bp.

EGF effect on HER2-GFP/EGFR-RFP redistribution and its sensitivity to a selective EGFR inhibitor

Figure 4a.

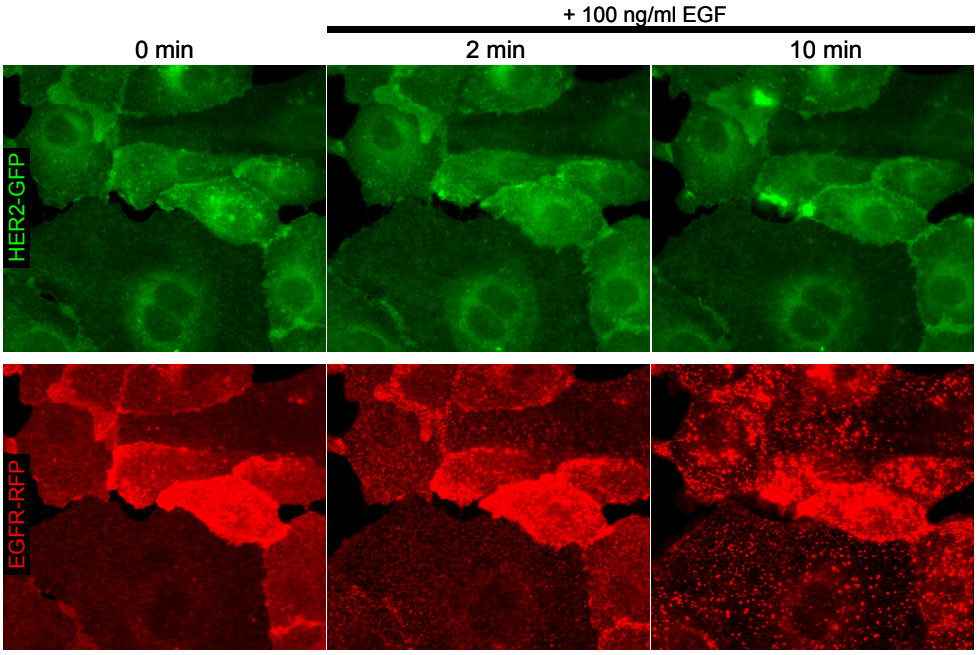
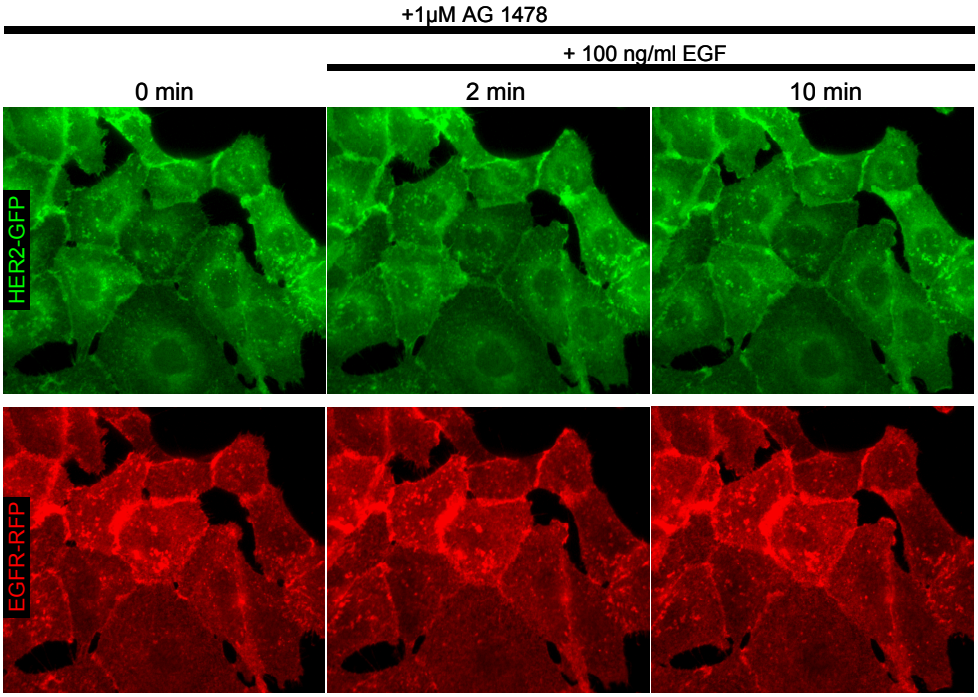


Figure 4b.



Fluorescence microscopy analysis demonstrates the EGFR-RFP fusion protein is translocated from the plasma membrane to endosomes after stimulation with 100 ng/ml of EGF (Catalog No. E9644), while HER2-GFP stays in the plasma membrane (see Figure 4a). Preincubation with 1 µM Tyrphostin AG 1478 (Catalog No. T4182), a selective EGFR inhibitor, for 20 minutes prior to the addition of EGF blocks the internalization of the EGFR-RFP as shown in Figure 4b.

Cell Line Description
Organism*: Homo sapiens* (human)

Tissue: ovary

Age: 64 years

Gender: Female

Ethnicity: Caucasian

Morphology: Epithelial

Growth properties: Adherent

DNA profile
Short Tandem Repeat (STR) analysis:
Amelogenin: X
CSF1PO: 11
D13S317: 8, 11
D16S539: 12
D5S818: 11
D7S820: 13,14
TH01: 9,9.3
TPOX: 8,11
vWA: 17,18

Parental Cell Line: ATCC® Catalog No. HTB-77™

Note: Please see HTB-77 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, SKOV3, 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
To make the complete growth medium, Add L-Glutamine, Catalog No. G7513, at a final concentration of 1.5 mM, and fetal bovine serum, Catalog No. F2442, at a final concentration of 10% in the base medium, McCoy's 5A medium, Catalog No. M8403. 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 5 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
6. Incubate cultures at 37 °C.

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

References

1. Sorkin, A. & Goh, L.K., Endocytosis and intracellular trafficking of ErbBs. Exp Cell Res., **315**, 683-696 (2009).
2. Liyong, C. et al., Mutational analysis of ErbB2 intracellular localization. Histochem Cell Biol., **128,** 473–483 (2007).
3. Ono, M. & Kuwano, M., Molecular mechanisms of epidermal growth factor receptor (EGFR) activation and response to gefitinib and other EGFR-targeting drugs. Clin. Cancer Res., **12**, 7242-7251 (2006).
4. Santin, A.D. et al., Trastuzumab treatment in patients with advanced or recurrent endometrial carcinoma overexpressing HER2/neu. Int J Gynaecol Obstet **102**, 128–131 (2008).
5. Olayioye, M.A., Update on HER-2 as a target for cancer therapy: intracellular signaling pathways of ErbB2/HER-2 and family members. Breast Cancer Res., **3,** 385–389 (2001).
6. Hashizume, T. et al., Cell type dependent endocytic internalization of ErbB2 with an artificial peptide ligand that binds to ErbB2. Cell Biol Int., **32**, 814-826 (2008).
7. Lurje, G. & Lenz, H.J., EGFR signaling and drug discovery. Oncology, **77**, 400-410 (2009).
8. Haslekås C. et al., The Inhibitory Effect of ErbB2 on Epidermal Growth Factor-induced Formation of Clathrin-coated Pits Correlates with Retention of Epidermal Growth Factor Receptor-ErbB2 Oligomeric Complexes at the Plasma Membrane. Mol Biol Cell., **16**, 5832–5842 (2005)
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).

Please see the Label License Agreement (LLA) for further details regarding the use of this product. The LLA is available on our website at www.wherebiobegins.com/biocells

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