

Applications	Reactivity	Sensitivity	MW (kDa)	Isotype
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W IP IHC-P IF-P IF-IC F H Endogenous 175 Rabbit IgG

Applications Key: W=Western Blotting IP=Immunoprecipitation IHC-P=Immunohistochemistry (Paraffin) IF-P=Immunofluorescence (Paraffin) IF-IC=Immunofluorescence (Immunocytochemistry) F=Flow Cytometry

Reactivity Key: H=Human

Species cross-reactivity is determined by western blot. Species enclosed in parentheses are predicted to react based on 100% sequence homology.

Protocols

Flow Cytometry Protocol

A. Solutions and Reagents

NOTE: Prepare solutions with purified water.

- 1X Phosphate Buffered Saline (PBS):** Dissolve 8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄ and 0.24 g KH₂PO₄ in 800 ml dH₂O. Adjust the pH to 7.4 with HCl and the volume to 1 L. Store at room temperature.
- Formaldehyde** (methanol free).
- 100% Methanol**
- Incubation Buffer:** Dissolve 0.5 g bovine serum albumin (BSA) in 100 ml 1X PBS. Store at 4 °C.

B. Fixation

- Collect cells by centrifugation and aspirate supernatant.
- Resuspend cells briefly in 0.5–1 ml PBS. Add formaldehyde to a final concentration of 2–4% formaldehyde.
- Fix for 10 min at 37 °C.
- Chill tubes on ice for 1 min.
- For extracellular staining with antibodies that do not require permeabilization, proceed to Section D, Step 1 or store cells in PBS with 0.1% sodium azide at 4 °C; for intracellular staining, proceed to permeabilization (Section C, Step 1).

C. Permeabilization

- Permeabilize cells by adding ice-cold 100% methanol slowly to pre-chilled cells, while gently vortexing, to a final concentration of 90% methanol. Alternatively, to remove fix prior to permeabilization, pellet cells by centrifugation and resuspend in 90% methanol.
- Incubate 30 min on ice.
- Proceed with immunostaining (Section D, Step 1) or store cells at –20 °C in 90% methanol.

D. Immunostaining

NOTE: Account for isotype matched controls for monoclonal antibodies or species matched IgG for polyclonal antibodies. Count cells using a hemocytometer or alternative method.

- Aliquot 0.5–1x10⁶ cells into each assay tube (by volume).
- Add 2–3 ml Incubation Buffer to each tube and rinse by centrifugation. Repeat.
- Resuspend cells in 100 µl Incubation Buffer per assay tube.
- Block in Incubation Buffer for 10 min at room temperature.

5. Add the unconjugated, biotinylated, or fluorochrome-conjugated primary antibody at the appropriate dilution to the assay tubes (see individual antibody datasheet for the appropriate dilution).
6. Incubate for 1 hr at room temperature.
7. Rinse as before in Incubation Buffer by centrifugation.
8. If using a fluorochrome-conjugated primary antibody, resuspend cells in 0.5 ml PBS and analyze on flow cytometer; for unconjugated or biotinylated primary antibodies, proceed to immunostaining (Section D, Step 9).
9. Resuspend cells in fluorochrome-conjugated secondary antibody or fluorochrome-conjugated avidin, diluted in Incubation Buffer at the recommended dilution.
10. Incubate for 30 min at room temperature.
11. Rinse as before in Incubation Buffer by centrifugation.
12. Resuspend cells in 0.5 ml PBS and analyze on flow cytometer; alternatively, for DNA staining, proceed to optional DNA stain (Section E, Step 1).

E. Optional DNA Stain

1. Resuspend cells in 0.5 ml of DNA dye (e.g. Propidium Iodide (PI)/RNase Staining Solution [#4087](#)).
2. Incubate for at least 5 min at room temperature.
3. Analyze cells in DNA stain on flow cytometer.

Immunohistochemistry Protocol (Paraffin)

Specific For Products:

- [EGF Receptor \(E746-A750del Specific\) \(6B6\) XP® Rabbit mAb #2085](#)
- [EGF Receptor \(L858R Mutant-Specific\) \(43B2\) Rabbit mAb #3197](#)

***IMPORTANT:** See product data sheets for the appropriate antibody dilution.

A. Solutions and Reagents

1. Xylene
2. Ethanol, anhydrous denatured, histological grade (100% and 95%)
3. Deionized water (dH₂O)
4. Hematoxylin (optional)
5. **Wash Buffer:**

1X TBS/0.1% Tween-20 (1X TBST): To prepare 1 L add 100 ml 10X TBS to 900 ml dH₂O. Add 1 ml Tween-20 and mix.

10X Tris Buffered Saline (TBS): To prepare 1 L add 24.2 g Trizma® base (C₄H₁₁NO₃) and 80 g sodium chloride (NaCl) to 1 L dH₂O. Adjust pH to 7.6 with concentrated HCl.

6. **Antibody Diluent:** [SignalStain® Antibody Diluent #8112](#)
7. **Antigen Unmasking:** EDTA: 1 mM EDTA: To prepare 1 L add 0.372 g EDTA (C₁₀H₁₄N₂O₈Na₂•2H₂O) to 1 L dH₂O. Adjust pH to 8.0.
8. **3% Hydrogen Peroxide:** To prepare, add 10 ml 30% H₂O₂ to 90 ml dH₂O.
9. **Blocking Solution:** TBST/5% normal goat serum ([#5425](#)): to 5 ml 1X TBST add 250 µl normal goat serum.
10. EnVision™+ HRP, Rabbit ([Dako North America](#), Inc. Carpinteria, CA #K4003)
11. **DAB Reagent or suitable substrate:** Prepare according to manufacturer's recommendations.

B. Deparaffinization/Rehydration

NOTE: Do not allow slides to dry at any time during this procedure.

1. Deparaffinize/hydrate sections:
 - a. Incubate sections in three washes of xylene for 5 minutes each.
 - b. Incubate sections in two washes of 100% ethanol for 10 minutes each.
 - c. Incubate sections in two washes of 95% ethanol for 10 minutes each.
2. Wash sections twice in dH₂O for 5 minutes each.

C. Antigen Unmasking

NOTE: This procedure describes the conditions that are recommended for the Biocare Medical Decloaking Chamber. Device-specific settings and operating instructions should be utilized for other pressure cookers.

1. Place slides in 250 ml room temperature EDTA unmasking solution in a 24-slide holder.
2. Place 500 ml dH₂O into the pressure cooker.
3. Place the slide holder into the pressure cooker, touching the heat shield. It may be advantageous to place a second 24-slide holder into the pressure cooker, filled with 250 ml water and blank slides.
4. Seal the chamber and proceed with retrieval. Settings for the Biocare Medical Decloaking Chamber follow.
 - a. SP1 125 °C 30 seconds
 - b. SP2 90 °C 10 seconds
5. Carefully vent the device, then remove the lid and cool the slides on the bench for 10 minutes.
6. Rinse the slides with dH₂O.

D. Staining

1. Wash sections in dH₂O three times for 5 minutes each.
2. Incubate sections in 3% hydrogen peroxide for 10 minutes.
3. Wash sections in dH₂O twice for 5 minutes each.
4. Wash section in wash buffer for 5 minutes.
5. Block each section with 100–400 µl blocking solution for 1 hour at room temperature.
6. Remove blocking solution and add 100–300 µl primary antibody diluted in [SignalStain® Antibody Diluent #8112](#) to each section. Incubate overnight at 4 °C. *Refer to product datasheets to determine the recommended dilution: [2085](#), [3197](#).*
7. Equilibrate EnVision™+ reagent to room temperature.
8. Remove antibody solution and wash sections in wash buffer three times for 5 minutes each.
9. Add 1–2 drops of EnVision™+ to each section. Incubate 30 minutes at room temperature.
10. Remove EnVision™+ solution and wash sections three times with wash buffer for 5 minutes each.
11. Add 100–400 µl DAB or suitable substrate to each section and monitor staining closely.
12. Upon completion of development, immerse slides in dH₂O.
13. If desired, counterstain sections in hematoxylin per manufacturer's instructions.
14. Wash sections in dH₂O two times for 5 minutes each.
15. Dehydrate sections:

- a. Incubate sections in 95% ethanol two times for 10 seconds each.
 - b. Repeat in 100% ethanol, incubating sections two times for 10 seconds each.
 - c. Repeat in xylene, incubating sections two times for 10 seconds each.
16. Mount coverslips.

Immunofluorescence General Protocol

IMPORTANT: Please refer to the APPLICATIONS section on the front page of product datasheet to determine if this product is validated and approved for use on cultured cell lines (IF-IC), paraffin-embedded samples (IF-P), or frozen tissue sections (IF-F). Please see product datasheet for appropriate antibody dilution and unmasking solution.

A. Solutions and Reagents

NOTE: Prepare solutions with purified water.

1. **10X Phosphate Buffered Saline (PBS):** To prepare 1 L add 80 g sodium chloride (NaCl), 2 g potassium chloride (KCl), 14.4 g sodium phosphate, dibasic (Na_2HPO_4) and 2.4 g potassium phosphate, monobasic (KH_2PO_4) to 1 L dH_2O . Adjust pH to 8.0.
2. **Formaldehyde:** 16%, methanol free, [Polysciences, Inc.](#) (cat# 18814), use fresh, store opened vials at 4 °C in dark, dilute in PBS for use.
3. **Blocking Buffer:** (1X PBS / 5% normal goat serum ([#5425](#)) / 0.3% Triton™ X-100): To prepare 25 ml, add 2.5 ml 10X PBS, 1.25 ml normal serum from the same species as the secondary antibody (e.g., normal goat serum, normal donkey serum) and 21.25 ml dH_2O and mix well. While stirring, add 75 µl Triton™ X-100.
4. **Antibody Dilution Buffer:** (1X PBS / 1% BSA / 0.3% Triton™ X-100): To prepare 40 ml, add 4 ml 10X PBS and 120 µl Triton™ X-100 to 0.4 g BSA. Bring to final volume of 40 ml with dH_2O and mix well.
5. **Fluorochrome-conjugated secondary antibody NOTE:** When using any primary or fluorochrome-conjugated secondary antibody for the first time, titrate the antibody to determine which dilution allows for the strongest specific signal with the least background for your sample.
6. **Prolong® Gold Anti-Fade Reagent ([#9071](#)),** with DAPI ([#8961](#)).

Reagents specific to IF-P application:

1. **Xylene**
2. **Ethanol**, anhydrous denatured, histological grade, 100% and 95%.
3. **Antigen Unmasking:**
 - a. **For Citrate:** 10 mM Sodium Citrate Buffer: To prepare 1 L add 2.94 g sodium citrate trisodium salt dihydrate ($\text{C}_6\text{H}_5\text{Na}_3\text{O}_7 \cdot 2\text{H}_2\text{O}$) to 1 L dH_2O . Adjust pH to 6.0.
 - b. **For EDTA:** 1 mM EDTA: To prepare 1 L add 0.372 g EDTA ($\text{C}_{10}\text{H}_{14}\text{N}_2\text{O}_8\text{Na}_2 \cdot 2\text{H}_2\text{O}$) to 1 L dH_2O . Adjust pH to 8.0.

B. Specimen Preparation

I. Cultured Cell Lines (IF-IC)

NOTE: Cells should be grown, treated, fixed and stained directly in multi-well plates, chamber slides or on coverslips.

1. Aspirate liquid, then cover cells to a depth of 2–3 mm with 4% formaldehyde in PBS. **NOTE:** Formaldehyde is toxic, use only in fume hood.
2. Allow cells to fix for 15 min at room temperature.
3. Aspirate fixative, rinse three times in PBS for 5 min each.
4. Proceed with Immunostaining (Section C).

II. Paraffin Sections (IF-P)

NOTE: Do not allow slides to dry at any time during this process.

1. **Deparaffinization/Rehydration:**

- a. Incubate sections in three washes of xylene for 5 min each.
- b. Incubate sections in two washes of 100% ethanol for 10 min each.
- c. Incubate sections in two washes of 95% ethanol for 10 min each.
- d. Rinse sections twice in dH₂O for 5 min each.

2. **Antigen Unmasking:**

NOTE: Consult product datasheet for specific recommendation for the unmasking solution.

2.

- a. **For Citrate:** Bring slides to a boil in 10 mM sodium citrate buffer pH 6.0, then maintain at a sub-boiling temperature for 10 min. Cool slides on bench top for 30 min.
- b. **For EDTA:** Bring slides to a boil in 1 mM EDTA pH 8.0 followed by 15 min at a sub-boiling temperature. No cooling is necessary.
3. Proceed with Immunostaining (Section C).

III. Frozen/Cryostat Sections (IF-F)

1. For fixed frozen tissue proceed with Immunostaining (Section C).
2. For fresh, unfixed frozen tissue, please fix immediately, as follows:
 - a. Cover sections with 4% formaldehyde in PBS.
 - b. Allow sections to fix for 15 min at room temperature.
 - c. Rinse slides three times in PBS for 5 min each.
 - d. Proceed with Immunostaining (Section C).

C. Immunostaining

NOTE: All subsequent incubations should be carried out at room temperature unless otherwise noted in a humid light-tight box or covered dish/plate to prevent drying and fluorochrome fading.

1. Block specimen in Blocking Buffer for 60 min.
2. While blocking, prepare primary antibody by diluting as indicated on datasheet in Antibody Dilution Buffer.
3. Aspirate blocking solution, apply diluted primary antibody.
4. Incubate overnight at 4 °C.
5. Rinse three times in PBS for 5 min each.

NOTE: If using primary antibodies directly conjugated with Alexa Fluor® fluorochromes, then skip to (Section C, Step 8).

6. Incubate specimen in fluorochrome-conjugated secondary antibody diluted in Antibody Dilution Buffer for 1–2 hr at room temperature in dark.
7. Rinse in PBS (Section C, Step 5).
8. Coverslip slides with Prolong® Gold Anti-Fade Reagent ([#9071](#)), with DAPI ([#8961](#)).
9. For best results, allow mountant to cure overnight at room temperature. For long-term storage, store slides flat at 4 °C protected from light.

Western Immunoblotting Protocol (Primary Ab Incubation In BSA)

For Western blots, incubate membrane with diluted antibody in 5% w/v BSA, 1X TBS, 0.1% Tween-20 at 4 °C with gentle shaking, overnight.

Products available from Cell Signaling Technology are linked by their respective catalog numbers.

A. Solutions and Reagents

NOTE: Prepare solutions with Milli-Q or equivalently purified water.

1. **1X Phosphate Buffered Saline (PBS).**
2. **1X SDS Sample Buffer:** ([#7722](#), [#7723](#)) 62.5 mM Tris-HCl (pH 6.8 at 25 °C), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue or phenol red.
3. **Transfer Buffer:** 25 mM Tris base, 0.2 M glycine, 20% methanol (pH 8.5).
4. **10X Tris Buffered Saline (TBS):** ([#9997](#)) To prepare 1 liter of 10X TBS: 24.2 g Tris base, 80 g NaCl; adjust pH to 7.6 with HCl (use at 1X).
5. **Nonfat Dry Milk:** ([#9999](#)) (weight to volume [w/v]).
6. **Blocking Buffer:** 1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk; for 150 ml, add 15 ml 10X TBS to 135 ml water, mix. Add 7.5 g nonfat dry milk and mix well. While stirring, add 0.15 ml Tween-20 (100%).
7. **Wash Buffer:** 1X TBS, 0.1% Tween-20 (TBS/T).
8. **Bovine Serum Albumin (BSA):** ([#9998](#)).
9. **Primary Antibody Dilution Buffer:** 1X TBS, 0.1% Tween-20 with 5% BSA; for 20 ml, add 2 ml 10X TBS to 18 ml water, mix. Add 1.0 g BSA and mix well. While stirring, add 20 µl Tween-20 (100%).
10. **Phototope®-HRP Western Blot Detection System:** ([#7071 anti-rabbit](#)) or ([#7072 anti-mouse](#)) Includes biotinylated protein ladder, secondary ([#7074 anti-rabbit](#)) or ([#7076 anti-mouse](#)) antibody conjugated to horseradish peroxidase (HRP), anti-biotin antibody conjugated to HRP, LumiGLO® chemiluminescent reagent and peroxide.
11. **Prestained Protein Marker, Broad Range (Premixed Format):** ([#7720](#)).
12. **Biotinylated Protein Ladder Detection Pack:** ([#7727](#)).
13. **Blotting Membrane:** This protocol has been optimized for nitrocellulose membranes, which CST recommends. PVDF membranes may also be used.

B. Protein Blotting

A general protocol for sample preparation is described below.

1. Treat cells by adding fresh media containing regulator for desired time.
2. Aspirate media from cultures; wash cells with 1X PBS; aspirate.
3. Lyse cells by adding 1X SDS sample buffer (100 µl per well of 6-well plate or 500 µl per plate of 10 cm diameter plate). Immediately scrape the cells off the plate and transfer the extract to a microcentrifuge tube. Keep on ice.
4. Sonicate for 10–15 seconds for complete cell lysis and to shear DNA (to reduce sample viscosity).
5. Heat a 20 µl sample to 95–100 °C for 5 minutes; cool on ice.
6. Microcentrifuge for 5 minutes.
7. Load 20 µl onto SDS-PAGE gel (10 cm x 10 cm). **NOTE:** CST recommends loading prestained molecular weight markers ([#7720](#), 10 µl/lane) to verify electrotransfer and biotinylated protein ladder ([#7727](#), 10 µl/lane) to determine molecular weights.

8. Electrotransfer to nitrocellulose or PVDF membrane.

C. Membrane Blocking and Antibody Incubations

NOTE: Volumes are for 10 cm x 10 cm (100 cm²) of membrane; for different sized membranes, adjust volumes accordingly.

1. (Optional) After transfer, wash nitrocellulose membrane with 25 ml TBS for 5 minutes at room temperature.
2. Incubate membrane in 25 ml of blocking buffer for 1 hour at room temperature.
3. Wash three times for 5 minutes each with 15 ml of TBS/T.
4. Incubate membrane and primary antibody (at the appropriate dilution) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4 °C.
5. Wash three times for 5 minutes each with 15 ml of TBS/T.

I. For Unconjugated Primary Antibodies

1. Incubate membrane with **appropriate** HRP-conjugated secondary antibody (1:2000) and HRP-conjugated anti-biotin antibody (1:1000) to detect biotinylated protein markers in 10 ml of blocking buffer with gentle agitation for 1 hour at room temperature.
2. Wash three times for 5 minutes each with 15 ml of TBS/T.

II. For HRP Conjugated Primary Antibodies

Skip to Detection of Proteins (Step D).

III. For Biotinylated Primary Antibodies

1. Incubate membrane with HRP-Streptavidin (at the appropriate dilution) in milk for one hour with gentle agitation at room temperature.
2. Wash three times for 5 minutes each with 15 ml of TBS/T.

D. Detection of Proteins

1. Incubate membrane with 10 ml LumiGLO® (0.5 ml 20X LumiGLO®, 0.5 ml 20X Peroxide and 9.0 ml Milli-Q water) with gentle agitation for 1 minute at room temperature. **NOTE:** LumiGLO® substrate can be further diluted if signal response is too fast.
2. Drain membrane of excess developing solution (do not let dry), wrap in plastic wrap and expose to x-ray film. An initial 10-second exposure should indicate the proper exposure time. **NOTE:** Due to the kinetics of the detection reaction, signal is most intense immediately following LumiGLO® incubation and declines over the following 2 hours.

* Product-specific protocol.

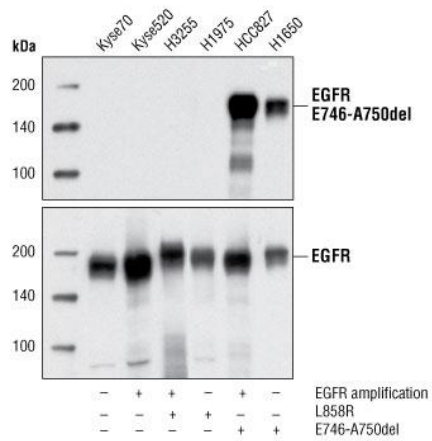
Specificity / Sensitivity

EGF Receptor (E746-A750del Specific) (6B6) XP® Rabbit mAb detects endogenous levels of EGFR E746-A750del mutant protein.

Source / Purification

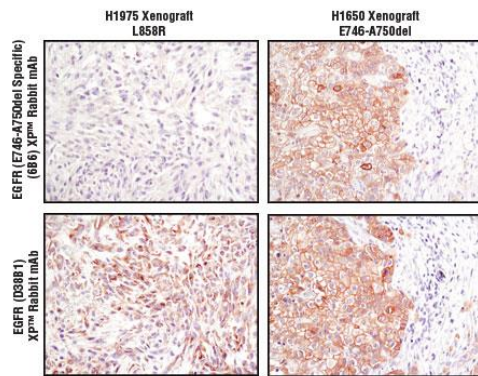
Monoclonal antibody is produced by immunizing animals with a synthetic peptide corresponding to E746-A750del mutant sequence of human EGFR.

Western Blotting



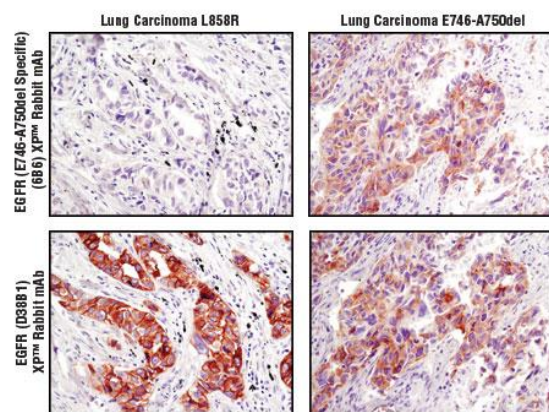
Western blot analysis of extracts from various EGFR expressing cell lines, showing the specificity of detection of mutant EGFR (E746-A750del) protein, using EGF Receptor (E746-A750del Specific) (6B6) XP[®] Rabbit mAb (upper), and total EGFR control antibody (lower).

IHC-P (paraffin)



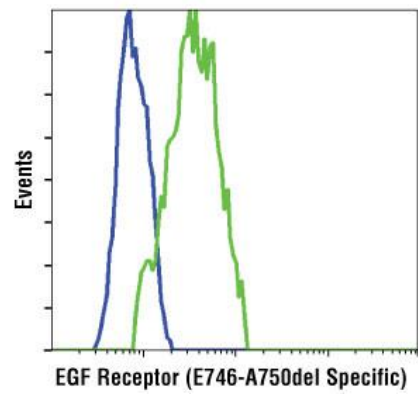
Immunohistochemical analysis of paraffin-embedded H1975 (left, EGFR L858R positive) and H1650 xenografts (right, EGFR deletion positive) using EGF Receptor (E746-A750del Specific) (6B6) XP[®] Rabbit mAb (upper) and total EGFR (D38B1) XP[®] Rabbit mAb #4267 (lower).

IHC-P (paraffin)



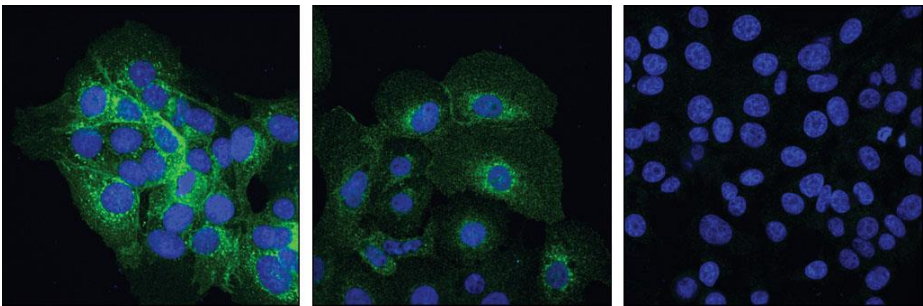
Immunohistochemical analysis of paraffin embedded human lung carcinoma of known mutational status using EGF Receptor (E746-A750del Specific) (6B6) XP[®] Rabbit mAb (upper) and total EGFR (D38B1) XP[®] Rabbit mAb #4267 (lower). EGFR L858R positive lung (left), EGFR deletion positive lung sections (right).

Flow Cytometry



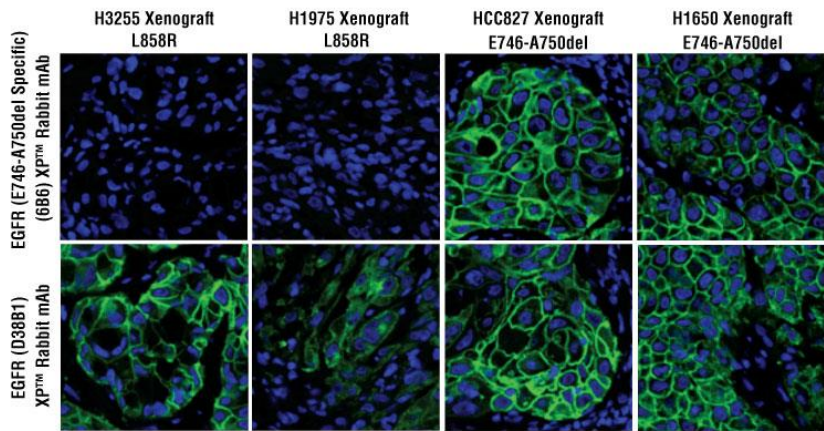
Flow cytometric analysis of Kyse450 cells (wildtype, blue) and HCC827 cells (exon 19 deletion (E746-A750del, green) using EGF Receptor (E746-A750del Specific) (6B6) XP[®]Rabbit mAb.

IF-IC



Confocal immunofluorescent analysis of HCC827, H1650 and Kyse450 cells using EGF Receptor (E746-A750del Specific) (D6B6) XP[®]Rabbit mAb (green). Blue pseudocolor = DRAQ5[®] #4084 (fluorescent DNA dye).

IF-P



Confocal immunofluorescent analysis of paraffin-embedded H3255, H1975, HCC827 and H1650 xenografts using EGF Receptor (E746-A750del Specific) (6B6) XP[®]Rabbit mAb (upper, green) and EGFR (D38B1) XP[®] Rabbit mAb #4267 (lower, green). Blue pseudocolor = DRAQ5[®] #4084 (fluorescent DNA dye).