Optimization and Validation of Activation-State Specific Antibodies for the Immunohistochemical Analysis of Fresh Frozen Tissues and Cells

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Introduction

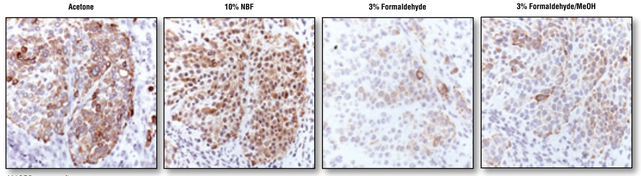
Activation-state specific antibodies are useful tools for pre-clinical research and may prove to be clinically relevant as predictive biomarkers of disease. To date, most of the immunohistochemical analyses performed with such antibodies have utilized formalin-fixed paraffin-embedded samples. In this study we analyzed a broad panel of phospho-specific and total antibodies directed against various signaling markers such as mTOR, Erk, S6 ribosomal protein and EGFR. To assess the utility of these antibodies on frozen sections, fresh frozen mouse tissue, xenograft models of human cancer and control or treated cell pellets were used. Along with optimizing fixatives and dilutions, other methods of validation were utilized, including peptide blocking and phosphatase-treatment.

Methods

Xenografts were initiated using 5-10 x 10^6 cells in 50% Matrigel™ injected subscutaneously into NCR/nu mice. Upon harvest tumors were snap frozen in liquid nitrogen and embedded in OCT. For pellet preparation, cells were obtained from ATCC and cultured in the recommended medium. After overnight serum starvation, treatments were performed as follows: 200 nM TPA (Phorbol-12-Myristate-13-Acetate) for 30 minutes at 37°C or 100 ng/ml EGF (Epidermal Growth Factor) for 5 minutes at 37°C. (TPA and EGF were both from CST.) Cells were scraped, washed in PBS, pelleted, snap frozen in liquid nitrogen and embedded in OCT. Efficacy of cell treatments was demonstrated by Western blot analysis using lysate prepared from the same stock of cells that were pelleted and frozen. Western blotting was performed according to the standard CST protocol. Frozen sections were cut at 7-8 µm and stored at -80°C. Immunohistochemical analysis was performed on sections taken from -80°C and brought to ambient temperature, fixed and stained according to standard CST protocols, with primary antibody incubations overnight at 4°C. All antibodies were from CST. Lambda phosphatase treatments were performed with 6% lambda protein phosphatase (400, 000 U/ml, from NEB) along with a lambda phosphatase reaction buffer control for 2 hours in an incubation chamber at 37°C for two hours. Peptide blocking was performed as follows: Twice the volume of peptide as volume of antibody were combined in 100 µl total volume. Antibody/peptide mixture was incubated for 30 minutes prior to adding the entire volume to the slide. Slides were then stained according to CST protocol. Staining was evaluated based on a 1+ - 3+ staining intensity scoring system. This was defined as any immunohistochemical staining of tumor or normal cells, depending on the expected localization, above background level.

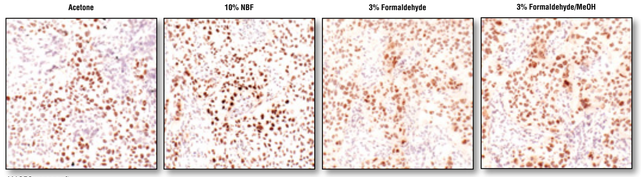
Fixation Optimization for Frozen Sections

Phospho-mTOR (Ser2448) (49F9) Rabbit mAb (IHC Specific) #2976



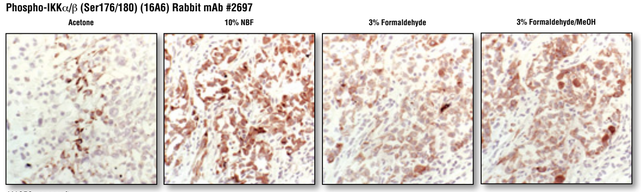
H1650 xenograft

Phospho-c-Jun (Ser63) (54B3) Rabbit mAb #2361



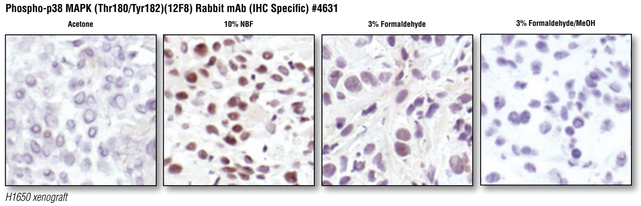
H1650 xenograft

Phospho-IKKα/β (Ser176/180) (16A6) Rabbit mAb #2697



H1650 xenograft

Phospho-p38 MAPK (Thr180/Tyr182)(12F8) Rabbit mAb (IHC Specific) #4631



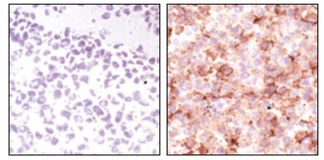
H1650 xenograft

Fixation Optimization Table

CST Product	Fixatives Tested: Acetone	10% NBF	3% Formaldehyde	Formaldehyde/MeOH	Optimal
#2148 α/β-Tubulin	++	-	+++	NT	Acetone
#2211 Phospho-S6 Ribosomal Protein (Ser235/236) Antibody	-	+++	-	-	10% NBF
#2234 Phospho-EGF Receptor (Tyr1068) Antibody	-	-	++	+++	Form/MeOH
#2242 HER2/ErbB2 Antibody	+++	-	-	NT	Acetone
#2243 Phospho-HER2/ErbB2 (Tyr1221/1222) (6B12) Rabbit mAb	-	+	+++	+++	Form/MeOH
#2361 Phospho-c-Jun (Ser63) (54B3) Rabbit mAb	+++	+++	+++	+++	Form/MeOH
#2401 Phospho-HSP27 (Ser82) Antibody	-	+++	+	NT	10% NBF
#2479 VEGF Receptor 2 (55B11) Rabbit mAb	-	++	NT	++	Form/MeOH
#2527 p53 (7F5) Rabbit mAb	-	+++	+	+	10% NBF
#2697 Phospho-IKKα/β (Ser176/180) (16A6) Rabbit mAb	-	+++	++	+++	Form/MeOH
#2764 Bcl-xL (54H6) Rabbit mAb	-	++	+	-	10% NBF
#2808 Survivin (71G4) Rabbit mAb	++	+++	++	NT	10% NBF
#2976 Phospho-mTOR (Ser2448) (49F9) Rabbit mAb (IHC Specific)	+++	-	NT	++	Acetone
#3195 E-Cadherin (24E10) Rabbit mAb	+++	+++	+++	+++	Form/MeOH
#3238 Caveolin-1	++	++	+++	NT	3% Form
#3512 Connexin 43	++	++	++	++	3% Form
#3787 Phospho-Akt (Ser473) (736E11) Rabbit mAb (IHC Specific)	-	-	++	+	3% Form
#4056 Phospho-Akt (Thr308) (244F9) Rabbit mAb	-	-	++	NT	3% Form
#4376 Phospho-p44/42MAPK (Thr202/Tyr204) (20G11) Rabbit mAb (IHC Preferred)	-	+++	++	-	10% NBF
#4407 Phospho-EGF Receptor (Tyr1173) (53A5) Rabbit mAb	-	-	++	+++	Form/MeOH
#4631 Phospho-p38 MAPK (Thr180/Tyr182)(12F8) Rabbit mAb (IHC Preferred)	-	+++	+	++	10% NBF
#4850 COX IV (3E11) Rabbit mAb	+++	+++	+++	NT	Acetone
#4857 Phospho-S6 Ribosomal Protein (Ser235/236) (91B2) Rabbit mAb (IHC Preferred)	-	+++	-	-	10% NBF
#4895 Mre11 Antibody	+	+++	++	++	10% NBF
#4968 Pan-Actin Antibody	+++	++	++	++	Acetone
#4970 β-Actin (13E5) Rabbit mAb	-	+++	++	-	10% NBF
#9145 Phospho-Stat3 (Tyr705) (D3A7) Rabbit mAb	-	++	+++	+++	3% Form
#9165 c-Jun (60A8) Rabbit mAb	+	+++	+++	NT	3% Form
#9197 CREB (48H2) Rabbit mAb	-	+++	++	-	10% NBF
#9198 Phospho-CREB (Ser133) (87G3) Rabbit mAb	-	+++	NT	+++	10% NBF
#9221 Phospho-ATF-2 (Thr71) Antibody	+	++	+++	+++	10% NBF
#9225 Phospho-ATF-2 (Thr69/71) Antibody	-	++	+++	+++	10% NBF
#9226 ATF-2 (20F1) Rabbit mAb	-	+++	+	+	10% NBF
#9251 Phospho-SAPK/JNK (Thr183/Tyr185) Antibody	-	+++	+	-	10% NBF
#9661 Cleaved Caspase-3 (Asp175) Antibody	-	+	+++	NT	3% Form
#9664 Cleaved Caspase-3 (Asp175) Rabbit mAb		+	+++	NT	3% Form
#9701 Phospho-Histone H3 (Ser10) Antibody	-	+++	NT	NT	10% NBF

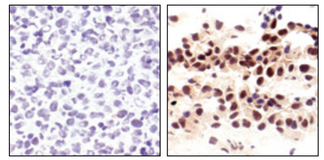
Untreated and Treated Frozen Cell Pellets

Phospho-EGF Receptor (Tyr1173) (53A5) Rabbit mAb #4407



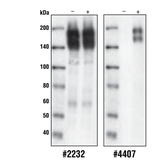
Immunohistochemical analysis of frozen, OCT-embedded MDA-MB-468 cells, untreated (left) or EGF-treated (right), using Phospho-EGF Receptor (Tyr1173) (53A5) Rabbit mAb #4407.

Phospho-p44/42MAPK (Thr202/Tyr204) (20G11) Rabbit mAb (IHC Preferred) #4376

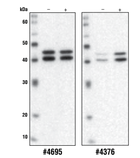


Immunohistochemical analysis of frozen ,OCT-embedded 3T3 cells, untreated (left) or TPA-treated (right), using Phospho-p44/42MAPK (Thr202/Tyr204) (20G11) Rabbit mAb (IHC Preferred) #4376.

Western Blots



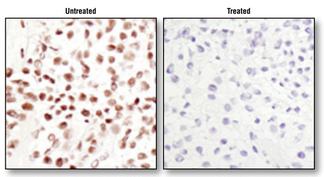
WB analysis of MDA-MB-468 cells untreated or treated with EGF, using EGF Receptor Antibody #2232 (left) or Phospho-EGF Receptor (Tyr1173) (53A5) Rabbit mAb #4407 (right).



WB analysis of 3T3 cells untreated or treated with TPA, using p44/42 MAPK (137F5) Rabbit mAb #4695 (left) or Phosph-p44/42 MAPK (Thr202/Tyr204) (20G11) Rabbit mAb (IHC Preferred) #4376 (right).

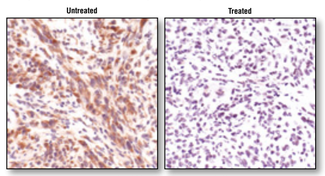
Phosphatase Treatments

Phospho-CREB (Ser133) (87G3) Rabbit mAb #9198



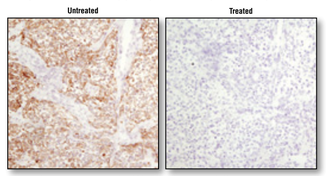
Immunohistochemical analysis of H1650 xenograft, untreated (left) or λ phosphatae-treated (right) using #9198.

Phospho-S6 Ribosomal Protein (Ser235/236) Antibody #2211



Immunohistochemical analysis of H1650 xenograft, untreated (left) or λ phosphatae-treated (right) using #2211.

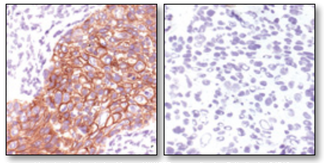
Phospho-mTOR (Ser2448) (49F9) Rabbit mAb (IHC Specific) #2976



Immunohistochemical analysis of H1650 xenograft, untreated (left) or phosphatae-treated (right) using #2976.

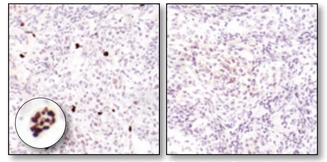
Peptide Blocking

E-Cadherin (24E10) Rabbit mAb #3195



Immunohistochemical analysis of frozen HCC827 xenograft using E-Cadherin (24E10) Rabbit mAb #3195 preincubated with control peptide (left) or E-Cadherin (24E10) Blocking Peptide #1056 (right).

Phospho-Histone H3 (Ser10) Antibody #9701



Immunohistochemical analysis of frozen HCC827 xenograft using Phospho-Histone H3 (Ser10) Antibody #9701 preincubated with control peptide (left) or Phospho-Histone H3 (Ser10) Blocking Peptide #1000 (right).

Conclusion

We observed that determining the optimal fixative for frozen sections is imperative for many antibodies. Use of a sub-optimal fixative often resulted in background staining, inappropriate localization of stain, or lack of staining entirely. Staining phosphorylated epitopes can be achieved in frozen samples without unmasking epitopes, as is often required for paraffin-embedded samples. Methods previously utilized with paraffin-embedded samples, such as phosphatase-treatment, peptide blocking and treated cell pellets, translated to frozen samples. Our results suggest that working with frozen platforms and activation-state specific antibodies is feasible and therefore offers an alternative method of analysis for research and clinical samples.

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