Immunohistochemistry protocol for γH2AX detection (formalin-fixed paraffin-embedded sections)

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Method Article

Keywords: γH2AX , immunohistochemistry, DNA damage checkpoints, DNA damage response, senescence

Posted Date: December 6th, 2006

DOI: https://doi.org/10.1038/nprot.2006.366

Abstract

Introduction

DNA damage accumulation and consequent DNA damage response activation may be the result of a variety of DNA insults and may contribute to the phenotypes associated with aging and cancer initiation. One of the most robust markers of the generation of a DNA double strand break and DNA damage response activation is the phosphorylation of the histone H2AX on serine 139. The modified form \(also known as γH2AX) labels the sites of DNA damage by spreading for megabases of chromatinised DNA. Its detection is therefore a reliable readout of the cellular events associated with DNA damage generation. We describe here a detailed protocol for the reliable detection of γH2AX in mammalian \(mouse or human) formalin-fixed paraffin embedded tissues.

Reagents

Formalin-fixed, paraffin-embedded tissue sections cut at 2µm. Histolemon-Erba, Carlo Erba Reagents, cat. 454912 EDTA 0.25mM pH8 H_2O_2 30%, Sigma cat. H1009 Ab mix: TBS1X – BSA4% - Tween20 0.02% γH2AX mouse Upstate Biotechnology \(cat.05-636) 1:200 in Ab mix anti-mouse DAKO Cytomation Envision+System Labelled Polymer-HRP, cat. K4001 DAKO Cytomation liquid DAB+ Substrate Chromogen System, cat. K3468 Harris Hematoxylin, EMS cat. 26041 Eukitt, O. Kindler GmbH&Co

Procedure

1.Deparaffinize tissue sections in Histolemon 2X10 min 2.Hydrate tissue sections through graded alcohol series \(100%,95%,70%, H_2O) 1X5 min 3.Incubate the slides in EDTA for 50 min at 95°C for antigen unmasking 4.Cool the slides for 20 min at room temperature \(RT), then wash once in H_2O 5. Incubate the slides in 3% H_2O_2 for 5 min at RT for quenching of endogenous peroxidases 6.Wash twice in TBS 1X 7.Preincubate the slides with Ab mix for 20 min at RT 8.Incubate the slides ON at 4˚C with anti-γH2AX diluted in Ab mix at indicated concentration, then wash twice in TBS 1X 9. Incubate the slides with secondary antibody for 30 min at RT, then wash twice in TBS 1X 10. Incubate the slides in peroxidase substrate solution for 5 min at RT. 11.Block the reaction in H_2O, counterstain with Hematoxilin 15 sec and wash the slides in running H_20 12.Dehydrate tissue sections through graded alcohol series and mount with Eukitt 13.Positive \(irradiated skin) and negative \(not irradiated skin) controls were included in each experiment

Timing

Approximately 5h

Critical Steps

The specificity of the antibody in immunohistochemistry must be verified by testing known positive and negative controls. A positive control is the tissue in which the molecule of interest is known to be expressed and in the specific case a X-rays irradiated tissue. On the other side, the simplest negative control is the absence of expression in tissues in which the molecule of interest is known not to be expressed. A better negative control is the elimination of the signal by pre-incubating the antibody with an excess of the peptide or protein with which it was raised, or by replacing the primary antibody with buffer in Ab mix. Each single experiment should run positive as well as negative controls simultaneously with the testing samples. Antigen unmasking can be optimized for different antibodies. DAB solution must be prepared just before the use according to manufacturer's protocol.

Troubleshooting

When tissue staining has not given the expected results, several experimental variables should be considered: NO STAINING 1) Confirm that no reagents were omitted and that were added in the correct order and for sufficient incubation times. 2) Check antibody titrations and dilutions. This is particularly important for the primary antibody. 3) Check reagent expiration dates and storage. 4) Check specimen storage. 5) Check that the microscope is adjusted correctly. 6) Do not let the slides air dry in any step of the procedure BACKGROUND 1. The pre-incubation time can be increased in order to reduce the background 2. Re-titer antibodies \(both primary and secondary) with a dilution series. 3. Incubate with chromogen for a shorter time. Some chromogens, such as DAB, develop very quickly. 4. Enzyme or biotin in the tissue is reacting with the reagent. This can be prevented by increasing the time or concentration of blocking, trying different types of blocking, or using a combination of more than one blocking, or changing the staining methods. 5. The incorrect blocking serum was used, or blocking serum was not used. The blocking serum should be from the species of the secondary antibody. 6. The secondary antibody cross-reacts with endogenous tissue proteins. 7. The embedding media may not be completely removed from the tissue. Review the removal procedure for possible changes.

References

Immunocytochemical Methods and Protocols \(second edition), edited by Lorette C. Javois, from _Methods in Molecular Medicine_, **volume 115**, Humana Press, 1999