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Validation of Commercial Histone Modification Antibodies to be Used for ChIP

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Overview: There are numerous issues that need to be addressed with respect to validating commercial histone modification antibodies used extensively for ChIP-chip and ChIP-seq analyses. The main issues are 1) specificity of antibodies with respect to other nuclear/chromatin proteins, 2) specificity with respect to unmodified histones and other modified histone residues (e.g. H3K9me and H3K27me), 3) specificity with respect to mono-, di-, and tri- methylation at the same residue (e.g. H3K9me1, me2 and me3), and 4) lot-to-lot variation. Validation of commercial antibodies is necessary to produce the high quality data sets desired by the modENCODE and ENCODE consortia. We propose that all commercial histone antibodies be validated by at least 2 independent methods, as described below. New lots of antibody must be analyzed independently. The proposed tests may be performed by ENCODE/modENCODE labs or by the companies that sell the antibodies, but only if the companies provide data for the specific lots of antibody.

Test #1: All antibodies must be checked for reactivity with non-histone proteins and with unmodified histone by performing Western blot analysis of total nuclear extract and recombinant histone. To enable visual quantification of reactivity, a concentration series of both extract and recombinant histone should be analyzed, using recombinant histone levels that are comparable to the respective histone in nuclear extract. Since cross-reactivity may vary between species, this test should be performed using nuclear extracts from each species to be studied by ChIP. In nuclear extracts, the specific nuclear histone band should constitute at least 50% of the nuclear protein signal, show at least 10-fold enrichment relative to any other single band, and show at least 10-fold enriched signal relative to unmodified histone.

Test #2 options: In addition to test #1, antibody specificity must be verified by at least one additional test. The tests and the pros and cons of each are described below, followed by the likely flow of tests.

A. Peptide binding tests. Histone tail peptides with particular modifications can be purchased commercially. Peptide binding and peptide competition assays provide a fast method to initially evaluate the specificity and relative strength of binding of antibodies to histone tails with different modifications (e.g. H3K9 and H3K27 and me1, me2, and me3 levels of methylation). A potential drawback is that antibodies may differ in their binding specificity toward histone tail peptides in vitro versus toward full-length histones in the context of chromatin in IP experiments. Nevertheless, observing at least a 10-fold enriched binding signal for the modification of interest relative to other modifications would contribute to confidence in antibody specificity.

B. Mass spec. For antibodies generated against related and historically problematic modifications, the ability of the antibody to effectively distinguish between similar histone

marks (e.g. H3K9me and H3K27me) and between different levels of methylation (e.g. H3K9me1, me2 and me3) should be tested by mass spec analysis of material IP'd from histone preparations. The target modification should constitute at least 80% of the IP'd histone signal, and contaminating bands should not contribute more than 20%.

C. Mutants defective in modifying histones. Mutants (in S. cerevisiae, S. pombe, Drosophila, C. elegans, or mammalian cells) that lack the ability to catalyze particular histone modifications offer the opportunity to test antibody specificity. Antibody signal should be reduced to below 10% of wild-type signal in mutant samples, compared to wild type. RNAi depletion of histone modifying activity may be substituted for mutants. Mutant or RNAi reduction of signal can be assayed by Western blot analysis or by immunofluorescence staining. Mutant/RNAi tests usually don't allow testing antibodies for the ability to discriminate between mono, di, and trimethylation. In cases where more than one enzyme modifies the same residue (e.g. K9 methylation in Drosophila), double mutants or RNAi may be required.

D. Mutant histones. Mutant histones (e.g. histone H3 with Lys4 mutated to Arg or Ala) expressed in yeast provide another avenue to alter histones for testing specificity by western blot analysis or even by ChIP. Since the modified residue is mutated, we expect at least a 10-fold reduction in signal relative to wild-type histone preparations. Mutant histone tests cannot distinguish whether antibodies discriminate between mono, di, and trimethylation.

Flow of tests: For antibodies to methylated histone residues, we envision the flow of tests will be: #1 western blot analysis to make sure the antibody does not show significant cross-reaction with unmodified histone or non-histone proteins; #2A peptide binding/competition tests to make sure the antibody does not interact with histone tail peptides lacking modifications or bearing other modifications; for similar and problematic modifications, #2B mass spec analysis to make sure the antibody does not IP unmodified histone or histone bearing other modifications.

For antibodies to histone modifications other than methylations, we envision the flow of tests will be: #1 western blot analysis to make sure the antibody does not show significant cross-reaction with unmodified histone or non-histone proteins; #2A peptide binding/competition tests to make sure the antibody does not interact with histone tail peptides lacking modifications or bearing other modifications OR one of the other #2 tests listed above.

Grandfathering old antibodies and testing new lots of antibodies: Once ChIP-chip or ChIP-seq results are obtained with antibodies validated as described above, then old or new antibodies can be validated by similar testing or by obtaining ChIP results using old/new antibodies that are statistically consistent with ChIP results using validated antibodies (based on ENCODE and modENCODE standards for statistical agreement between replicates).

Use of 2 different antibodies: Even if antibodies pass the specificity tests described above, observing very similar ChIP results with 2 independent antibodies would give added confidence in the results. Therefore, we encourage using 2 independent antibodies whenever possible, providing statistical comparisons of the results, and presenting the intersection of the peak sets obtained with the 2 antibodies.