ENCODE fRIP Antibody Characterization Guidelines

Approved on November 4, 2016

Background

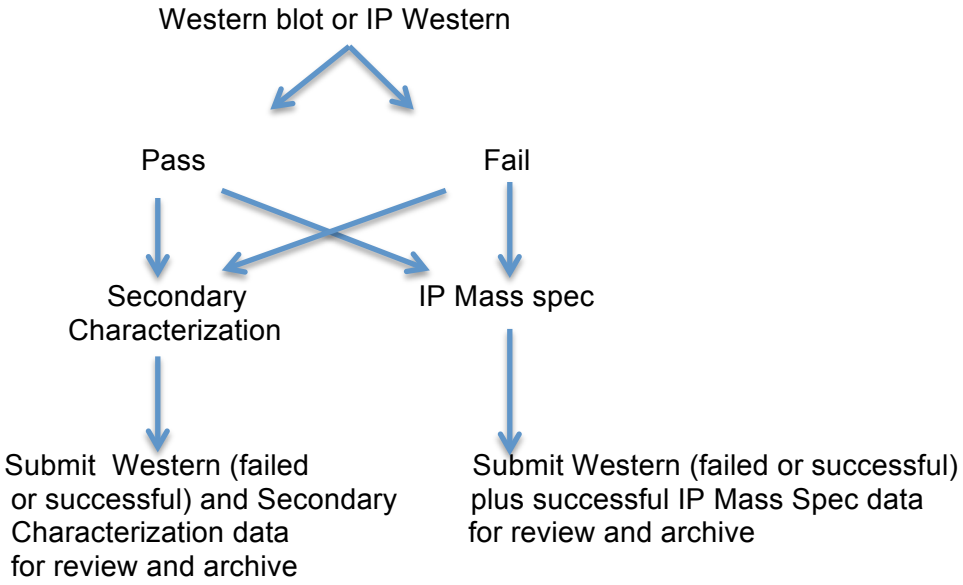
An integral part of the ENCODE Project is to characterize the antibodies used in the experiments. This document describes the guidelines used by the ENCODE Consortium for characterizing antibodies used in **fRIP** experiments. We expect that these antibody characterization data will be pertinent to users of ENCODE data as well as to future users of these antibody reagents.

As detailed below, the antibodies that pass our working thresholds for Primary and, as appropriate, a supporting Secondary Characterization method, are believed by ENCODE data producers to recognize the target factor and to be active in immunoprecipitation in the context of the cell type(s) tested.

The antibody characterization data themselves provide users with richer and more nuanced information than can be captured by pass/fail threshold parameters alone. Therefore, all antibody characterization data are available at the ENCODE data portal. As part of the ENCODE fRIP data finalization process, the antibody characterization data are reviewed by a panel of ENCODE investigators and analysts, and successfully validated antibodies are designated as such. In some instances, the characterization data fall short of current ENCODE thresholds or assay specifications, yet the antibody has generated fRIP data that we judge potentially useful to the community. We flag such antibodies and corresponding data as having a quality concern, and the nature of the characterization deficiency is reported. Because antibody reagents can vary considerably from one specific lot number to another, each lot is characterized independently.

It is important to recognize that our necessarily arbitrary thresholds might ultimately prove to have been either too strict or too relaxed for particular antibody/factor pairs. There may also be confounding differences between antibody-epitope recognition in fRIP experiments versus the denatured (Western blot) or native (mass spec) states that are required by the Primary Characterization methods. *We therefore recommend that users review our primary antibody characterization data in light of their own expert knowledge, any additional data, or their own specific requirements.*

I-A. Characterization flowchart:



Primary characterization by Western blot or Immunoprecipitation/blot

For every antibody used in fRIP, ENCODE data producers first perform an immunoblot characterization. This can be either a standard Western blot, or an immunoprecipitation followed by a Western blot ("IP Western"). If the blot results do not meet the parameters and thresholds given below, then Primary Characterization Method 2 (IP mass spec) is performed. In the latter case, the failed (or partially failed) immunoblot that preceded IP/mass spec is included in the report so that researchers and data users can independently evaluate the data for each antibody.

Immunoblot parameters:

a. ENCODE developed a set of working parameters and thresholds to identify antibodies with a high likelihood of being specific for the target factor. The parameters allow for modest variation in gel migration characteristics and in band number to accommodate known behaviors of typical nuclear factors. Current acceptable parameters are that the major band is within 20% of the size predicted by the size of the coding region and corresponds to >50% of all bands on the gel (excluding the antibody bands in the case of an immunoprecipitation). If the western or IP-western results meet these criteria, we consider the antibody to meet expectations for the primary characterization. The immunoblot results (which must include appropriate size markers) are submitted as evidence for each cell type or tissue tested. For IP-westerns, a control IgG precipitation is also performed and analyzed on the same gel.

b. If the antibody fails to pass the immunoblot tests because the bands observed are too numerous, or too far from the predicted migration behavior, it can be “rescued” by a secondary characterization that supports the conclusion that the band(s) detected correspond to the correct protein (e.g. all bands are reduced by treatment with a specific siRNA to that protein; see secondary characterizations).

c. If the antibody passes the immunoblot tests, a further characterization is required to support the successful immunoblot. This can be Primary Characterization Method 2 (IP mass spec) or any one of the Secondary Characterization methods.

d. Additional IP critieria specific for fRIP.

Owing to the cross-linked nature of fRIP, antibodies are also tested using the following pipeline. Each targeted Chromatin Associated Protein (CAPs) is immunoprecipitated under fRIP conditions and analyzed by western. Two independent IPs are performed and compared to 10% of lysate, under fRIP conditions. Antibodies are designated as passing this QC step if (i) both independent immunoprecipitations show a band of appropriate size; (ii) there is not a larger band than the targeted CAP (smaller bands are allowed as we suspect many of the smaller bands are degradation products caused by the shearing and cross-linking conditions; and (iii) the targeted CAP that has the strongest signal on the blot corresponds to the size of the protein to which the antibody has been raised.

Primary Characterization Method 2: Immunoprecipitation followed by mass spectrometry ("IP mass spec")

If the immunoblot characterization data was not successful (ranging from no bands to patterns that do not meet the thresholds given above), then Mass spec of an immunoprecipitation can be performed. The failed or ambiguous immunoblot is, however, shown as part of the antibody characterization dataset. Because the IP/mass spec assay provides explicit evidence about the identity of the proteins detected, it can also be used in lieu of Secondary methods after a successful Immunoblot (see flowchart above).

For fRIP mass spec, a cell or nuclear extract is immunoprecipitated with the same antibody used to perform fRIP. That IP is then fractionated on a denaturing polyacrylamide gel, and the fractions are prepared and analyzed by mass spec as described below.

What is reported for IP mass spec:

- IP-western blot of gel image with outline of gel slices submitted for mass spec.
- All peptides (with peptide counts) from all immunoreactive bands.
- Fold enrichment of all peptides in the immunoreactive bands vs either mock IP or a set of proteins that have been immunoprecipitated from the same cell type using a collection of other antibodies from the same host species (the list of proteins used as the set of IP contaminants list must be provided).
- Indication as to which proteins above the target protein on the ranked list (ranked by fold enrichment) are TFs and which TFs are members of the same TF family as the target protein.

IP mass spec requirements to be considered fully validated for ENCODE data:

- The target protein should be enriched in the IP when compared to a mock IP or to a set of proteins that have been immunoprecipitated from the same cell type using a collection of other antibodies from the same host species.
- The target should be in the top 25 ranked proteins and the top most-enriched CAP (by fold enrichment) in the immunoreactive band, unless the higher ranked CAPs are known interacting partners of the target CAP and/or a known interacting partner of one of the other higher ranked CAPs that is a known partner of the target CAP. Evidence for interaction can come from publications or refer to records in interaction databases such as BioGRID, or other sources.
- The target should be the top ranked member of that family of CAPs (exceptions will be

allowed if a publication is provided that demonstrates that a higher ranked family member is known to dimerize with the target protein).

- In situations for which the target protein has 0 peptides in the mock IP, a ranking by enrichment can not be performed. In that case, the following criteria are used:
    - If the target CAP is the top CAP as ranked by number of detected peptides, then the antibody passes this characterization method.
    - If the target CAP is not the top CAP but the CAPs having more counts have previously been documented to be in the same complex and/or interact directly with the target CAP, then the antibody passes this characterization method.
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    - If the target CAP is not the top CAP but the non-target CAPs (having a greater number of detected peptides) were detected using mass spec analysis of two different antibodies to the target CAP, then the antibody passes this characterization method (with the assumption that the other CAPs are bona fide interacting CAPs that have not yet been documented in the literature).
    - If the target CAP is not the top CAP and the CAPs having more counts have never been linked to the target CAP then this antibody is flagged, with the explanation that enrichment could not be determined due to the lack of detected peptides in the IgG and that no published data exists linking the target to the non-target CAPa.
- If an antibody doesn't meet these characteristics, the antibody characterization document can be submitted for consideration as a special request (see Note 3).

Additional situations for Primary Characterization

a. Guidelines for using the same lot number of a previously characterized antibody in a new cell type

If a specific lot number for an antibody has previously passed characterization in another cell type, and if the banding pattern on the immunoblot or immunoprecipitation is the same in the new cell type as in the characterized cell type, then no further characterization is needed for the antibody in that new cell type. If the banding pattern is different in the new cell type, a secondary characterization is performed in the new cell type. Exceptions to this guideline will be considered for studies of human tissues (due to the fact that it is often hard to obtain sufficient tissue for the antibody characterization and a subsequent experiment). If an antibody has passed characterization criteria in 2 different human cell lines and/or tissues, it does not have to be characterized in each tissue type.

b. Guidelines for using a different lot number of a previously characterized antibody

For the first time that a new lot number is used for a previously-characterized antibody, a Primary Characterization method (immunoblot or IP mass spec) is performed with one of the same cell types used to characterize the previous lot number plus the cell type for which data will be deposited for the new lot number; the ENCODE antibody accession number of the specific previously characterized lot that should be used for comparison is indicated. If the patterns for the new lot number are the same in the previously characterized cell type and in the cell type for which data will be deposited as shown in the characterization of the original lot number of that antibody, then no further characterization is required. If the banding patterns are different, a secondary characterization is performed.

Other primary characterization methods. If other methods not specified above are used for primary characterization of an antibody, the antibody characterization document is submitted as a special request and is so annotated and flagged.

IB. Secondary Characterization Methods. These methods are used to support and clarify the Immunoblot data. In particular, they aim to verify that a band or bands observed on the prior blot correspond to the intended CAP. At least one successful Secondary Characterization (or alternatively IP/Mass spec as shown in figure 1 above) is required to support a successful Western or IP/Western.

Secondary Characterization Method 1: siRNA or shRNA against the mRNA of the target protein

For siRNA or shRNA knockdown characterization, the band(s) detected by the antibody on a western blot should be reduced by at least 50% of the control signal. These methods are especially intended to address instances where the Western or IP Western data give multiple bands and unpredicted migration patterns. The sequence or vendor and catalog number of the oligonucleotide(s) reagent should be provided. A control knockdown should also be performed. Cell types will be labeled and size markers should be included on the immunoblot. A brief description of the transfection protocol will also be provided.

Secondary Characterization Method 2: data from a previously characterized antibody that was validated for ChIP-seq.

In some cases, antibodies to CAPs that were previously validated for ChIP-seq are used in fRIP. Antibodies that have passed the current characterization standards for use in ChIP-seq are considered acceptable for use in fRIP if they also pass the fRIP-specific primary characterization requirement described in I-A, part d; see current antibody characterization standards for transcription factors and antibody characterization standards for histone modifications and chromatin-associated proteins for details.