Antibody Characterization Standards
ENCODE 3
February 2014

Characterization of an antibody for a transcriptional regulator (including TFs, chromatin remodelers, and chromatin modifiers)

Before ChIP-seq data can be submitted for any transcriptional regulator (including TFs, RNA-binding proteins and chromatin remodelers), the specific lot number of the antibody used to collect the data must be approved for use in that cell type by the antibody characterization committee of the ENCODE DCC. A primary and secondary characterization must both be submitted for review and deemed COMPLIANT, in order for an antibody lot to be approved for use.

1. In all cases, a primary characterization of the antibody must be provided for each lot number and each specific cell type. This can be either an immunoblot (Western blot) or an immunoprecipitation (IP-Western). [slide 1]

a. If the major band is within 20% of the size predicted by the size of the coding region of the protein and corresponds to >50% of all bands on the gel (excluding the antibody bands in the case of an immunoprecipitation), then the antibody passes this initial characterization. Each immunoblot should indicate which cells types were tested and include size markers. For immunoprecipitations, a control IgG precipitation should also be included.

b. If the antibody does not pass this initial characterization (e.g. due to multiple splice variants or multiple modifications that alter the electrophoretic properties of the protein), 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 upon treatment with siRNA to that protein).

2. Use of 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 both primary and secondary 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 must be performed in the new cell type. [slide 2]

3. Use of a new lot number of a previously characterized antibody. If this is the first time that a new lot number has been used for a previously characterized antibody, a primary characterization must be performed. However, if the pattern is the same as for the old lot number of the previously characterized antibody, then no further characterization is required. If the banding pattern is different, a secondary characterization must be performed. [Slide 3]

Allowable Secondary Characterization Methods:

1) **Secondary characterization using siRNA or shRNA against the mRNA of the target protein**. For siRNA or shRNA characterization, the band(s) detected by the antibody should be reduced to no more than 50% of the original signal. The sequence or vendor and catalog

number of the oligonucleotide(s) should be provided. A control knockdown should also be performed. Cell types should be labeled and size markers should be included on the immunoblot. A brief description of the transfection protocol should also be provided.

2) **Secondary characterization using ChIP-seq data obtained using a previously characterized antibody for that factor.** If ChIP-seq data for a different lot number of a previously characterized antibody or a previously characterized, but different, antibody for a given transcriptional regulator is available, this ChIP-seq data can be used for characterization of a new antibody or new lot number. In addition, ChIP-seq data obtained using an epitope-tagged version of the target protein and an antibody that recognizes the tag can be used for comparison. The ChIP-seq data from the new antibody or new lot number should be compared to the previous ChIP-seq data. If the two datasets pass the ENCODE IDR cuts-offs for narrow peak ChIP-seq reproducibility (see below for current IDR standards), then the secondary characterization of the new antibody/lot number is compliant with this defined standard.

3) **Secondary characterization using overexpressed, tagged proteins.** For proteins that are resistant to knockdown using siRNAs (e.g. very stable proteins), comparison to overexpressed, tagged proteins can be used for characterization. In this case, the primary characterization of the antibody must first show the appropriate specificity. Then, two side-by-side immunoblots should be performed using control cells and cells overexpressing the factor. The first immunoblot should employ the antibody to the tag to show the position of the exogenous factor and the second immunoblot should employ the antibody to the endogenous factor to show that the band in the control cells that is identified by the endogenous antibody is the same size as the exogeneous protein.

Notes:

- These methods refer to characterization of antibodies that recognize endogenously expressed proteins. The requirements for characterization of epitope-tagged proteins are described elsewhere.
- It was generally agreed that the method of immunoprecipitation followed by mass spectrometry would be appropriate for use as a secondary characterization. However, the details of the requirements for this method have not yet been finalized.
- At the current time, motif enrichment in the ChIP-seq peaks is not considered a valid antibody characterization method.
- Current IDR standards for a narrow-peak ChIP-seq dataset are:

  Rescue Ratio RR_new = | Np U Nt | / | Np ^ Nt |
  Self consistency ratio SR_new = | N1 U N2 | / | N1 ^ N2 |
  where
  ^ = intersection (common) of 2 peak sets
  U = union (merge) of 2 peaks sets

  If (RR_new > 2) AND (SR_new > 2) then the replicates are proclaimed to have low reproducibility (failed) and flagged with -1 quality score
  If (RR_new > 2) OR (SR_new > 2) but not both, then the replicates are proclaimed to have moderate reproducibility (passed) and flagged with a 0 quality score
  If (RR_new <= 2) AND (SR_new <= 2) then the replicates are proclaimed to have high reproducibility (passed) and flagged with +1 quality score

Characterization of an antibody for an RNA Binding Protein

Before binding data can be submitted for any RNA binding protein (RBP), the specific lot number of the antibody used to collect the data must be approved for use in that cell type by the antibody characterization committee of the ENCODE DCC. A primary and secondary characterization must both be submitted for review and deemed COMPLIANT, in order for an antibody lot to be approved for use.

1. In all cases, a primary characterization of the antibody must be provided <u>for each lot number and each specific cell type</u>. This can be either an immunoblot (Western blot) or an immunoprecipitation (IP-Western). [slide 1]

a. If the major band is within 20% of the size predicted by the size of the coding region of the protein and corresponds to >50% of all bands on the gel (excluding the antibody bands in the case of an immunoprecipitation), then the antibody passes this initial characterization. Each immunoblot should indicate which cells types were tested and include size markers. For immunoprecipitations, a control IgG precipitation should also be included.

b. If the antibody does not pass this initial characterization (e.g. due to multiple splice variants or multiple modifications that alter the electrophoretic properties of the protein), 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 upon treatment with siRNA to that protein).

2. Use of the same lot number of a previously characterized antibody in a new cell type. If a <u>specific lot number</u> for an antibody has previously passed both primary and secondary 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 must be performed in the new cell type. [slide 2]

3. Use of a new lot number of a previously characterized antibody. If this is the first time that a new lot number has been used for a previously characterized antibody, a primary characterization must be performed. However, if the pattern is the same as for the old lot number of the previously characterized antibody, then no further characterization is required. If the banding pattern is different, a secondary characterization must be performed. [Slide 3]

Allowable Secondary Characterization Methods:

1) **Secondary characterization using siRNA or shRNA against the mRNA of the target protein**. For siRNA or shRNA characterization, the band(s) detected by the antibody should be reduced to no more than 50% of the original signal. The sequence or vendor and catalog number of the oligonucleotide(s) should be provided. A control knockdown should also be performed. Cell types should be labeled and size markers should be included on the immunoblot. A brief description of the transfection protocol should also be provided.

2) **Secondary characterization using overexpressed, tagged proteins.** For proteins that are resistant to knockdown using siRNAs (e.g. very stable proteins), comparison to

overexpressed, tagged proteins can be used for characterization. In this case, the primary characterization of the antibody must first show the appropriate specificity. Then, two side-by-side immunoblots should be performed using control cells and cells overexpressing the factor. The first immunoblot should employ the antibody to the tag to show the position of the exogenous factor and the second immunoblot should employ the antibody to the endogenous factor to show that the band in the control cells that is identified by the endogenous antibody is the same size as the exogeneous protein.

Notes:

- These methods refer to characterization of antibodies that recognize endogenously expressed proteins. The requirements for characterization of epitope-tagged proteins are described elsewhere.
- It was generally agreed that the method of immunoprecipitation followed by mass spectrometry would be appropriate for use as a secondary characterization. However, the details of the requirements for this method have not yet been finalized.

Characterization of a Histone Antibody

Before ChIP-seq data can be submitted for any antibody that detects a modified histone, the specific lot number of the antibody used to collect the data must be approved by the antibody characterization committee of the ENCODE DCC. A primary and secondary characterization must both be submitted for review and deemed COMPLIANT, in order for an antibody lot to be approved for use. [slide 4]

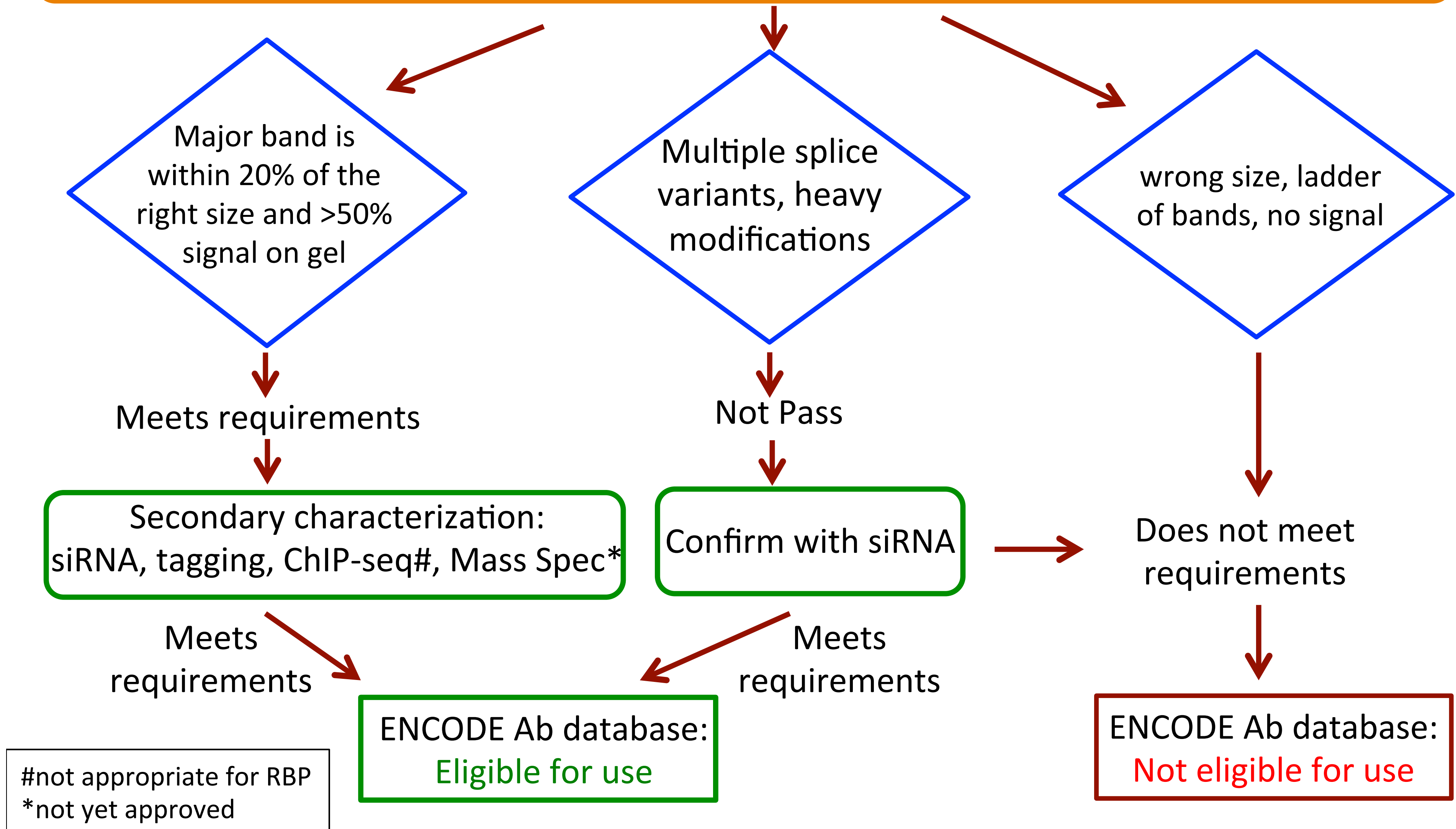
1. **In all cases, a primary characterization of each lot number of an antibody for a modified histone must be performed**. Primary characterization of a specific lot number for a histone antibody should include a immunoblot using nuclear or whole cell extract (to show a positive band at the size of the histone) plus a recombinant unmodified version of that histone (to show lack of reactivity with the unmodified histone). Extracts from at least 3 cell lines/types should be analyzed, including the species in which the antibody will be used. The specific histone band detected in the extracts should constitute at least 50% of the protein signal and show at least 10-fold enrichment relative to any other single band. In addition, this signal should be at least 10-fold enriched relative to that detected using the unmodified recombinant histone. Each immunoblot should label the cells and include size markers.

2. **Secondary characterization** using dot blots, peptide arrays or peptide competitions. Commercial arrays are acceptable and the vendor and catalog number should be provided. If arrays that include small numbers of peptides are used, it is critical that the array contain the most relevant peptides for the antibody being tested. If peptide competition is used to validate a histone antibody, it is also important that the most relevant peptides for the antibody being tested are used in the competition series. A 10-fold enriched binding signal for the modification of interest relative to other modifications is required. An annotated map of the peptides present on the blot or array should be provided.

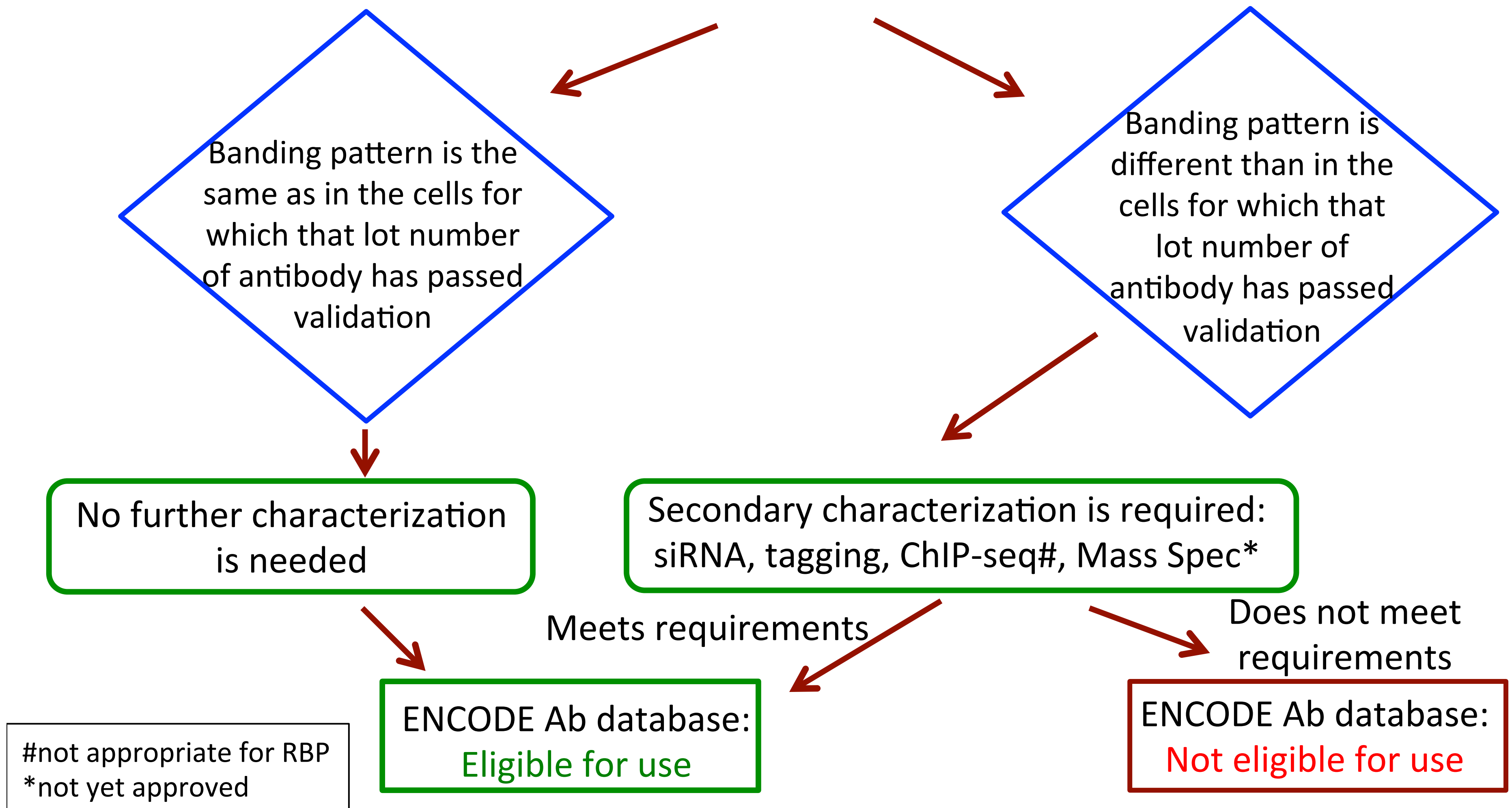
3**. Additional cell types**. Once a specific lot number for an antibody to a modified histone has passed both the primary and secondary characterizations, it is eligible for use in all cell types (with the caveat that the species must have been included in the primary characterization).

Note. It was generally agreed that it should be allowable to use similarity of ChIP-seq data of a different lot number of the same histone antibody, a different antibody for the same histone modification, or a component of the histone modifying complex that puts on the histone mark as a characterization method. However, a quantitative measure of how closely the patterns must match has not yet been decided; these quantitative measures may not be the same for all histone marks. Once quantitative measures have been determined, this characterization method may be allowed.

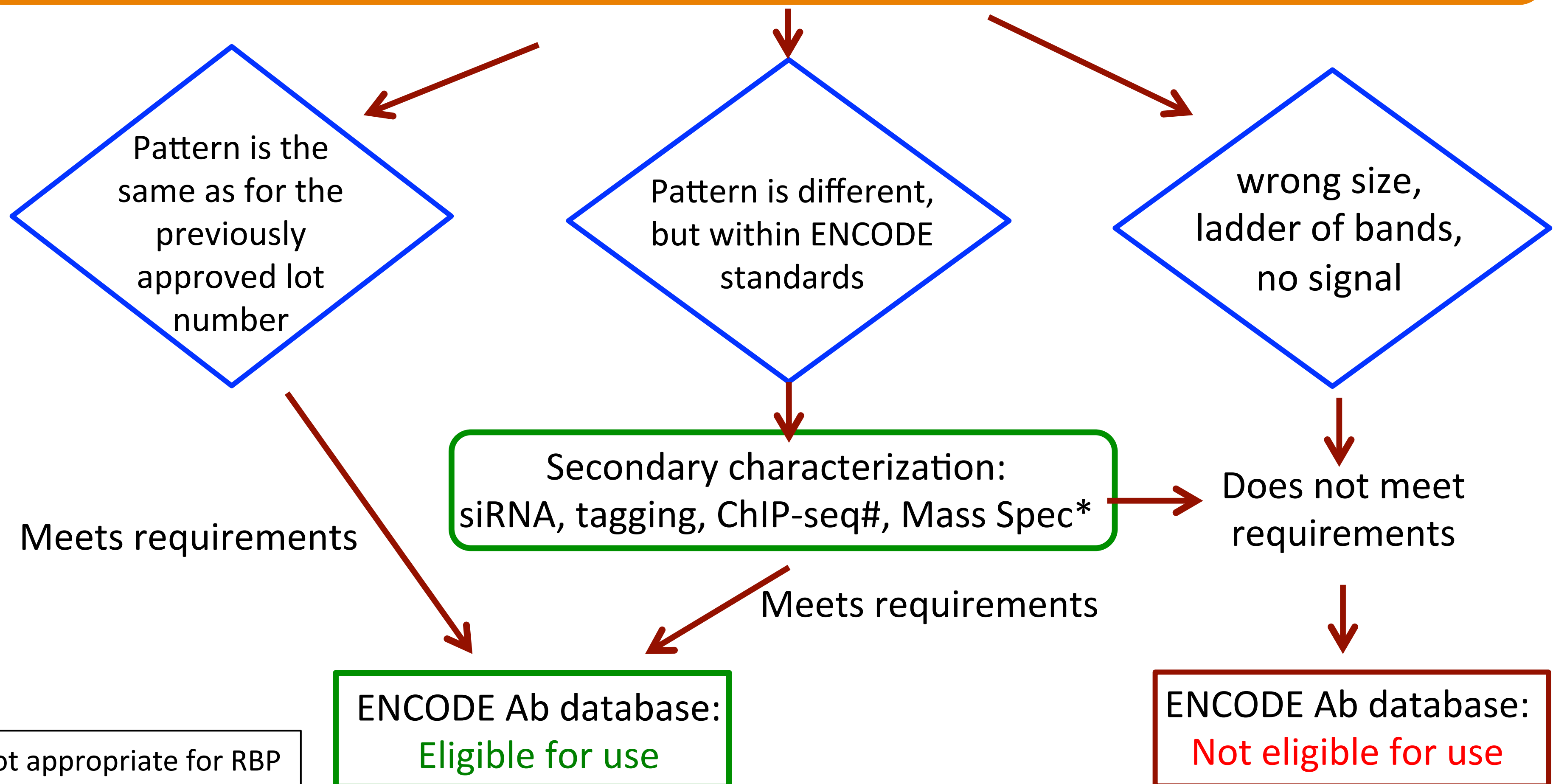
Initial characterization of an antibody (for TFs, chromatin remodelers, chromatin modifiers, RBPs)



Use of a previously characterized lot number of an antibody in a new cell type (for TFs, chromatin remodelers, chromatin modifiers, RBPs)



Use of a new lot # of a previously characterized antibody (for TFs, chromatin remodelers, chromatin modifiers)



#not appropriate for RBP
*not yet approved

Characterization of a modified histone antibody