Accelerating eCLIP Studies: Importance of Antibody Validation and Pre-Validated Antibodies for RNA Binding Proteins

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Abstract

Enhanced cross-linking and immunoprecipitation (eCLIP) has been used to determine the RNA binding sites for hundreds of RNA binding proteins (RBPs) with many of these datasets publicly available in ENCODE. However, bioinformatic analysis has predicted that there are over 1,000 RBPs that have yet to be successfully eCLIP'd. eCLIP has been a successful method to identify RBP binding sites with more robust washes, improved ligations, and use of a sized matched input control, however identifying an antibody that can work successfully in these conditions has been a challenge. Even antibodies labeled IP-grade have failed to yield eCLIP results. To help the community quickly access which antibodies are eCLIP validated, we have partnered with antibody provider, Cell Signaling Technology (CST). Here we present data demonstrating the importance of antibody validation and how using prevalidated eCLIP antibodies can expedite a successful eCLIP experiment.

Antibody Validation Steps and eCLIP QC Guidelines

- The RBP-eCLIP assay relies on the use of an antibody capable of specifically and robustly binding to its target protein in a cell lysate. Therefore, all eCLIP-validated antibodies are first tested in IP-Western blot (see Eclipsebio Antibody IP Validation Kit #E-CAV00001). Antibodies that work for IP-Western are more likely to work in eCLIP, but it is not guaranteed that all IP-Western antibodies will work. We will discuss the additional parameters that Eclipsebio evaluates to determine if an antibody is suitable for eCLIP in the subsequent points below.

- A successful eCLIP enrichment should yield enough RNA to generate a sequenceable library with fewer than 18 rounds of PCR amplification. While the optimal number of amplification cycles is highly specific to the RNA-binding protein (RBP), eCLIP enrichments with lower RNA yields may indicate a failed experiment. These libraries will require more PCR amplification cycles and have higher PCR duplication rates..

- To demonstrate reproducibility, each antibody is tested in duplicate and must generate an acceptable irreproducible discovery rate (IDR), as previously described for ChIP-seq data analysis.
Li, Q., et al, Ann. Appl. Stat. (2011).

- For sequence-specific RBPs, antibody specificity can be determined by performing motif analysis of enriched RNA fragments.

- Antibody specificity can also be determined by comparing enrichment across the transcriptome using multiple antibodies against distinct target protein epitopes or antibodies against different subunits of a multiprotein complex.

- eCLIP peaks are determined by analyzing the signal:noise ratio of RBP enrichment across the transcriptome in antibody:input control comparisons. The antibody must provide an acceptable minimum number of defined enrichment peaks and a minimum signal:noise threshold compared to input.

- Antibody specificity is further confirmed by comparing enrichment across the transcriptome to previously published CLIP or RNA-IP-seq data (i.e. from ENCODE).

Questions?
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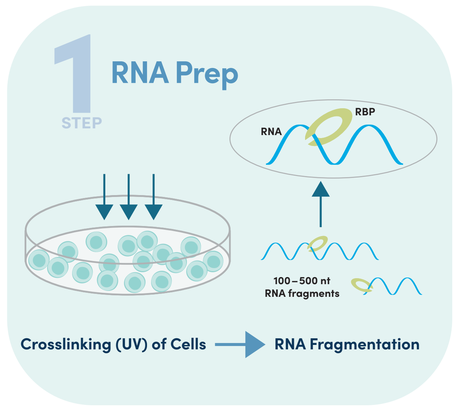
RBP-eCLIP Assay



Antibody IP Validation

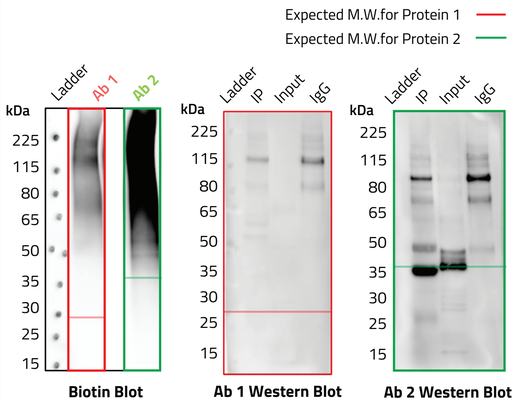
Eclipsebio's Antibody IP Validation Kit was used in HEK-293s cells, for 2 proteins of interest.

Antibody 1 results in a failed validation test.
Biotin Blot: RNA smear begins at incorrect molecular weight.

Western Blot: No bands around expected molecular weight. IP & IgG lane are same binding patern.

Antibody 2 resuls in a passed validation test.
Biotin Blot: RNA smear is robust and begins at expected molecular weight.

Western Blot: Bands appear at expected molecular weight in both IP and input lanes. Bands are distinct from IgG control.



Improved Library Yield

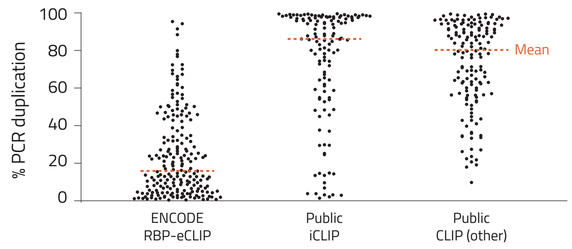


Figure 1. PCR duplication rates comparing many samples across methodologies, eCLIP, iCLIP, and other CLIP methodologies. RBP-eCLIP yields significantly lower PCR duplications rates on average.

Antibody Reproducibility and IDR

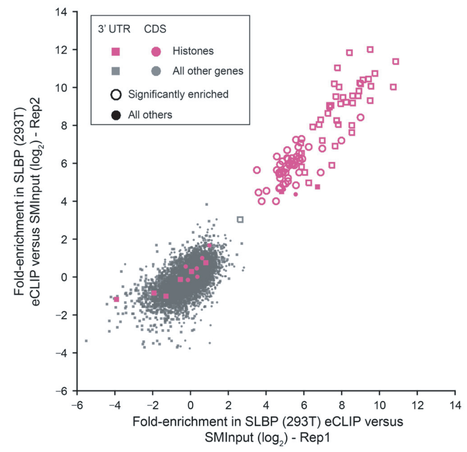


Figure 2. SLBP eCLIP fold-enrichment over SMInput at histone RNAs is reproducible across biological replicate experiments. Each point indicates eCLIP fold-enrichment over paired SMInput for the CDS (circle) or 3'UTR (square) of genes profiled in independent biological replicate SLBP eCLIP experiments. Histone genes are indicated in pink, with open circles indicating significantly enriched regions (fold-enrichment ≥ 4-fold, p-value ≤ 10^{-5} in eCLIP vs SMInput). Both CDS (R^2 = 0.50) and 3' UTR (R^2 = 0.73) show significant correlation ($p < 10^{-300}$, all significance determined by standard conversion of r values to t-statistic), and show enrichment at most histones.

Binding Motif Identification

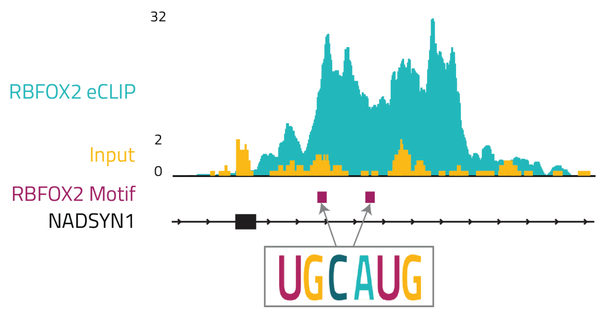


Figure 3. Read coverage of an RBFOX2 eCLIP peak on the gene NADSYN1 containing the canonical RBFOX2 RNA binding motif, UGCAUG.

Confirm Antibody Specificity with Distinct Antibody Epitopes

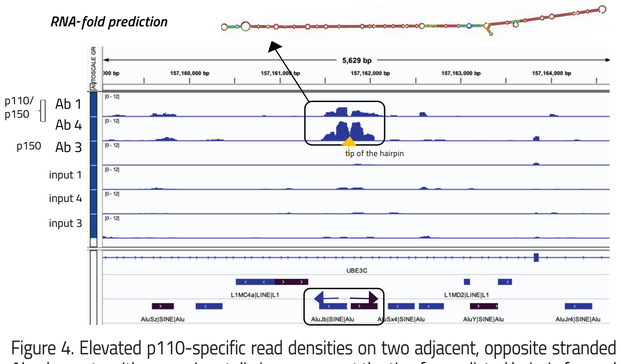


Figure 4. Elevated p110-specific read densities on two adjacent, opposite stranded Alu elements with a prominent dip in coverage at the tip of a predicted hairpin formed by the two divergent Alu elements. The predicted fold of the gray shaded region is shown below, with yellow highlighted hairpin region boxed on the folded RNA.

eCLIP Enrichment Peaks and Signal:Noise Ratio

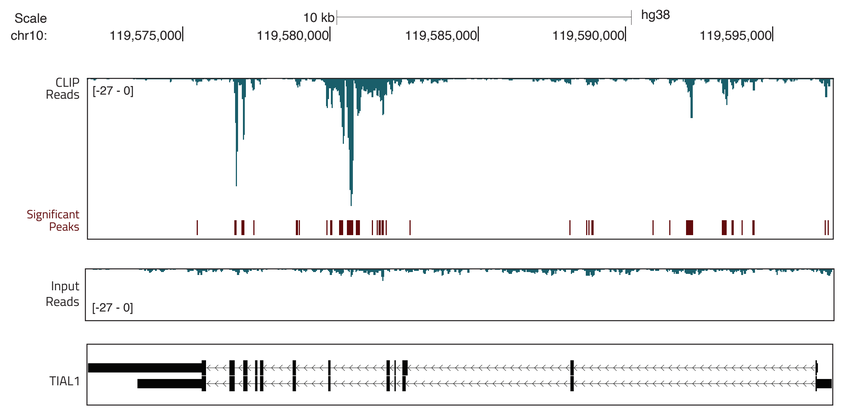


Figure 5. eCLIP was performed with RNA from K-562 cells and CST TIAR (D32D3) XP® Rabbit mAb # 8509 using a protocol based on the RBP-eCLIP Kit from Eclipsebio. The figure shows an acceptable minimum number of defined enrichment peaks and a minimum signal:noise threshold compared to the input across the TIAL1 transcript.

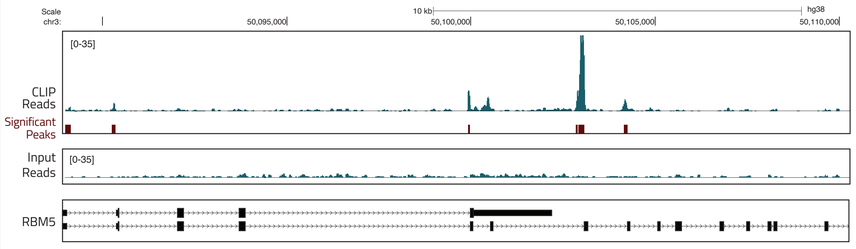


Figure 6. eCLIP was performed with RNA from K-562 cells and CST RBM5 (E8G8K) Rabbit mAb #86425 using a protocol based on the RBP-eCLIP Kit from Eclipsebio. The figure shows an acceptable minimum number of defined enrichment peaks and a minimum signal:noise threshold compared to the input across the RBM5 transcript.

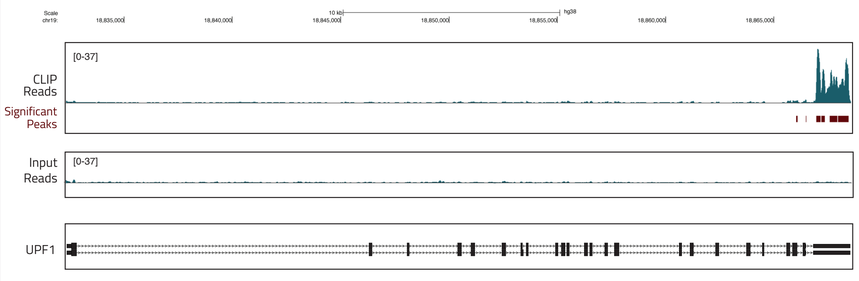


Figure 7. eCLIP was performed with RNA from K-562 cells and CST UPF1 (D15G6) Rabbit mAb #12040 using a protocol based on the RBP-eCLIP Kit from Eclipsebio. The figure shows an acceptable minimum number of defined enrichment peaks and a minimum signal:noise threshold compared to the input across the UPF1 transcript.

Comparable to ENCODE Data

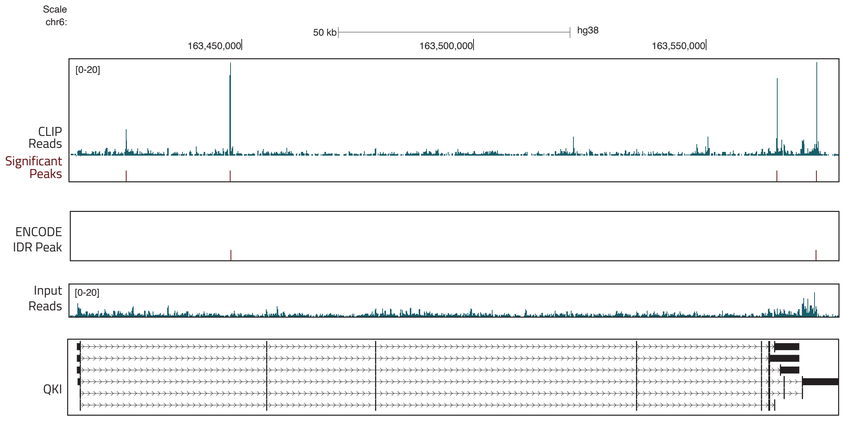


Figure 8. eCLIP was performed with RNA from K-562 cells and CST QKI (E7O4A) Rabbit mAb #23065 using a protocol based on the RBP-eCLIP Kit from Eclipsebio. The figure shows an acceptable minimum number of defined enrichment peaks and a minimum signal:noise threshold compared to the input across the QKI transcript, with compared eCLIP data downloaded from ENCODE.