Antibody-Free Reagents outperform Antibodies in Alida Bio's EpiPlex™ Assay for Epitranscriptomics

Measure m6A and inosine with high accuracy and < 20 ng of mRNA input

EPIPLEX: YOUR KEY TO ACCESSIBLE EPITRANSCRIPTOMICS

The epitranscriptome is the set of chemical modifications (mods) found naturally on RNA which play key roles in regulating nearly all aspects of RNA biology, including RNA-protein interactions, splicing, secondary and tertiary structure, transcript lifetime, trafficking, and translation.[1] The distribution and number of mods, and their % abundance at each locus, changes between cell types, during development, and in disease.[2] The ability to profile and quantify multiple RNA mods transcriptome-wide will enable basic and applied research, including the advancement of new types of biomarkers for diagnostics and improved efficacy and safety of RNA-centered medicine.[3] With EpiPlex we are announcing a new, user-friendly epitranscriptome analysis solution using custom non-antibody binding agents, a 1-day workflow, and data analysis tools that provide best-in-class capabilities for reading RNA modifications.

HIGHLIGHTS

Multiplexed Detection of RNA Modifications

Alida Bio's barcoding technology enables the detection of multiple RNA mods simultaneously.

Higher Accuracy than MeRIP

Binders derived from small, native RNA binding proteins detect m6A and inosine with significantly less off-target signal than antibodies.

Exceptionally low RNA Input

RNA input of < 20 ng mRNA facilitates the analysis of blood and tissue samples.

User Friendly End-to-End Workflow

From purified RNA to sequencing in under one day, followed by analysis with Alida's software.

Quantitative Data

Spike-in standards enable relative RNA mod quantification at each locus.

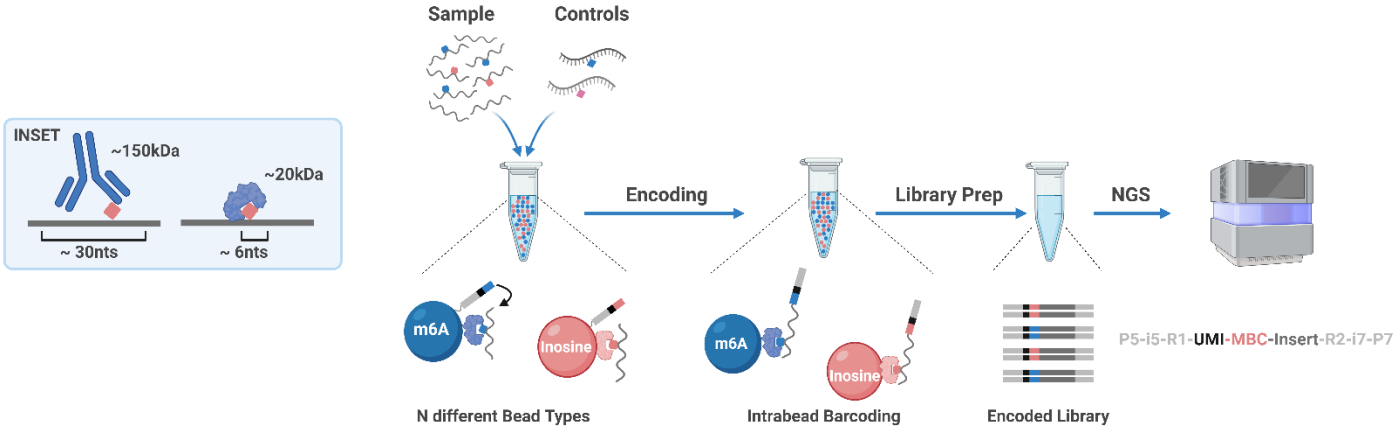

FIGURE 1: The EpiPlex workflow. Fragmented RNA is captured by a bead pool where each bead type displays a sequencing adapter with a unique RNA modification barcode (MBC) and a non-antibody binder (NAB). The inset illustrates the size difference of antibodies and NABs, each bound to a modified RNA target. The bead pool is scalable for additional RNA modifications. To generate an encoded library, the adapter is enzymatically transferred from the bead surface to the captured RNA using assay conditions that limit crosstalk between beads. Reverse transcription followed by PCR completes the library. The resulting library architecture comprises the forward and reverse Illumina adapters (P5-i5-R1 and P7-i7-R2, respectively), a unique molecular identifier (UMI) and the RNA modification barcode (MBC). Sequencing infers the presence of an RNA modification based on the MBC.

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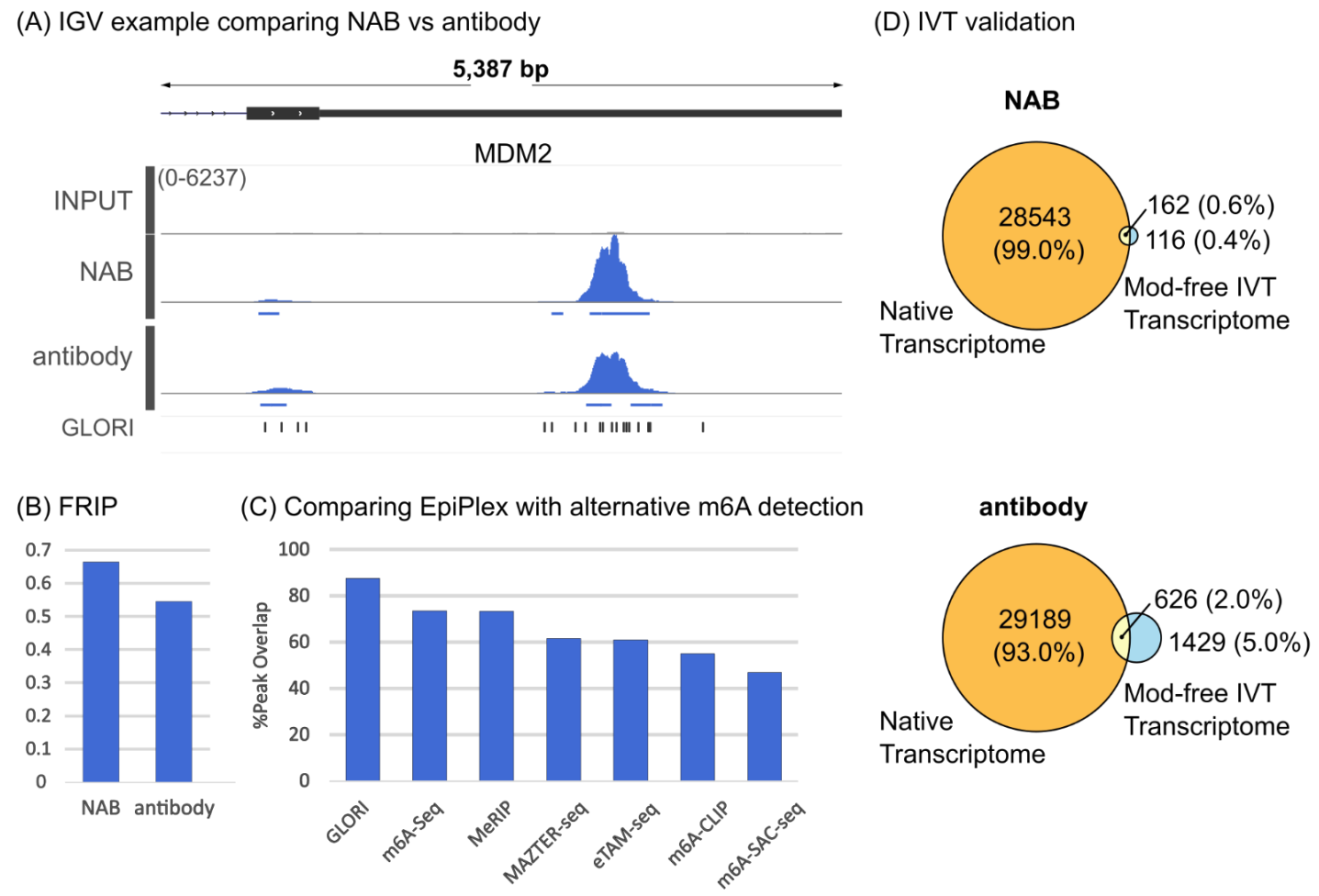

FIGURE 2: EpiPlex data shows that the m6A NAB provides multiple advantages over antibodies. (A) The IGV view of the MDM2 gene shows peaks called when using a NAB or an antibody to recognize m6A. Blue bars show sites of m6A peaks called by Alida's peak detection algorithm. Single base m6A calls by GLORI,[4] a chemical conversion method for detection of m6A, are shown for comparison. (B) The improved properties of the m6A NAB allow sequencing reads to be used more efficiently as measured by the fraction of reads in peaks (FRIP). (C) Intersection plot comparing EpiPlex with published m6A assays shows the highest concordance with GLORI. (D) Venn diagrams show the overlap in m6A peak calls for mRNA derived from A549 cells vs. a modification-free *in vitro transcribed* (IVT) transcriptome. The use of an anti-m6A antibody results in > 7-fold more peak calls in the unmodified IVT transcriptome as compared with the use of a NAB; these are false positives.

The EpiPlex assay employs a proprietary barcoding technology to record the presence of mods on fragmented RNA (Figure 1). Molecular recognition of the mods using custom binding agents enriches modified fragments onto beads specific to each mod type. The February 2024 release of EpiPlex interrogates N^6-methyladenosine (m6A) and inosine, which are significant mods of mRNA and ncRNA. Alida's intra-bead barcoding chemistry appends a mod-specific barcode (MBC) during library preparation, recording the identity of each mod in the fragment. Unique molecular identifiers (UMIs) are included to improve the accuracy of deduplication. The superior sensitivity and specificity of EpiPlex provide excellent assay performance with as little as 20ng of polyA-enriched RNA or 250ng total RNA input.

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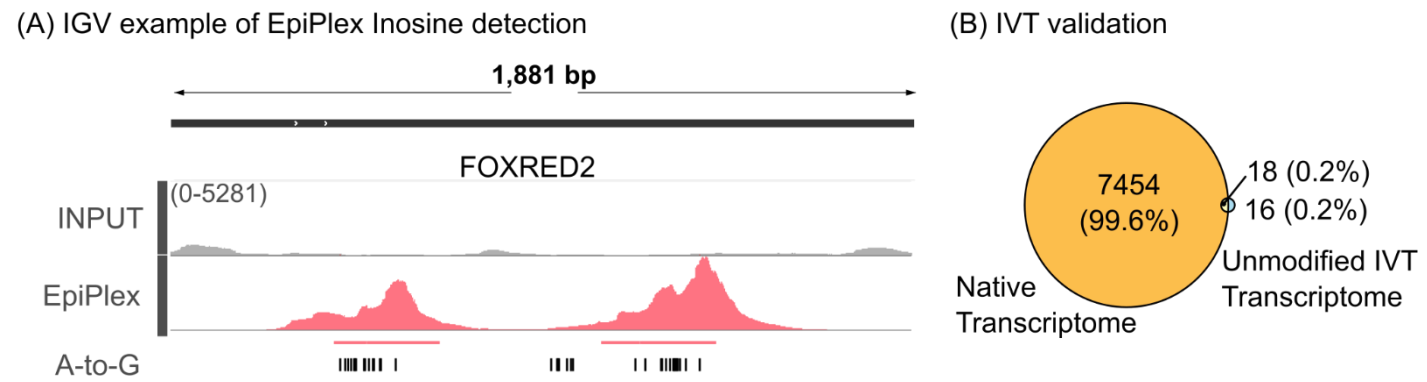

FIGURE 3: Accuracy of EpiPlex using NABs for inosine detection. (A) An IGV plot showing accurate and strong signal for inosine detection. Black bars show sites of A to G variants where G calls make up ≥ 20% of the reads. (B) Venn diagrams showing minimal false positive detection by reference to a modification-free IVT transcriptome.

EXCEPTIONAL SENSITIVITY AND SPECIFICITY THROUGH NON-ANTIBODY BINDERS

Molecular recognition of RNA modifications using antibodies, e.g. in MeRIP-Seq, is a prominent method for their detection. However, existing RNA mod-targeting antibodies vary widely in their affinity and specificity.[5] Antibody evolution by immunization does not provide an efficient mechanism for counter-selection against off-targets and the size of antibodies is much larger than many native RNA-binding proteins. Accordingly, antibodies make contacts with the RNA backbone, and these contacts are non-specific in nature.

To address the performance limits of antibodies for RNA modification detection, we established a state-of-the-art protein engineering program designed to evolve the affinity and specificity of RNA binding proteins. The first two products of this program are non-antibody binders (NABs) for N^6-methyladenosine (m6A) and inosine. These Alida NABs have high affinity and exhibit minimal binding to canonical bases or off-target modifications. We expected and observed substantial performance gains when comparing NABs with antibodies in Alida's EpiPlex technology.

EPIPLEX NAB PERFORMANCE

The advantages of NABs over antibodies in the EpiPlex epitranscriptomic assay are demonstrated by the following four measurements.

First, an examination of representative regions of the transcriptome shows read pileups at known mod sites with low background for both m6A and inosine when using EpiPlex NABs (Figures 2A and 3A). For m6A, similar peaks are detected for NABs and antibody. Second, we performed a global analysis of fraction of reads in peaks (FRIP), which measures the efficient allocation of sequencing reads to *bona fide* signals. The FRIP for EpiPlex NAB shows more than a 20% increase as compared with the antibody version (Figure 2B). The use of NABs therefore allows a substantial improvement in the efficient use of sequencing reads, lowering the required sequencing depth and improving signal-to-noise.

Third, a comparison of 6,001 high-confident m6A sites called by EpiPlex NAB is in good agreement with other methods (Figure 2C). EpiPlex NAB shows especially strong concordance with GLORI,[4] a chemical conversion method for absolute detection of m6A. Inosine calls are corroborated by being read as A→G mutations (Figure 3A), which provides a ground truth. Because the EpiPlex assay enriches modified fragments of RNA, the sensitivity for inosine detection

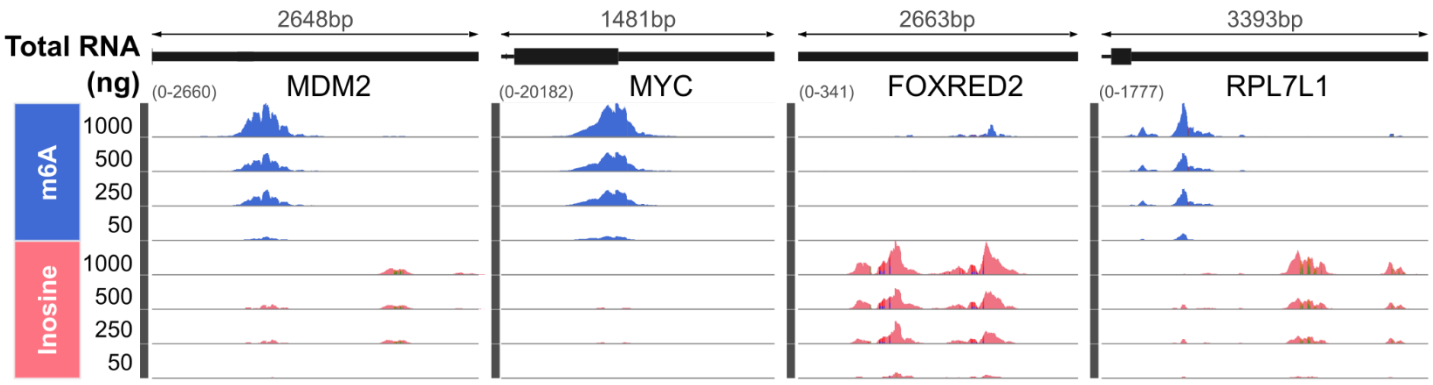

FIGURE 4: RNA input dependence of EpiPlex raw data. An RNA input titration was performed by varying total RNA between 1 µg and 50 ng (a ribosomal RNA depletion step was included after the assay before sequencing). MDM2, MYC, FOXRED2, and RPL7L1 are shown as representative genes where the raw read pileups titrate down with total RNA input. N^6-methyladenosine tracks are shown in blue, inosine tracks in light red.

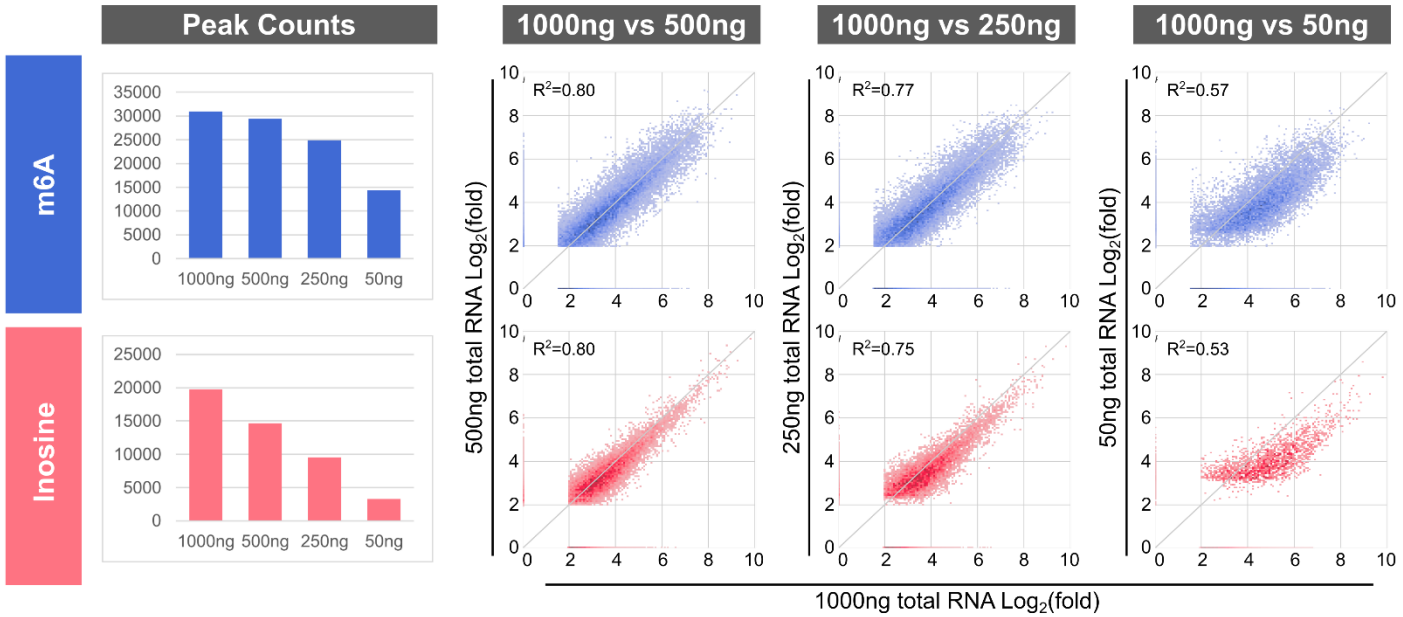

FIGURE 5: EpiPlex enables analysis of samples with low RNA input. As expected, the number of m6A and inosine peaks depends on the total RNA input. Fewer peaks are detected at lower RNA input. Peaks recorded at lower RNA input are reliable, as demonstrated by the correlation plots. Currently, we recommend using >= 250 ng of total RNA or >= 20 ng mRNA as assay inputs.

is substantially greater than that available from A→G mutation analysis alone.

Last, to assess the accuracy of peak calling, we ran parallel assays with mRNA isolated from A549 cells vs. a modification-free *in vitro* transcribed (IVT) transcriptome generated from the same mRNA. Because the IVT transcriptome was generated using only canonical nucleobases, any signals resulting from enrichment in the IVT sample are considered

false positives. A best-in-class anti-m6A antibody calls 2055 m6A peaks in an IVT sample, while our m6A NAB calls only 278, a reduction of 87%. A Venn diagram for A549 vs. its corresponding IVT shows an overlap of 162 peak calls out of more than 28,500 m6A peaks in A549 sample, suggesting a 0.6% false positive rate (Figure 2D). A similar EpiPlex analysis of inosine shows that the NAB has a false positive rate of only 0.2% (Figure 3B).

UNPRECEDENTED SENSITIVITY

RNA input requirements are a critical parameter when the user wants to analyze samples that are not derived from cell culture. Tissue samples, clinical biopsies, and other samples of native biological material typically afford only small amounts of RNA, often including less than 500 ng of total RNA. mRNA makes up typically 1–3% of this total.

The efficient use of sequencing reads offered by the NABs' affinity and specificity is expected to enable high quality peak calling when RNA samples are limited and sequencing is shallow. To measure the effect of RNA input amount on assay performance, we titrated down the total RNA input and carried out the complete assay workflow and analysis, which includes a ribosomal RNA depletion step after the assay but before sequencing (Figure 4). While an expected decrease in the magnitude of read pileups is observed, mod peak signal intensity and unambiguous peak calling is available in both the m6A and inosine channels with only 50 ng of RNA input (< 1 ng mRNA). The number of m6A and inosine peaks called decreases only slowly with decreasing input (Figure 5A) and, importantly, peak intensities are strongly correlated down to 250 ng total RNA input (and, in the m6A channel, down to 50 ng; Figure 5B). This high sensitivity is significant because it enables epitranscriptomic studies on precious clinical samples.

ORDERING INFORMATION

Alida Bio is currently offering the EpiPlex reagent kit as part of an early access program. To become an early access user please email orders@alidabio.com.

REAGENT KIT SPECIFICATIONS

Kit size	8 samples*
RNA mods	m6A, inosine
RNA input	>= 20 ng polyA+ RNA >= 250 ng total RNA
Assay time	< 1 day
Sequencing requirement	>= 25M per sample 200 cycle sequencing
Analysis	performed by Alida Bio, beta software expected for July 2024

*Sample = 1 enriched sample + 1 solution control

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