Name	Sequence (5'to 3')
MALDI_Probe_A	CGUGGACUGGCU-biotin
MALDI_Probe_m^{6}A	CGUGGm^{6}ACUGGCU-biotin
Validation_Probe_NNm^{6}ANN	UCGACGUNNm^{6}ANNGGCAUUGCU
Validation_Probe_GGam^{6}ACU (am^{6}A = allylic m^{6}A)	CUCUCGACGUGGam^{6}ACUGGCAUUGCGCUCUC
Validation_Probe_GGa^{6}ACU (a^{6}A = allylic A)	CUCUCGACGUGGa^{6}ACUGGCAUUGCGCUCUC
Validation_Probe_NNam^{6}ANN	UCGACGUNNam^{6}ANNGGCAUUGCU
Validation_Probe_NNa^{6}ANN	UCGACGUNNa^{6}ANNGGCAUUGCU
Validation_Probe_m^{6}A	CGUGGm^{6}ACUGGCU-biotin
Validation_Probe_8 A :1 m^{6}A	CGCAAAUGCUUCUAGGCGGm^{6}ACUAUGACUUAGUUGCGUUAC-biotin
Calibration_spike-in_1 (0% m^{6}A)	UAUCUGUCAUCGCUCUCGACGUGGACUGGCAUUGCGCUCUC
Calibration_spike-in_2 (25% m^{6}A)	UAUCUGUCUAGCCUCUCGACGUGGm^{6}ACUGGCAUUGCGCUCUC
Calibration_spike-in_3 (50% m^{6}A)	UAUCUGUCCGAUCUCUCGACGUGGm^{6}ACUGGCAUUGCGCUCUC
Calibration_spike-in_4 (100% m^{6}A)	UAUCUGUCGCUACUCUCGACGUGGm^{6}ACUGGCAUUGCGCUCUC
Calibration_NN_spike-in_1 (0% m^{6}A)	UA UCU GUC AUC G UCG ACG UNNANN GGC AUU GCU
Calibration_NN_spike-in_2 (25% m^{6}A)	UA UCU GUC UAG C UCG ACG UNNm^{6}ANN GGC AUU GCU
Calibration_NN_spike-in_3 (50% m^{6}A)	UA UCU GUC CGA U UCG ACG UNNm^{6}ANN GGC AUU GCU
Calibration_NN_spike-in_4 (100% m^{6}A)	UA UCU GUC GCU A UCG ACG UNNm^{6}ANN GGC AUU GCU

Table S1. RNA Probes used in this study. Related to "Before you begin" "Preparation of spike-in probes" Step 9 and "Quality control of MjDim1 activity" Step 17.

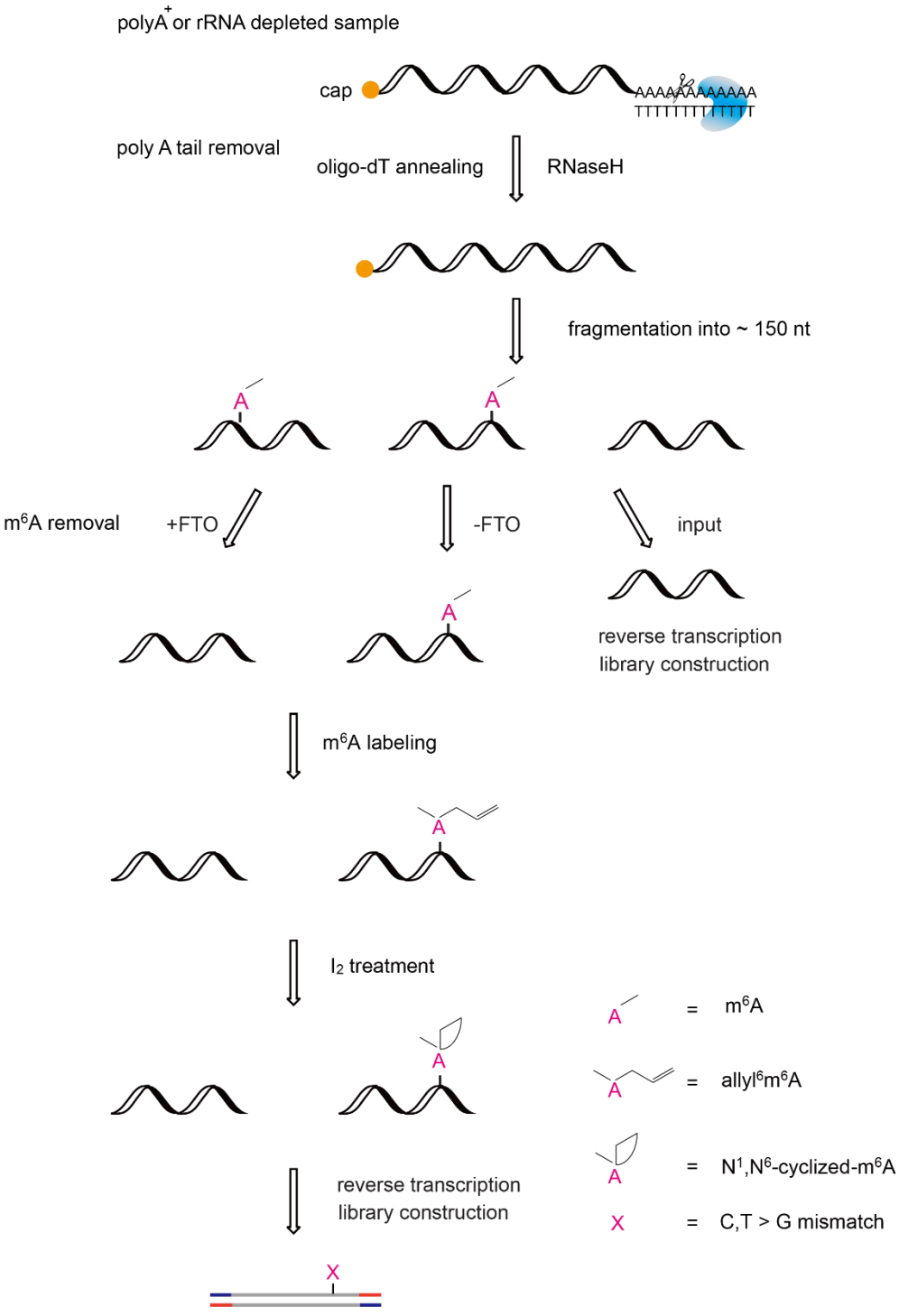

Figure S1. Schematic diagram of m⁶A-SAC-seq, related to Steps 3-5 (Library preparation).

Fragmented RNA sample is divided into 3 groups: Input group (reference), experimental group (FTO-), and control group (FTO+, m⁶A demethylase treatment). A comparison of results from treated RNA versus reference and control group could accurately identify bona fide m⁶A sites in the transcriptome. Note that the control group (FTO+ treatment) might be omitted for accurate identification of more than 90% m⁶A sites in the transcriptome.

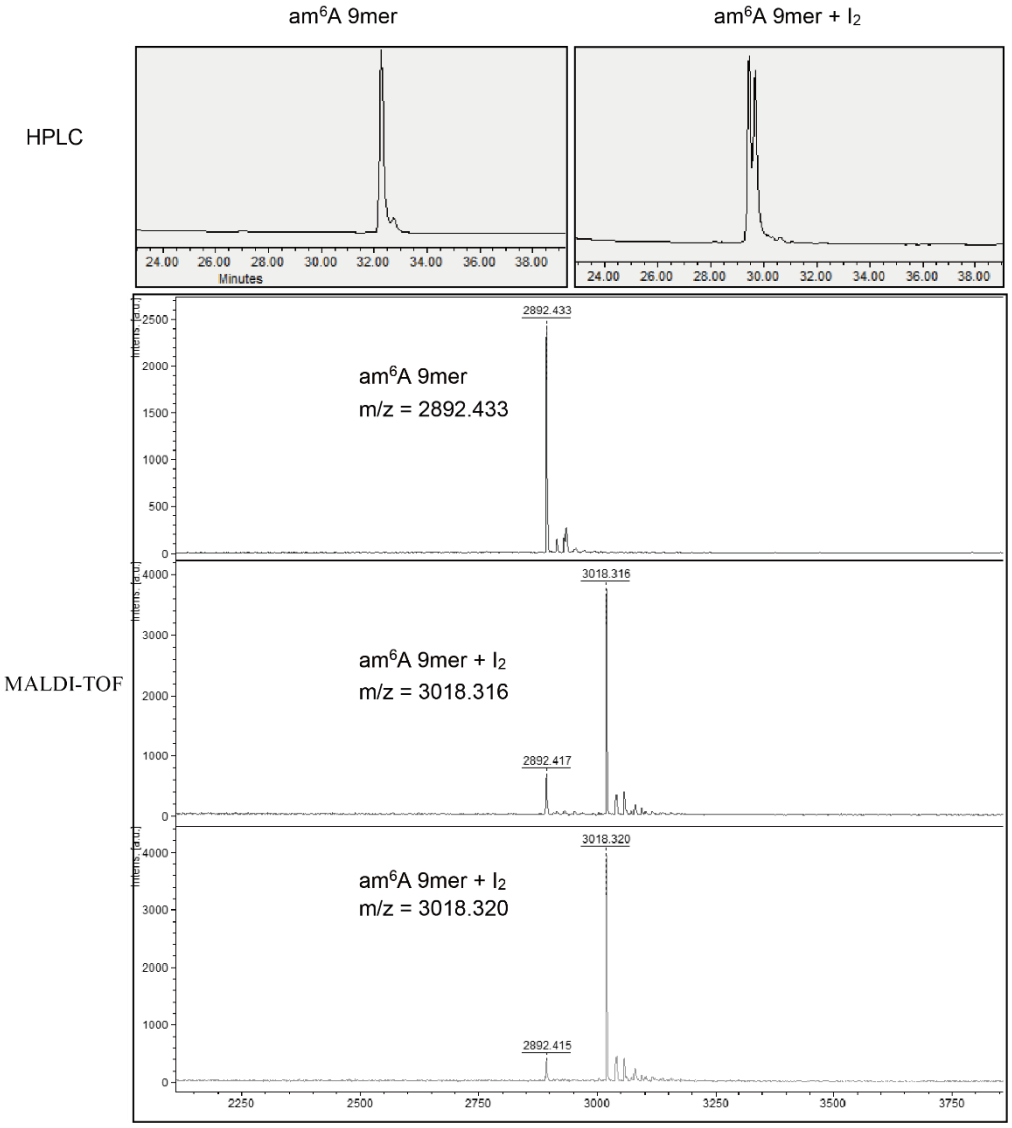

Figure S2. I_2-induced am^{6}A cyclization is of 100% efficiency, related to Step 4e (Library preparation).

A specific 9-mer RNA probe containing the am^{6}A modification in the middle was treated by 125mM I_2 dissolved in 200mM KI for 1 h at RT in the darkness (It's suitable to put the reaction in the drawer beneath the bench). MALDI-TOF results show the 100% cyclization efficiency.

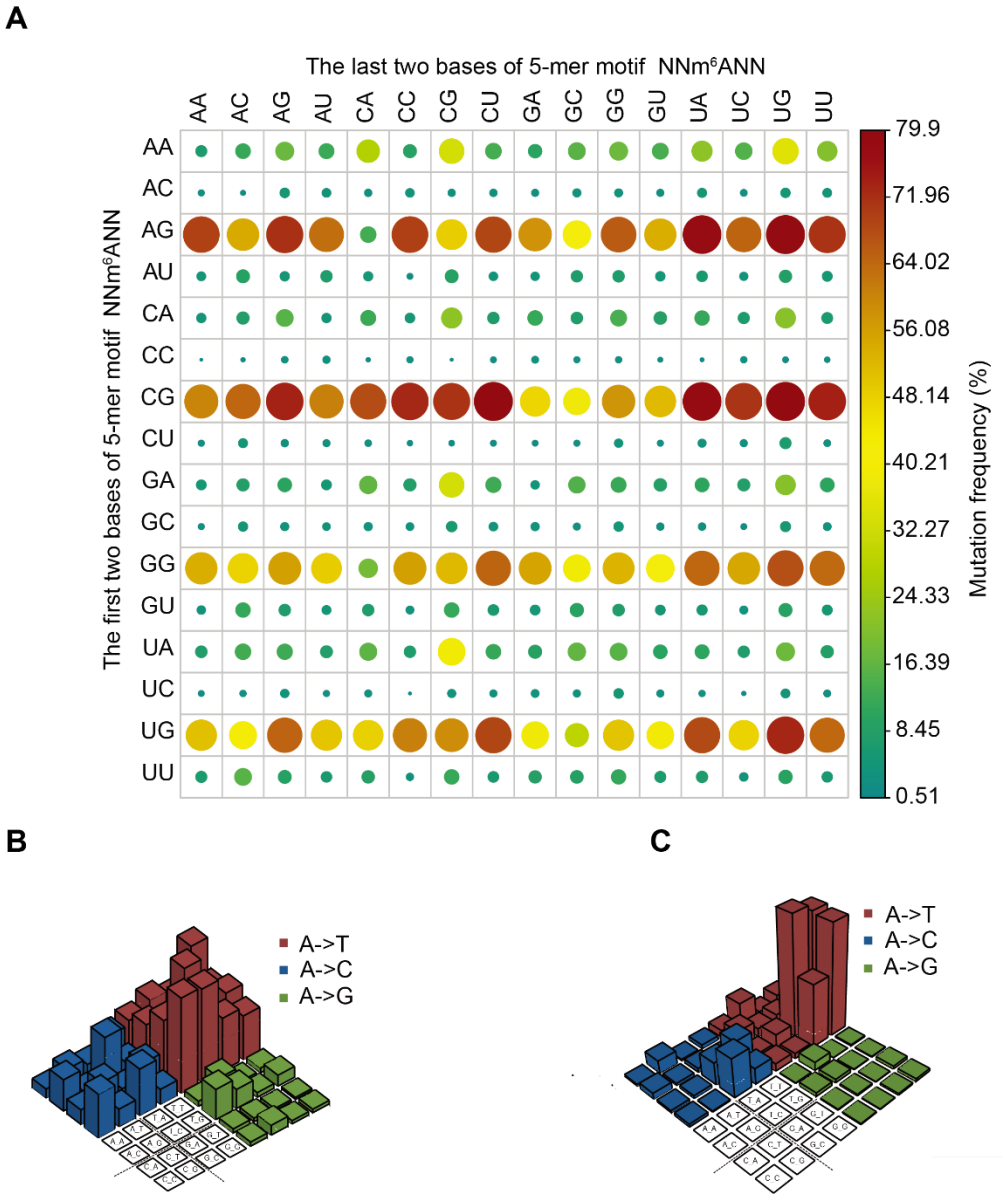

Figure S3. Motif preference of MjDim1 methyltransferase and HIV reverse transcriptase validated with RNA probes, related to Step 4f (Library preparation).

(A) Mutation frequency distribution of the RNA probes containing 5-mer motif (NNm⁶ANN) (Validation_Probe_NNm⁶ANN, Table S1) generated by m⁶A-SAC-seq. The 2-mer sequence of each row represents the first two base in the 5-mer motif and that of each column represents the last two bases in the 5-mer motif.

(B) Sequence context-dependent mutation pattern of HIV reverse transcriptase. Validation_Probe_NNa⁶m⁶ANN (Table S1) was cyclized by I_2 and subjected to RT reaction followed by next-generation sequencing. HIV reverse transcriptase shows negligible sequence context preference. The mismatch pattern is A into T and C > G.

(C) Mutation pattern of m⁶A-SAC-seq. Validation_Probe_NNm⁶ANN (Table S1) was subjected to m⁶A-sac -seq. The Mjdim1 enzyme prefers GA.

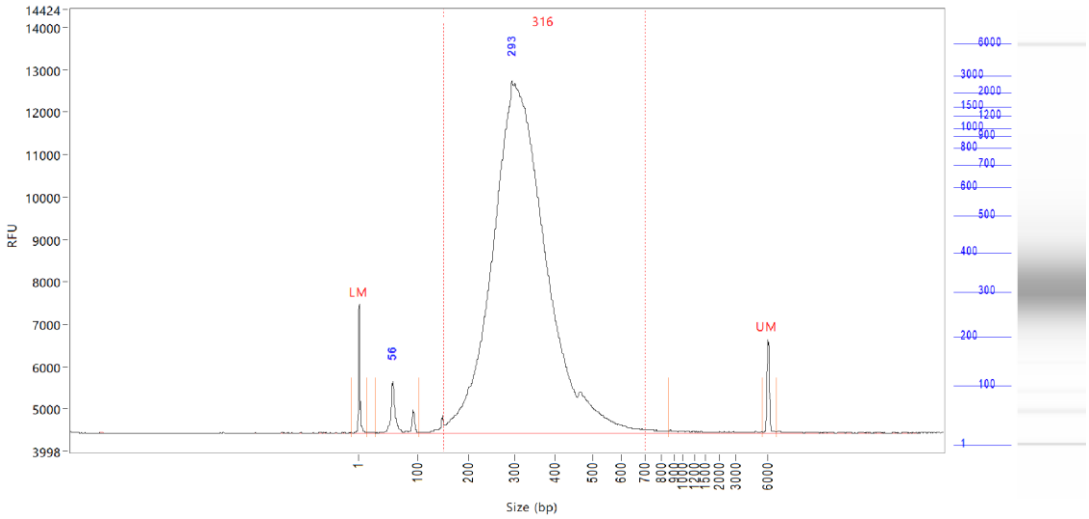

Figure S4. Bioanalyzer profile of one typical m^{6}A-SAC-seq library, related to Step 5h (Library preparation).

Bioanalyzer profile of one typical m^{6}A-SAC-seq library. The average size of the library is about 300bp.