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Methylated RNA Immunoprecipitation Assay to Study m5C Modification in Arabidopsis
--Manuscript Draft--

1 **TITLE:**
2 Methylated RNA Immunoprecipitation Assay to Study m^{5}C Modification in Arabidopsis
3
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17 **KEYWORDS:**
18 RNA methylation; 5-methylcytosine (m^{5}C); immunoprecipitation (IP); Arabidopsis;
19 epitranscriptomics; mRNA; RNA sequencing
20

21 **SUMMARY:**
22 The methylated RNA immunoprecipitation assay is an antibody-based method used to enrich for
23 methylated RNA fragments. Coupled with deep sequencing, it leads to the identification of
24 transcripts carrying the m^{5}C modification.
25

26 **ABSTRACT:**
27 Secondary base modifications on RNA, such as m^{5}C, affect the structure and function of the
28 modified RNA molecules. Methylated RNA Immunoprecipitation and sequencing (MeRIP-seq) is
29 a method that aims to enrich for methylated RNA and ultimately identify modified transcripts.
30 Briefly, sonicated RNA is incubated with an antibody for 5-methylated cytosines and precipitated
31 with the assistance of protein G beads. The enriched fragments are then sequenced and the
32 potential methylation sites are mapped based on the distribution of the reads and peak
33 detection. MeRIP can be applied to any organism, as it does not require any prior sequence or
34 modifying enzyme knowledge. In addition, besides fragmentation, RNA is not subjected to any
35 other chemical or temperature treatment. However, MeRIP-seq does not provide single-
36 nucleotide prediction of the methylation site as other methods do, although the methylated area
37 can be narrowed down to a few nucleotides. The use of different modification-specific antibodies
38 allows MeRIP to be adjusted for the different base modifications present on RNA, expanding the
39 possible applications of this method.
40

41 **INTRODUCTION:**
42 In all three kingdoms of life, RNA species undergo post-transcriptional modifications and research
43 on these functionally relevant biochemical modifications is called "epitranscriptomics".
44 Epitranscriptomics is a growing field and various methods are being developed to study and map

45   the modifications on RNA molecules (reviewed in [1,2]). More than a hundred RNA modifications
46   have been found, detected in rRNAs, tRNAs, other ncRNAs, as well as mRNAs[3,4]. Although the
47   presence and function of chemically diverse post-transcriptional modifications in tRNAs and
48   rRNAs are extensively studied[5–8], only recently have mRNA modifications been characterized. In
49   plants, many mRNA modifications have been identified to date, including m^7G at the cap
50   structure[9], m^1A[10], hm^5C[11,12], and uridylation[13]. However, only m^6A[10,14,15], m^5C[11,16,17], and
51   pseudouridine[18] have been mapped transcriptome-wide in Arabidopsis. Post-transcriptional
52   mRNA base modifications are involved in several developmental processes[19,20].
53
54   One of the most commonly used approaches in epitranscriptomics is the methylated RNA
55   immunoprecipitation coupled with deep sequencing (MeRIP-seq). MeRIP-seq was developed in
56   2012 to study m^6A in mammalian cells[21,22]. It requires the use of an antibody for the desired
57   modification and aims to enrich for RNA fragments carrying the modified nucleotide(s). It is
58   usually followed by deep sequencing to identify and map the enriched fragments or quantitative
59   PCR to verify specific RNA targets. The accuracy of MeRIP is based on the specificity of the
60   antibody to recognise the modified nucleotide over similar modifications (e.g., m^5C and
61   hm^5C[11,23]). Besides m^6A, MeRIP-seq has been also applied to study m^1A and m^5C RNA methylation
62   in several organisms[11,17,23–25].
63
64   Methylation of the cytosine at the fifth carbon position (m^5C) is the most prevalent DNA
65   modification[26,27] and one of the most common RNA modifications too[3,4]. While m^5C was detected
66   in eukaryotic mRNAs in 1975[28], only recently have studies focused on mapping the modification
67   transcriptome-wide, in coding and non-coding RNAs[11,16,17,23,29–34].
68
69   Alternative methods used in m^5C RNA research include chemical conversion of non-methylated
70   cytosines into uracils (bisulfite sequencing) and immunoprecipitation assays based on an
71   irreversible binding of a known RNA cytosine methyltransferase to its RNA targets (miCLIP, aza-
72   IP). In brief, bisulfite sequencing exploits the feature of 5-methylated cytosine to be resistant to
73   sodium bisulfite treatment that deaminates unmodified cytosines to uracil. The method was first
74   developed for DNA but adapted for RNA too and many studies have chosen this approach to
75   detect m^5C sites in RNA[16,23,29,32,34,35]. Both miCLIP and aza-IP require previous knowledge of the
76   RNA cytosine methyltransferase and use of the respective antibody. In the case of miCLIP
77   (methylation individual-nucleotide-resolution crosslinking and immunoprecipitation), the
78   methyltransferase carries a single amino acid mutation so that it binds to the RNA substrate but
79   cannot be released[30]. In aza-IP (5-azacytidine–mediated RNA immunoprecipitation), the
80   irreversible binding is formed between the 5-azaC nucleoside and the RNA cytosine
81   methyltransferase when exogenously provided 5-azaC is incorporated by RNA polymerases into
82   a target RNA molecule[31].
83
84   The main advantage of these three methods is that they allow single nucleotide resolution
85   mapping of m^5C. In addition, miCLIP and aza-IP provide information about the specific targets of
86   a selected RNA cytosine methyltransferase deciphering deeper the mechanism and role of post-
87   transcriptional RNA modifications. However, the MeRIP-seq approach can identify
88   transcriptome-wide m^5C regions without any previous knowledge required and avoids harsh

89 chemical and temperature conditions, such as bisulfite treatment or incubation with 5-azaC. Both
90 MeRIP and bisulfite sequencing can be inhibited by secondary RNA structures[36]. The
91 fragmentation step that is included in the MeRIP assay prior to immunoprecipitation aims to
92 facilitate antibody binding and increase the resolution of m^5C identification.
93
94 Another method worth mentioning is mass spectrometry (MS) of RNA nucleosides. MS can detect
95 and distinguish any type of modification both on DNA and on RNA. Briefly, RNA is extracted and
96 DNase digested, then desalted and digested to single nucleosides. The RNA nucleosides are
97 analyzed by a mass spectrometer. This method can be used to quantify the levels of each
98 modification and it does not rely on an antibody or a chemical conversion. However, a major
99 drawback is that it provides bulk information about the presence of RNA modifications. In order
100 to map the modifications, MS needs to be combined with RNase digestion and sequencing
101 information about specific RNA molecules, as in the case of human $tRNA^{Leu}_{(CAA)}$[37].
102
103 Here, we describe and discuss the MeRIP assay as used to study m^5C RNA methylation in
104 Arabidopsis[17].
105
106 **PROTOCOL:**
107
108 **1   Preparing the RNA**
109
110 1.1   Grind 200 mg of plant tissue to powder in liquid nitrogen, making sure that the tissue
111 remains frozen throughout the procedure.
112
113 1.2   Extract the RNA from the desired plant tissue following an acid guanidinium thiocyanate-
114 phenol-chloroform extraction protocol. To decrease the possibility to contaminate RNA with DNA
115 during the phase separation, use 1-bromo-3-chloropropane instead of chloroform.
116
117 1.2.1   Add 1 mL of RNA extraction reagent containing guanidine thiocyanate and acid phenol to
118 the grinded plant tissue (500 µL per 100 mg tissue). Mix well by inverting and make sure all the
119 tissue is wet. Incubate for 10 min at room temperature to dissociate the ribonucleoprotein
120 complexes.
121
122 1.2.2   Centrifuge for 10 min at 12,000 x g at 4 °C and transfer the supernatant to a new 1.5 mL
123 tube.
124
125 1.2.3   Add 200 µL of 1-bromo-3-chloropropane (100 µL per 500 µL RNA extraction reagent) and
126 vortex vigorously.
127
128 1.2.4   Centrifuge for 15 min at 12,000 x g at 4 °C and transfer the upper aqueous phase (approx.
129 500 µL) to a new 1.5 mL tube.
130
131 1.2.5   Add 1 volume of isopropanol (500 µL) and 0.1 volume of 3 M sodium acetate pH 5.5 (50
132 µL), mix well by inverting and precipitate 10 min at -20 °C.

133

NOTE: The use of sodium acetate (NaOAc) is recommended in order to enhance RNA precipitation. The protocol can be paused at this point by prolonging the precipitation of RNA for a few hours or even overnight.

1.2.6   Centrifuge for 30 min at 12,000 x g at 4 °C and discard the supernatant.

1.2.7   Wash the pellet twice with 500 µL of 80% EtOH, centrifuge for 5 min at 12,000 x g at 4 °C and discard.

1.2.8   Wash the pellet once with 500 µL 99% EtOH, centrifuge for 5 min at 12,000 x g at 4 °C and discard.

1.2.9   Dry the pellet for 5-10 min and dissolve in 30 µL of RNase-free H_2O.

NOTE: Instead, use any RNA extraction protocol of choice (e.g., a column-based system). If a DNase digestion is included in the protocol, skip it in the following step (step 1.3).

1.3   Measure the RNA concentration (e.g., with the use of a spectrophotometer) and digest 20 µg of RNA with DNase.

NOTE: DNA is rich in m5C and the antibody does not distinguish between DNA and RNA.

1.3.1   In a typical DNase reaction, treat 10 µg of RNA in a 50 µL reaction. Mix the following components and incubate the reaction(s) at 37 °C for 30 min:
10 µg of RNA   x µL
10x DNase buffer   5 µL
DNase   1 µL (2 units)
RNase-free H_2O up to 50 µL

1.3.2   Remove the enzyme either by adding an appropriate volume of DNase inactivation reagent (if it is included in the DNase kit and according to manufacturer's instructions) or by performing a cleanup step (e.g., column purification or phenol/chloroform extraction).

1.4   Check the quality and purity of the isolated RNA by capillary electrophoresis and proceed if the RNA integrity number (RIN) is higher than 7, to ensure the samples are of good quality.

1.5   OPTIONAL: Remove the ribosomal RNA to enrich the samples in mRNA content using an rRNA removal kit and according to manufacturer's protocol.

1.5.1   Use the DNase treated RNA from the previous step for the rRNA depletion reaction(s). Perform multiple reactions if the amount of total RNA is more than the maximum amount suggested for the reaction.

177 1.5.2   Note that only 5-10% of the input amount will be recovered after rRNA depletion. Proceed
178 with the rRNA depleted RNA (equal amount for all samples) and ignore the amounts mentioned
179 in the following steps, as they refer to total RNA.
180
181 NOTE: For a comparison and description of available rRNA depletion methods see here[38–40].
182 rRNA is the major part of total RNA and is m5C methylated in many organisms.
183
184 1.6   Prepare in advance in vitro transcripts (IVT) to be used as control RNA sequences and add
185 them in the samples.
186
187 1.6.1   Produce two distinct IVTs using an in vitro transcription kit, one with non-methylated
188 nucleosides and one where rCTP is replaced by 5-methyl-rCTP, to serve as negative and positive
189 controls in MeRIP, respectively. The transcripts prepared were those of *EGFP* and *Renilla*
190 luciferase.
191
192 NOTE: The IVTs should not exist in the transcriptome of the organism you are analysing. If the
193 IVTs are from the same template (e.g., both EGFP), then add the positive and negative control in
194 two different samples. If their sequence is different (e.g., EGFP and Renilla), they can be added
195 to the same sample.
196
197 1.6.2   Spike in each sample 0.1 ng IVT per 3 µg of RNA, as controls.
198
199 1.7   Sonicate the RNA to approximately 100 nt fragments.
200
201 NOTE: The conditions for RNA shearing must be adjusted in advance and they differ for each
202 sonicator. For the model used here, sonication is performed with the following conditions: Peak
203 power 174, Duty factor 10, Cycles/burst 200, 17 min.
204
205 1.7.1   Sonicate the same amount of RNA for all samples, at least 12 µg RNA per sample in 80 µL
206 of total volume (min 60 µL, max 100 µL), filled up with RNase-free H_2O.
207
208 1.8   Confirm the efficiency of sonication and the concentration of the RNA samples by capillary
209 electrophoresis. The average size of fragmented RNA should be around 100 nt.
210
211 **2   Methylated RNA Immunoprecipitation (MeRIP)**
212
213 2.1   In low-binding tubes, add 9 µg of sonicated RNA and RNase-free H_2O up to 60 µL (or more,
214 depending on the concentration).
215
216 2.2   Dissociate the secondary structures by heating the RNA at 70 °C in a water bath for 10
217 min and cooling down for an additional 10 min in an ice-water mix.
218
219 2.3   Split the sample in three parts: one-third (20 µL, if 60 µL were taken in step 2.1) is saved
220 in a separate tube at -80 °C as the Input sample. Fill the remaining 40 µL with RNase-free H_2O up

221 to 860 µL and then split in two low-binding tubes: one for IP and one for the Mock control (430
222 µL each).

224 2.4   Add to both tubes:
225   50 µL of 10x MeRIP buffer
226   10 µL of RNase inhibitor
227   10 µL of α-m^{5}C antibody (10 µg) in the IP sample per 10 µL of H$_2$O in the Mock sample

229 NOTE: The antibody clone used previously is not commercially available anymore. However, any
230 anti-5-methylcytosine monoclonal antibody should work similarly. Antibodies should be tested
231 for specificity before used for MeRIP[11,23].

233 2.5   Seal the tubes with parafilm and incubate for 12-14 hours at 4 °C, with over-head rotation.

235 2.6   The next day, prepare the protein G magnetic beads for binding.

237 2.6.1 For each tube (either IP or Mock control), use 40 µL of beads. Add the total amount of
238 beads (# of tubes x 40 µL, e.g., for 2 IP and 2 Mock samples, 160 µL of beads are needed) in a 15
239 mL tube and wash three times with 800 µL of 1x MeRIP buffer per sample (# of tubes x 800 µL
240 buffer, e.g., 3.2 mL for 2 IP and 2 Mock samples).

242 2.6.2 Perform washes at room temperature for 5 min with overhead rotation, collect the beads
243 with the help of a magnetic rack and discard the washing buffer. After the third wash, resuspend
244 the beads in the same volume of 1x MeRIP buffer as the initial volume of beads taken (# of tubes
245 x 40 µL, e.g., 160 µL of 1x MeRIP buffer for 2 IP and 2 Mock samples).

247 NOTE: The amount of protein G beads used is determined by the binding capacity of the beads
248 for the specific antibody type and the amount of antibody used. In this case, the beads have a
249 binding capacity of approx. 8 µg of mouse IgG per mg of beads and 30 mg/mL concentration.
250 Therefore, 40 µL are enough to bind approx. 9.6 µg of antibody.

252 2.7   Add 40 µL of resuspended beads to each IP and Mock sample and incubate for additional
253 2 hours at 4 °C, with over-head rotation.

255 2.8   Place the tubes on a magnetic rack for 1 min and discard the supernatant or save it as a
256 control (non-bound RNA sample).

258 2.9   Wash the beads 5 times by resuspending in 700 µL of 1x MeRIP buffer supplied with 0.01%
259 Tween 20 and incubating for 10 min at room temperature with overhead rotation.

261 2.10   Resuspend the washed beads in 200 µL of Proteinase K digestion buffer and add 3.5 µL of
262 Proteinase K. Incubate for 3 hours at 50 °C, shaking at 800 rpm. Occasionally, flick manually the
263 bottom of the tube if a sediment of beads is forming during the incubation.
264

3   Downstream analysis

3.1   Submit the Input and IP samples for single end sequencing with 50 bases read length (SE50).

3.2   Trim 3' end adaptors using cutadapt[41] and discard reads that are shorter than 48 nt.

3.3   Map trimmed reads to the Arabidopsis genome (TAIR10 annotation) using STAR[42] with a cutoff of 6% for mismatches and maximum intron size of 10 kb. Keep uniquely mapped reads for further analysis.

3.4   Identify enriched RNA fragments in IP samples compared to Input using two distinct methods and consider those that are found significantly enriched by both.

3.4.1   First, detect MeRIP-seq peaks using MACS2 peak caller[43] on pooled IPs versus Input.

3.4.2   Secondly, follow the analysis for MeRIP-seq peak calling as described in Meyer et al.[22] and Yang et al.[17].

3.4.2.1 Using custom R scripts, divide the genome in distinct 25 nt windows and count the number of uniquely mapped reads for each window based on the position of the last mapped nucleotide (since the reads originate from 100 nt RNA fragments).

3.4.2.2 Calculate significantly enriched windows in IP samples compared to Input with the Fisher Exact Test. The Benjamini-Hochberg procedure was used to correct for multiple testing.

3.4.2.3 Keep the significantly enriched peaks that span over at least two consecutive windows and discard peaks that cover only one window.

3.5   Identify annotated regions of the genome (transcripts) with significantly enriched peaks found by both methods.

3.6   Alternatively or complementarily, test specific RNA targets for their enrichment in the IP samples.

3.6.1   Reverse transcribe the RNA (same volume of Input, IP and Mock samples) with random hexamers.

309   3.6.2   Perform quantitative real-time PCR on the chosen targets, comparing Input, IP and Mock
310   via the ΔΔCt method.
311
312   NOTE: The generated product should not be longer than 100 bp, as this is the average
313   fragmentation size.
314
315   **REPRESENTATIVE RESULTS:**
316   A schematic of the method is provided in **Figure 1**. The first critical steps of the protocol are to
317   obtain RNA of good quality (RIN ≥ 7) and sonicate it to approximately 100 nt fragments. The
318   efficiency of both steps is examined by a chip-based capillary electrophoresis machine. In **Figure**
319   **2A**, a representative run of a good RNA sample is shown. The sample is diluted 1:10 before
320   loading on the chip in order to have a concentration that is in the range of detection of the kit
321   used (5-500 ng/µL). The same sample is also run after sonication and is shown in **Figure 2B**. Notice
322   the presence of one uniform peak shifted to the left of the diagram, at a size of around 100
323   nucleotides. The lower concentration is caused both by loss of RNA during fragmentation but also
324   because of the increased volume of the samples (60-100 µL, step 1.7.2).
325
326   The quality of the IP and Mock samples can be evaluated by qRT-PCR. To this end, the spiked-in
327   IVTs serve as positive and negative controls: the methylated IVT, where in all cytosine positions
328   there is m^5C, is expected to be highly enriched in the IP sample; on the contrary, the non-
329   methylated IVT should not have a difference between IP and Mock. The primers used in the qRT-
330   PCR assay for the two control IVTs (*EGFP* and *Renilla* luciferase) are listed in **Table 1**. Indeed, as
331   shown in **Figure 3**, around 80% of the methylated IVT was recovered in the IP sample, and only
332   approximately 2% in the Mock. For the non-methylated control, the recovery was below 1% in
333   both IP and Mock samples. This verifies the efficiency of MeRIP that methylated RNA fragments
334   were precipitated and enriched, and is a good indicator that the samples can be used for
335   downstream analysis. In addition, the fold enrichment of the non-methylated IVT (IP to Mock
336   ratio) can be applied as a threshold to estimate significance of enrichment in the qRT-PCR assays.
337
338   After aligning the reads to the genome (**Figure 4**), the peak calling algorithms described in steps
339   3.1.4 and 3.1.5 are applied to identify the statistically significant windows, enriched in the IP
340   samples compared to the Input. The sequences that correspond to these windows can be used
341   further, for example to search for conserved methylation-related motifs[11,17].
342
343   **FIGURE AND TABLE LEGENDS:**
344   **Figure 1: Schematic representation of MeRIP-seq protocol**. RNA samples are incubated with an
345   antibody for 5-methylated cytosines and the complexes are pulled down with protein G magnetic
346   beads that capture the antibodies along with the bound RNA. The eluted RNA samples are
347   analyzed by deep sequencing and qRT-PCR.
348
349   **Figure 2: Representative results from quality analysis of RNA samples.** (**A**) Representative
350   profile of a qualified total RNA plant sample. (**B**) Representative profile of an RNA sample after
351   sonication to 100 nt fragments. Output files from capillary electrophoresis software.
352

353 **Figure 3: qRT-PCR analysis of control IVTs**. Methylated and non-methylated in vitro transcripts
354 were used as positive and negative controls of the MeRIP assay, respectively. After
355 immunoprecipitation, the methylated IVT is highly enriched in the IP sample (green) but not in
356 the Mock sample (without anti-m^{5}C antibody; purple). The non-methylated IVT showed no
357 enrichment and no difference between IP and Mock.
358
359 **Figure 4: Read alignment before and after MeRIP around a representative transcript.** Reads
360 aligned to a specific transcript in the Input sample (top row) and in two IP replicates (middle and
361 bottom row). The black box shows an identified enriched 50 nt long window based on MACS2[43]
362 and MeRIP-seq[22] peak-calling analyses.
363
364 **Table 1: Information about the primers and generated products for the qRT-PCR analysis.**
365
366 **DISCUSSION:**
367 RNA carries more than one hundred distinct base modifications[4] that form the
368 epitranscriptome[44]. These modifications add an additional layer of regulation of translation and
369 signalling (reviewed in [5,6,8,20,45,46]). Early studies were able to detect the presence of post-
370 transcriptional modifications on RNA[28,47] but the specific modified RNAs need to be identified in
371 order to understand the role of epitranscriptomics. MeRIP was designed as a method to map RNA
372 methylation sites transcriptome-wide[21,22]. It can be adapted to any modification, if a specific
373 antibody is available.
374
375 The main strength of this protocol is that is relatively simple, safe for the RNA and the user (e.g.,
376 5-azaC is highly toxic for plants and humans) and does not require sequence or modifying enzyme
377 information. Moreover, the enrichment of methylated RNAs by the IP increases the chances of
378 low abundant mRNAs to be detected, unlike bisulfite sequencing that does not contain an
379 enrichment step. When two serial rounds of MeRIP are performed, enrichment in RNA fragments
380 containing methylation sites increases further[22]. One of the limitations of MeRIP, especially when
381 applied to mRNA methylation studies, is the high quantity of RNA required as input for the assay.
382 The ribodepletion – or poly(A) enrichment – step will reduce the background caused by the
383 heavily modified ribosomal RNA but it removes more than 90% of total RNA. DNA must also be
384 completely removed as it is rich in 5-methylated cytosines. Another drawback is the lower
385 resolution of the exact position of methylation. Sonication of the RNA prior to incubation with
386 the antibody helps towards this direction by narrowing down the region containing the
387 modification to 100-200 nucleotides. When MeRIP is combined with deep sequencing, the
388 resolution of m^{5}C site prediction increases as the sequencing reads form a Gaussian distribution
389 around the potential methylation site. Additionally, the specificity of the antibody needs to be
390 confirmed prior to the assay (e.g., with RNA dot blot assays, performed with oligos synthesized
391 with modified nucleotides), however, to what extent an antibody can actually distinguish
392 between closely related modifications (e.g., m^{6}A and m^{6}Am) is a point of argument in the
393 field[48,49]. Moreover, highly structured RNAs might interfere with the antibody–antigen
394 interaction, another restriction that is mostly addressed with fragmentation and denaturation of
395 RNA prior to IP. On the contrary, bisulfite sequencing that is also affected by secondary
396 structures, does not include a fragmentation step and this might be one reason that causes

397   discrepancy between the m^{5}C sites and mRNAs predicted by bisulfite sequencing[16] and MeRIP-
398   seq[11,17]. Other cytosine modifications (e.g., hm^{5}C) are also resistant to bisulfite-mediated
399   deamination[35].
400

401   Modifications of MeRIP-seq include a crosslinking step, either with the introduction of a
402   photoactivatable ribonucleoside (photo-crosslinking-assisted m^{6}A-seq, PA-m^{6}A-seq [50]) or using
403   UV light to create antibody-RNA crosslinks after the IP (miCLIP[49], different method than the
404   miCLIP described in introduction[30], but also with individual-nucleotide resolution). In the future,
405   and as knowledge on RNA methylation is accumulating, more targeted approaches might be
406   preferable, based on the modifying enzymes and/or the consensus sequences where methylation
407   is appearing. The identification of reader proteins is essential to the understanding of the
408   molecular and signalling function of post-transcriptional modifications. Nanopore sequencing
409   technology already allows the direct identification of modified nucleotides without prior
410   treatment of the RNA[17] but there is still room for improvement on this field regarding sequence
411   depth and bioinformatic analysis. Overall, MeRIP-seq is currently an established, reliable, and
412   unbiased approach to identify methylated RNA transcripts.
413

419

420   **DISCLOSURES:**
421   The authors have no conflict of interest to disclose.
422

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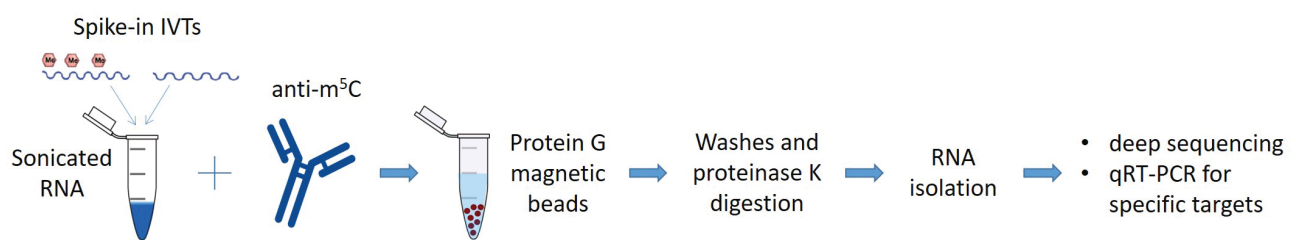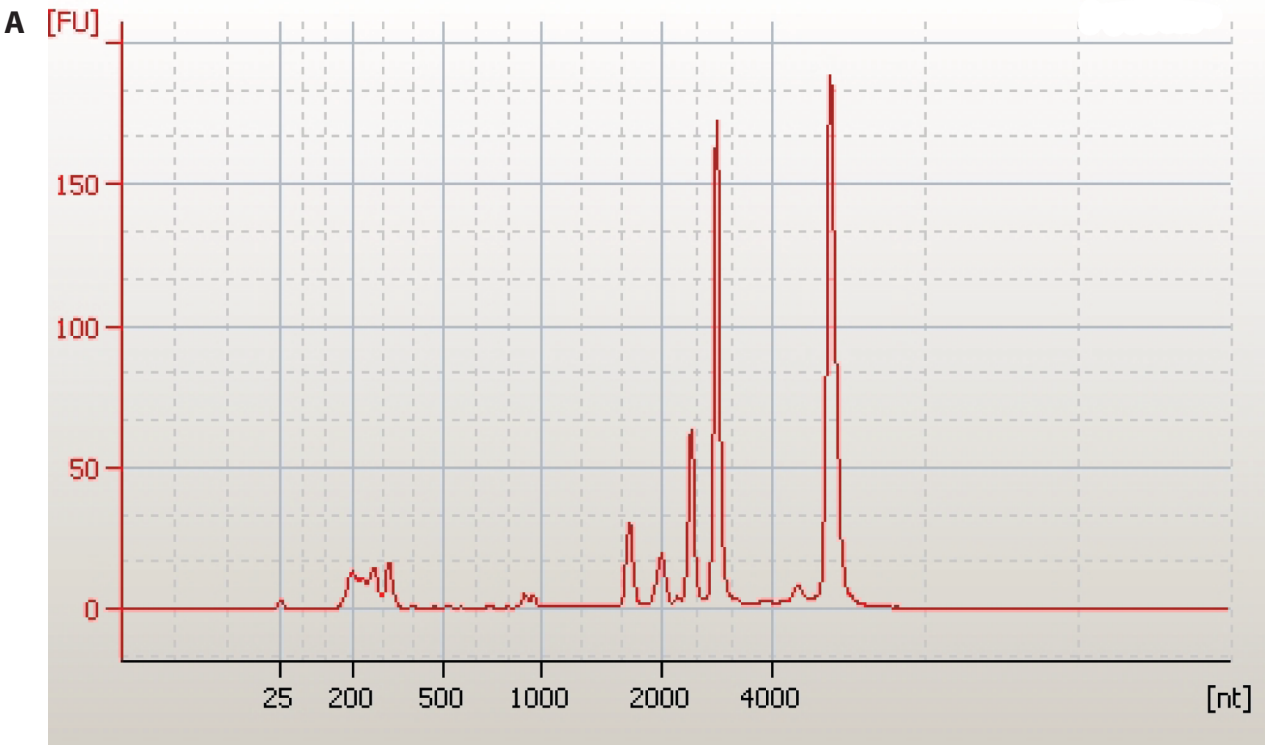
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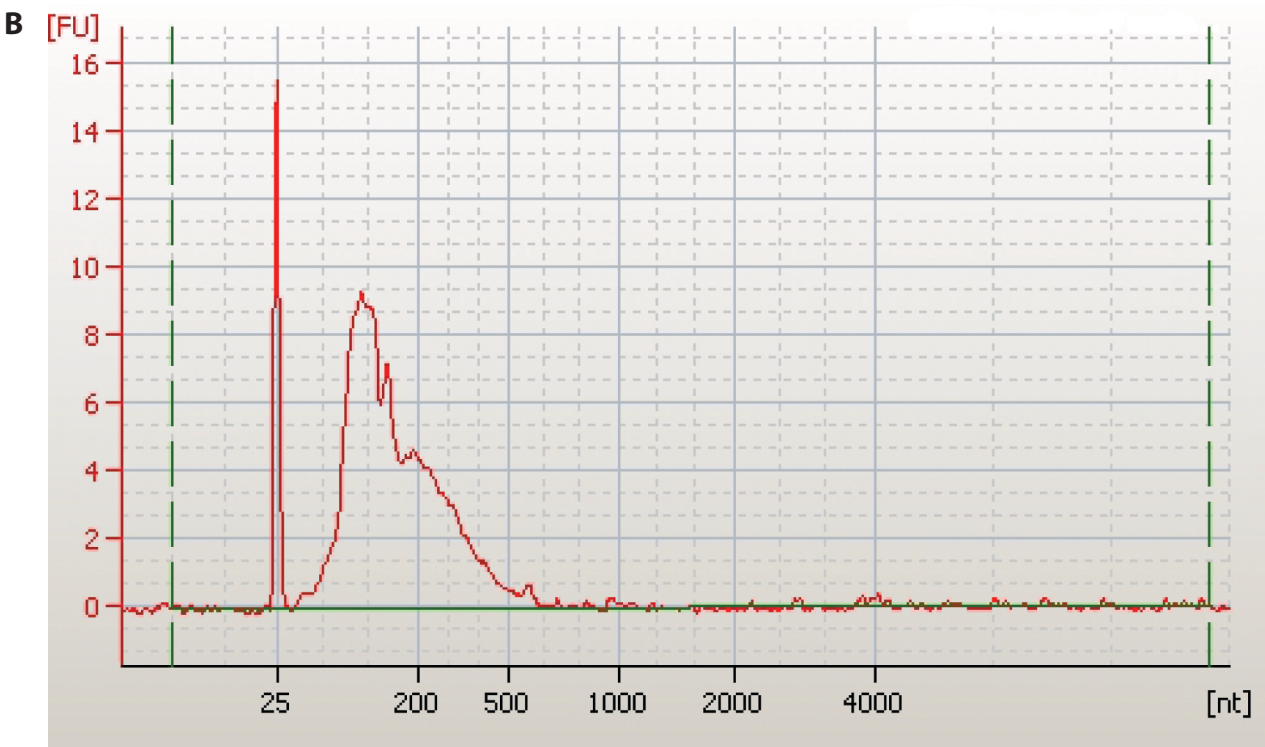484   stem cells and brain. *Genome Biology*. **18** (1), 1–16 (2017).

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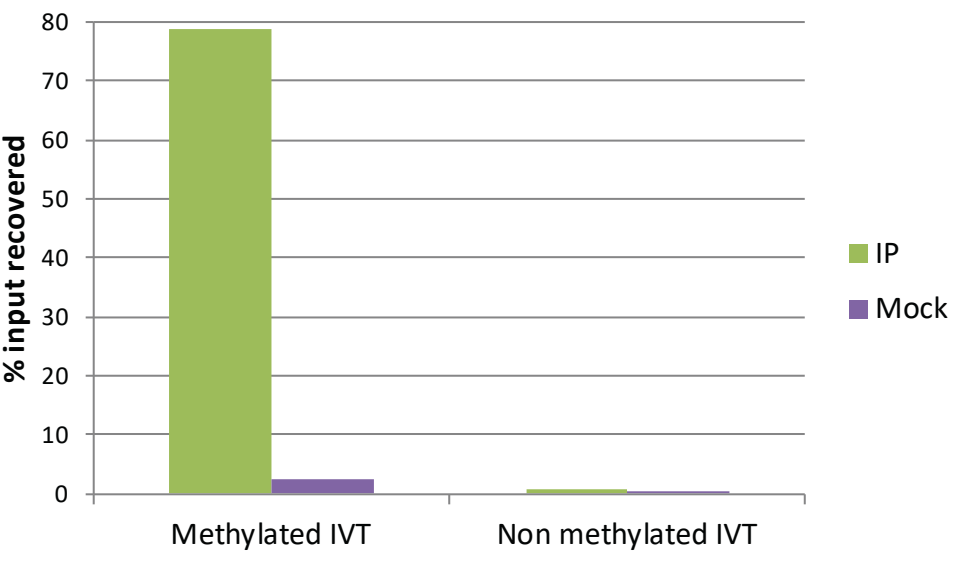530   (5), 1587–1590 (2015).
531

Spike-in IVTs

Sonicated RNA

anti-m^{5}C

Protein G magnetic beads

Washes and proteinase K digestion

RNA isolation

- deep sequencing
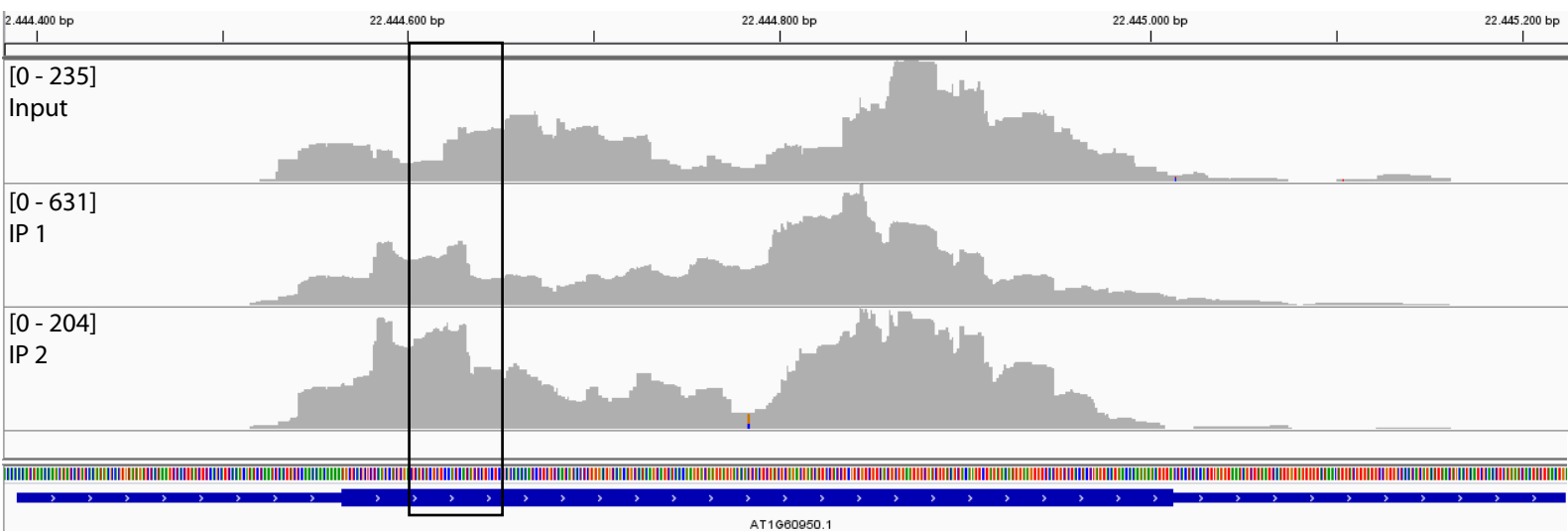- qRT-PCR for specific targets

A

RNA Concentration: 497 ng/µL   RNA Integrity Number (RIN):   8.20
rRNA Ratio [25s / 18s]:   1.4

B

RNA Concentration: 34 ng/µL   RNA Integrity Number (RIN):   2.50
rRNA Ratio [25s / 18s]:   0.0

Sequence of the 50 nt window of the peak: AACUUCAUUCAUCCGUCGUUCCCCAGCUCCAAUCAGUCUCCGUUCCCUUC

Table

Target	Primer pair
EGFP	For: 5'- GGCAACTACAAGACCCGCGCC -3'
	Rev: 5'- GCCCTTCAGCTCGATGCGGTT -3'
Renilla luciferase	For: 5'- GGAGAATAACTTCTTCGTGGAAAC -3'
	Rev: 5'- GCTGCAAATTCTTCTGGTTCTAA -3'

Product sequence	Product length
GGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCG ACACCCTGGTGAACCGCATCGAGCTGAAGGGC	72 bp
GGAGAATAACTTCTTCGTGGAAACCATGTTGCCATCAAAAAT CATGAGAAAGTTAGAACCAGAAGAATTTGCAGC	75 bp

Table of Materials

Name of Material	Company	Catalog Number	Comments/Description
α-m5C antibody	ZymoResearch	A3001 (discontinued), A3002 available	Clone 10G4 (discontinued), 7D21 available
Chip and reagents for capillary electrophoresis	Agilent	5067-1511	RNA 6000 Nano kit
Dnase	Ambion	AM1907	TURBO DNA-free kit
Co-precipitant of nucleic acids	Ambion	AM9515	Glycoblue, 15 mg/mL
In vitro transcription kit	Promega	P1300	T7 RiboMAX Large Scale RNA Production System
Low-binding reaction tubes	Kisker Biotech	G017	1.7 ml microcentrifuge tubes
Protein G magnetic beads	Invitrogen	10003D	Dynabeads Protein G
Proteinase K	Ambion	AM2546	20mg/mL stock solution, RNA grade
RNase inhibitor	Promega	N2615	RNasin Plus 40 U/µl
rRNA removal kit	Epicentre	RZPL11016	Ribo-Zero rRNA removal kit (Plant leaf)
Sonication tubes	Covaris	520045	microtube AFA Fiber Pre-Slit Snap-Cap 6x16mm
RNA extraction reagent	Ambion	15596018	TRIzol reagent
Equipment			
Capillary electrophoresis machine	Agilent	G2939BA	2100 Bioanalyzer System
Magnetic rack	Invitrogen	12321D	DynaMag-2
Microentrifuge	Eppendorf	discontinued	5417R model
Rotator	Benchmark	R4040	RotoBot Mini
Sonicator	Covaris	500217	S220 Focused-ultrasonicator
Spectrophotometer	ThermoFisher Scientific	ND-ONEC-W	NanoDrop OneC
Waterbath	GFL	1012	1012 Incubation bath

Manuscript No: JoVE61231

"Methylated RNA Immunoprecipitation Assay to Study m5C Modification in Arabidopsis"

We would like to thank the editor and the reviewers for their work and time to comment on the manuscript. We hope we addressed sufficiently the kind and helpful suggestions and we believe the implemented changes upgrade the manuscript. Below follows a point-by-point response to editorial and reviewers' comments.

With kind regards,

Eleftheria Saplaoura and Fritz Kragler (for all authors)

Response to editorial and reviewers' comments:

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We went through the manuscript and made the necessary corrections.

2. Figure 2: Please capitalize the L in the microliter abbreviation. Please use periods as decimals instead of commas.

Figure 2 was corrected.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

We apologize for this. We went through the text and removed all commercial language.

4. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

5. 1.1: How much tissue is ground?

More details were filled in.

6. 1.2: How is this done? Please provide explicit information here if this is to be filmed.

More details were filled in.

7. 1.3: How is this done? Please provide a reaction recipe and the temperatures/times used.

More details were filled in.

8. 1.5: How is this done?

References were added for comparisons of available methods that also describe the protocols when required. When a kit is used instead (for example in our case), the protocol is described by the manufacturer and it would be commercial language / property.

9. 1.6.1: What transcripts are prepared? Please provide sequences whenever possible.

The transcripts were filled in and a table with the primers used for the qPCR, as well as the generated product, was included in the results section.

10. Please note that if any of step 3 is to be filmed, more explicit details are required. We would need all user terminal commands used and/or GUI button clicks.

The main analysis pipeline is based on established methods. Thus, our aim was to just briefly describe the main steps that are necessary to understand the downstream analysis. For further details, we refer to the original publications and we would prefer not to include any of those steps in the film.

Reviewers' comments:
Reviewer #1:

Manuscript Summary:

This manuscript describes the protocol to capture and analyze RNA modifications mainly m5c, which is known to affect its function and the structure. This manuscript focusses mostly on MeRIP-seq approach using antibody that recognizes the RNA base modification to capture the molecule and subsequently next-generation sequencing is employed to identify and catalog them.

This is a detailed and well-described protocol and it warrants publication. This will be very useful for a large audience in general.

Major Concerns:
My only criticism is that the bioinformatics pipeline could be described in little more detailed. I am sure many "biologists" are well aware of the molecular and wet-biology part but not with the analysis part.

We thank the reviewer for this comment and we added a few more details in the bioinformatics pipeline and also included a fourth figure, as recommended by Reviewer 2, that shows a read alignment with an identified peak. However, the focus of this manuscript is the wet-biology part of the MeRIP protocol, and as mentioned above, we wanted to just briefly describe the main steps that are necessary to understand the downstream analysis. The main analysis pipeline is based on established methods and for further details, we refer to the original publications of the peak calling analyses.

Reviewer #2:

Manuscript Summary:
In this work by Saplaoura et al., the protocol of MeRIP is described in detail to be shared with the scientific community of RNA biology. I find this article timely and well achieved. Said that, I have some comments that can help to improve the MS before publication.

Major Concerns:

Points 2.4 and 2.6: according to Thermo/Invitrogen, for 50 ul of Dynabeads, 1 to 10 ug of antibody should be used, depending on the Ab. Here the authors propose to use 20 ug of Ab for 40 ul of beads, what represents a ratio of 20/1 to 6/1 of what is recommended by the manufacturer. This would not represent any problem if the Ab was coated to the beads and washed prior to incubation with the sample (although it may mean a waste of Ab). However, an excess of Ab in the sample may result in blocking the beads with free Ab. This makes me think that this amount of Ab is in fact needed for the amount of RNA used, what is in high excess related to the beads used. As a potential user of this protocol, I'd like to have a NOTE discussing this to know the opinion of the authors.

This is indeed a very interesting discussion point. The manual for the beads mentions that Dynabeads™ Protein G have a binding capacity of ~8 µg of human IgG per mg of beads. The amount of Ab captured depends on the concentration of beads and Ab in the starting sample, as well as the type of immunoglobulin being bound (see Table).

Ig origin	Affinity for Protein G
Human IgG1,2,4	+++
Human IgD	-
Human IgA, E, M	-
Human IgG3	+++
Mouse IgG1	+++
Mouse IgG2, 2b, 3	+++
Mouse IgM	+

Our Ab is mouse IgG1 monoclonal, which has similar affinity to human IgG. Dynabeads Protein G have a concentration of 30 mg/mL, therefore the 40 µl we used are 1.2 mg and have a binding capacity of 9.6 µg antibody. In the IP, we use 10 µg Ab (10 µL of Ab with stock concentration 1 mg/mL). Since it seems that mentioning the final concentration of the Ab in step 2.4 is confusing, we changed it to mentioning the final amount (10 µg) and making a note about the binding capacity of the beads used.

Figures: I suggest to add a plot of the Seq approach derived BED or WIG file using a browser (e.g. IGB or IGV) showing a peak identified by sequencing.

We thank the reviewer for the suggestion. This has now been included as Figure 4.

Minor Concerns:

Comments are related to protocol points:

1.1 give an estimation of how much plant material.

More details were filled in.

1.2 is TRIzol (Ambion-Invitrogen used here) specially recommended or any similar product by other companies are equally useful? If yes, then I suggest using a more generic name for the product or, even better, mention those brands that were tried and work well in your hands. If the authors think that any equivalent product can be used instead, I think this should be noted. The same comment is applicable to the brand of the Ab.

We thank the reviewer for the comment. We routinely perform TRIzol extraction for RNA and did not test other brands than the one mentioned. However, we changed to a generic name for the reagent and added a note that any other RNA extraction protocol of choice could be used at this step.

Regarding the antibody, we only checked the clone mentioned but other publications have used different Ab brand efficiently, too. We added a note citing the other publications and mentioning that another Ab can be used as well. Especially since the clone we used in our study is not available anymore by the company and has been replaced (also mentioned in the Table of Materials now).

2.11 no AcNA is added for precipitation?

We thank the reviewer for noticing that this was missing. We perform the elution of the RNA from the antibody by adding TRIzol, which already contains the salts needed for the precipitation. However, we do add salts to enhance the precipitation. It is now corrected by referring to the RNA extraction protocol in step 1.2 that is more thoroughly explained.

Intro and discussion: I suggest devoting a few more phrases linking RNA modifications to the fate or function of RNAs. Also, express in the authors opinion what is missing in Arabidopsis regarding post-transcriptional modifications of RNAs.

We added a part in the introduction as suggested and updated the literature with some more recent reviews focusing on the function of RNA modifications in plants (Chmielowska-Bak et al., 2019, Liang et al., 2020).