**Please cite this article as:** Lisha *et. al.*, (2017). Dot Blot Analysis of N$^6$-methyladenosine RNA Modification Levels, *Bio-protocol* 7 (1): e2095. DOI: 10.21769/BioProtoc.2095.

bio-protocol www.bio-protocol.org/e2095 Vol 7, Iss 01, Jan 05, 2017
DOI:10.21769/BioProtoc.2095

# Dot Blot Analysis of N$^6$-methyladenosine RNA Modification Levels

Lisha Shen, Zhe Liang and Hao Yu*****

Temasek Life Sciences Laboratory and Department of Biological Sciences, National University of Singapore, Singapore
*****For correspondence: dbsyuhao@nus.edu.sg

**[Abstract]** N$^6$-methyladenosine (m$^6$A) is the most prevalent internal modification of eukaryotic messenger RNA (mRNA). The total amount of m$^6$A can be detected by several methods, such as dot blot analysis using specific m$^6$A antibodies and quantitative liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Fu *et al.*, 2014; Shen *et al.*, 2016). Here we describe the method for fast detection of total m$^6$A levels in mRNA by dot blot analysis using a specific m$^6$A antibody.
**Keywords:** Dot blot, RNA modification, m$^6$A

**[Background]** Dot blot analysis for detecting total m$^6$A levels in mRNA is relatively easy, fast, and cost-effective as compared to other methods, such as two-dimensional thin layer chromatography and LC-MS/MS. This approach can be used, in a qualitative manner, to evaluate temporal and spatial changes in m$^6$A levels in various plant tissues or plants at different developmental stages. This is particularly useful for initial examination of changes in m$^6$A levels in relevant mutants prior to detailed investigations by other complex and quantitative approaches.

## Materials and Reagents

1. Amersham Hybond-N+ membrane (GE Healthcare, catalog number: RPN203B)
2. Plastic wrap
3. Amersham Hyperfilm ECL (GE Healthcare, catalog number: 28906835)
4. Total RNA
5. Dynabeads® mRNA Purification Kit (Thermo Fisher Scientific, Ambion™, catalog number: 61006)
6. RNase-free water
7. Anti-m$^6$A antibody (Synaptic Systems, catalog number: 202 003)
8. Goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology, catalog number: sc-2004)
9. ECL Western Blotting Substrate (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 32106)
10. 1x phosphate buffered saline (1x PBS), pH 7.4
11. Tween 20 (Sigma-Aldrich, catalog number: P9416)
12. Non-fat milk (Bio-Rad Laboratories, catalog number: 1706404)
13. Wash buffer (see Recipes)
14. Blocking buffer (see Recipes)

**Please cite this article as:** Lisha *et. al.*, (2017). Dot Blot Analysis of $N^6$-methyladenosine RNA Modification Levels, *Bio-protocol* 7 (1): e2095. DOI: 10.21769/BioProtoc.2095.



15. Antibody dilution buffer (see Recipes)

## Equipment

1. NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Thermo Scientific™, model: NanoDrop™ 2000 Spectrophotometer)
2. Heat block
3. Stratalinker 2400 UV Crosslinker (Stratalinker)
4. Shaker

## Software

1. ImageJ

## Procedure

1. mRNA purification
   a. Isolate mRNA from total RNA using the Dynabeads® mRNA Purification Kit following the manufacturer's instructions. For one dot blot assay, we recommend purifying at least 20 µg of total RNA.
   b. Determine the concentration of purified mRNA with NanoDrop and make a serial dilution of mRNA to 50 ng/µl, 10 ng/µl and 2 ng/µl using RNase-free water.
2. Dot blotting
   a. Denature the serially diluted mRNA at 95 °C to disrupt secondary structures in a heat block for 3 min.
   b. Chill on ice immediately after denaturation to prevent the re-formation of secondary structures of mRNA.
   c. Drop 2 µl of mRNA directly onto the Hybond-N+ membrane optimized for nucleic acid transfer (Figure 1).

**Please cite this article as:** Lisha *et. al.*, (2017). Dot Blot Analysis of $N^6$-methyladenosine RNA Modification Levels, *Bio-protocol* 7 (1): e2095. DOI: 10.21769/BioProtoc.2095.



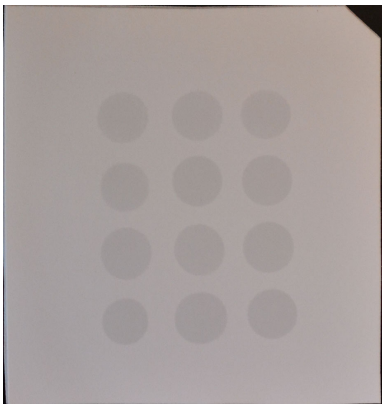

**Figure 1. Example of mRNA dots on a membrane**

d. Crosslink spotted mRNA to membrane in a Stratalinker 2400 UV Crosslinker twice using the Autocrosslink mode (1,200 microjoules [x100]; 25-50 sec).
e. Wash the membrane in 10 ml of wash buffer in a clean washing tray, which is unnecessary to be RNase-free, for 5 min at room temperature with gentle shaking to wash off the unbound mRNA.
f. Incubate the membrane in 10 ml of blocking buffer for 1 h at room temperature with gentle shaking.
g. Incubate the membrane with anti-$m^6A$ antibody (1:250 dilution; 2 μg/ml) in 10 ml of antibody dilution buffer overnight at 4 °C with gentle shaking.
h. Wash the membrane three times for 5 min each in 10 ml of wash buffer with gentle shaking.
i. Incubate the membrane with goat anti-rabbit IgG-HRP (1:10,000 dilution; 20 ng/ml) in 10 ml of antibody dilution buffer for 1 h at room temperature with gentle shaking.
j. Wash the membrane four times for 10 min each in 10 ml of wash buffer with gentle shaking.
k. Incubate the membrane with 3 ml of ECL Western Blotting Substrate for 5 min in darkness at room temperature. Please note that the volume of ECL solution added is dependent on the size of the membrane. According to the manufacturer's instructions, 0.125 ml ECL solution per $cm^2$ of the membrane is recommended.
l. Wrap the membrane in plastic wrap and expose with Hyperfilm ECL for a proper exposure period.
m. Develop the film.

## Data analysis

As dot blot analysis is a semi-quantitative approach, the analysis should be repeated through the above procedures using independent biological materials. Only the repeatable changes in $m^6A$ levels observed in independent materials as compared to the wild-type control are considered 'positive' results, which may be further investigated by other quantitative approaches. In addition,

the signals from the dot blot images can be quantified by ImageJ and the statistical analysis should be based on at least three biological replicates.

## Representative data

For representative data, please see the paper of Shen *et al.*, 2016.

## Notes

This protocol is also applicable to detect other types of RNA modifications if the corresponding specific primary antibodies and secondary antibodies are available.

## Recipes

1. Wash buffer
   1x PBS
   0.02% Tween-20
2. Blocking buffer
   1x PBS
   0.02% Tween-20
   5% non-fat milk
3. Antibody dilution buffer
   1x PBS
   0.02% Tween-20
   5% non-fat milk

*Note: It is unnecessary to use RNase-free water to prepare the above solutions.*

## Acknowledgments

This work was supported by Academic Research Fund (MOE2015-T2-1-002) from the Ministry of Education-Singapore, the Singapore National Research Foundation Investigatorship Programme (NRF-NRFI2016-02), and the intramural research support from National University of Singapore and Temasek Life Sciences Laboratory.

## References

1. Fu, Y., Dominissini, D., Rechavi, G., and He, C. (2014). Gene expression regulation mediated through reversible m6A RNA methylation. Nat Rev Genet 15(5): 293-306.

bio-protocol www.bio-protocol.org/e2095 Vol 7, Iss 01, Jan 05, 2017
DOI:10.21769/BioProtoc.2095

2. Shen, L., Liang, Z., Gu, X., Chen, Y., Teo, Z.W., Hou, X., Cai, W.M., Dedon, P.C., Liu, L., and Yu, H. (2016). N6-methyladenosine RNA modification regulates shoot stem cell fate in *Arabidopsis*. Dev Cell 38(2): 186-200.