TRAINING COURSE IN

Computational Methods for Epitranscriptomics

Bari, 11th-13th September 2024

Methods for RNA modifications profiling

Logan Mulroney

Slides adapted from Mattia Furlan and Mattia Pelizzola

Outline

- Background on RNA modifications
- Methods to profile m6A
  - Bulk levels – Dot-blot, ELISA/colorimetric, MassSpec
  - Genome-wide, Antibody-based – MeRIP-seq, miCLIP
  - Genome-wide, not Antibody-based – MAZTER-seq, GLORI
  - Single cell – scDART-seq
  - Single molecule – Nanopore native RNA-seq
- Other endogenous modifications – m5C, Ψ, m1A, Nm
- Exogenous modifications – 4sU-seq, SLAM-seq, nano-ID

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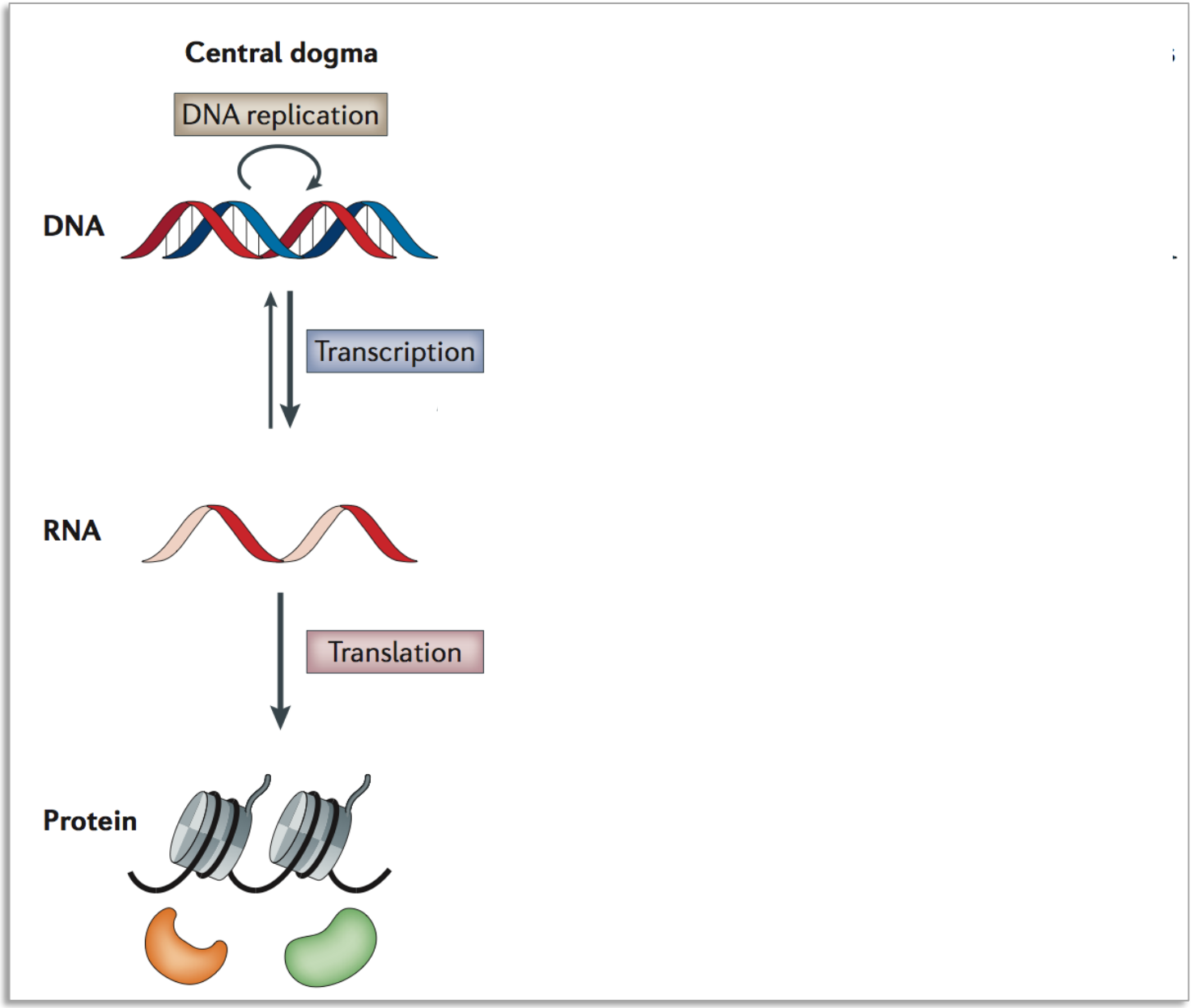
The epitranscriptome



Adapted from: Fu Y *et al*, *Nature Review Genetics* 2014

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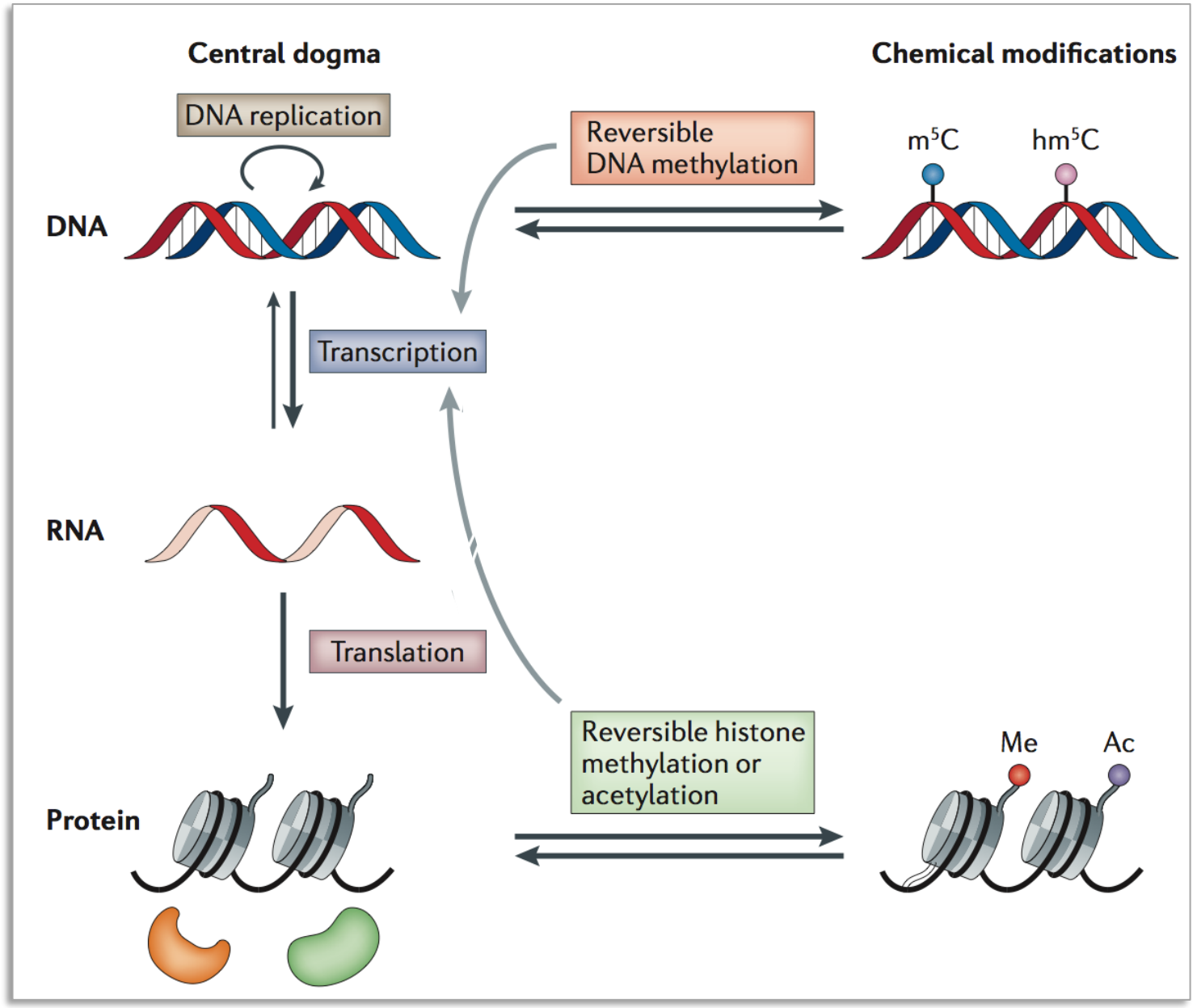

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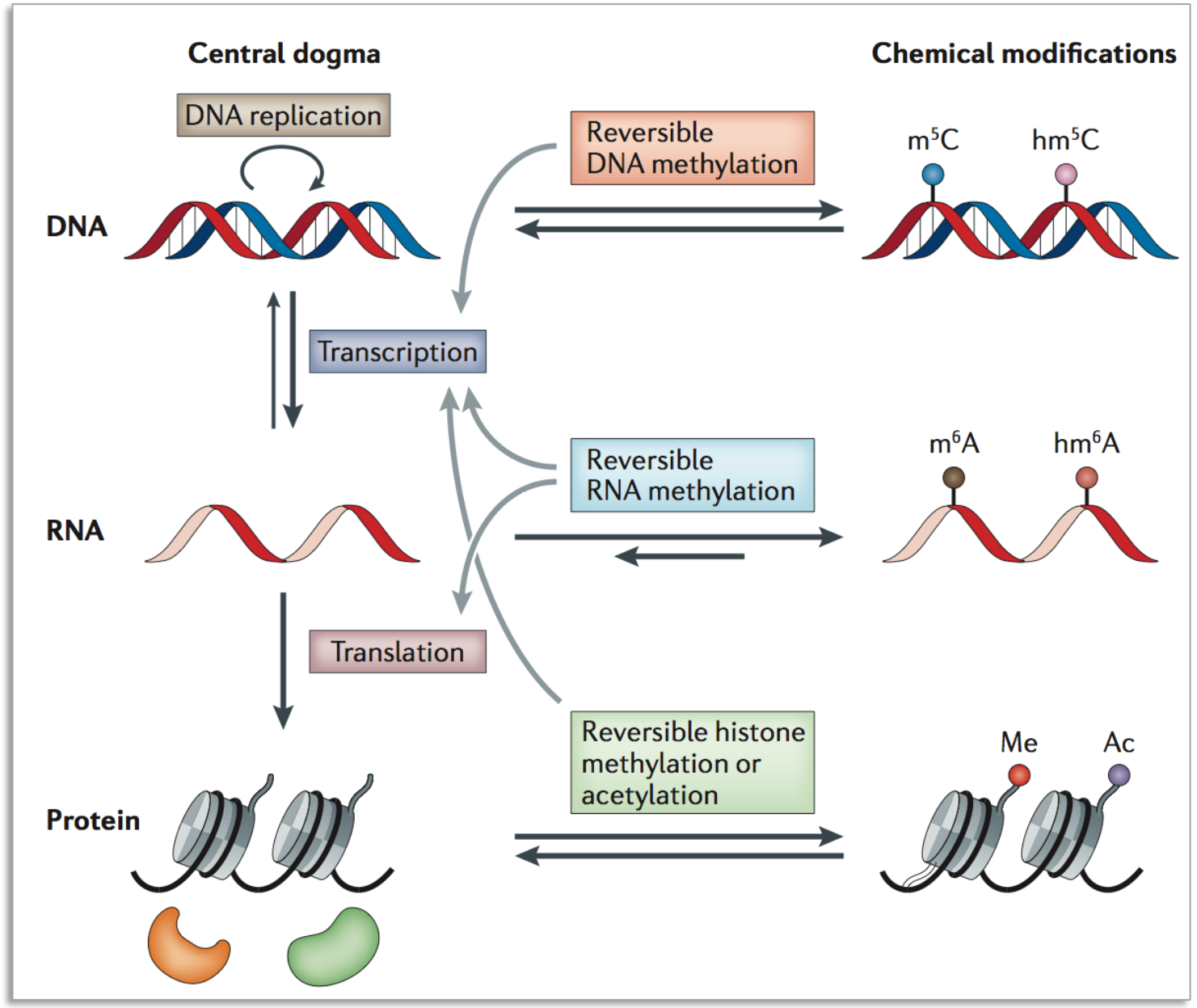

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The tRNA epitranscriptome

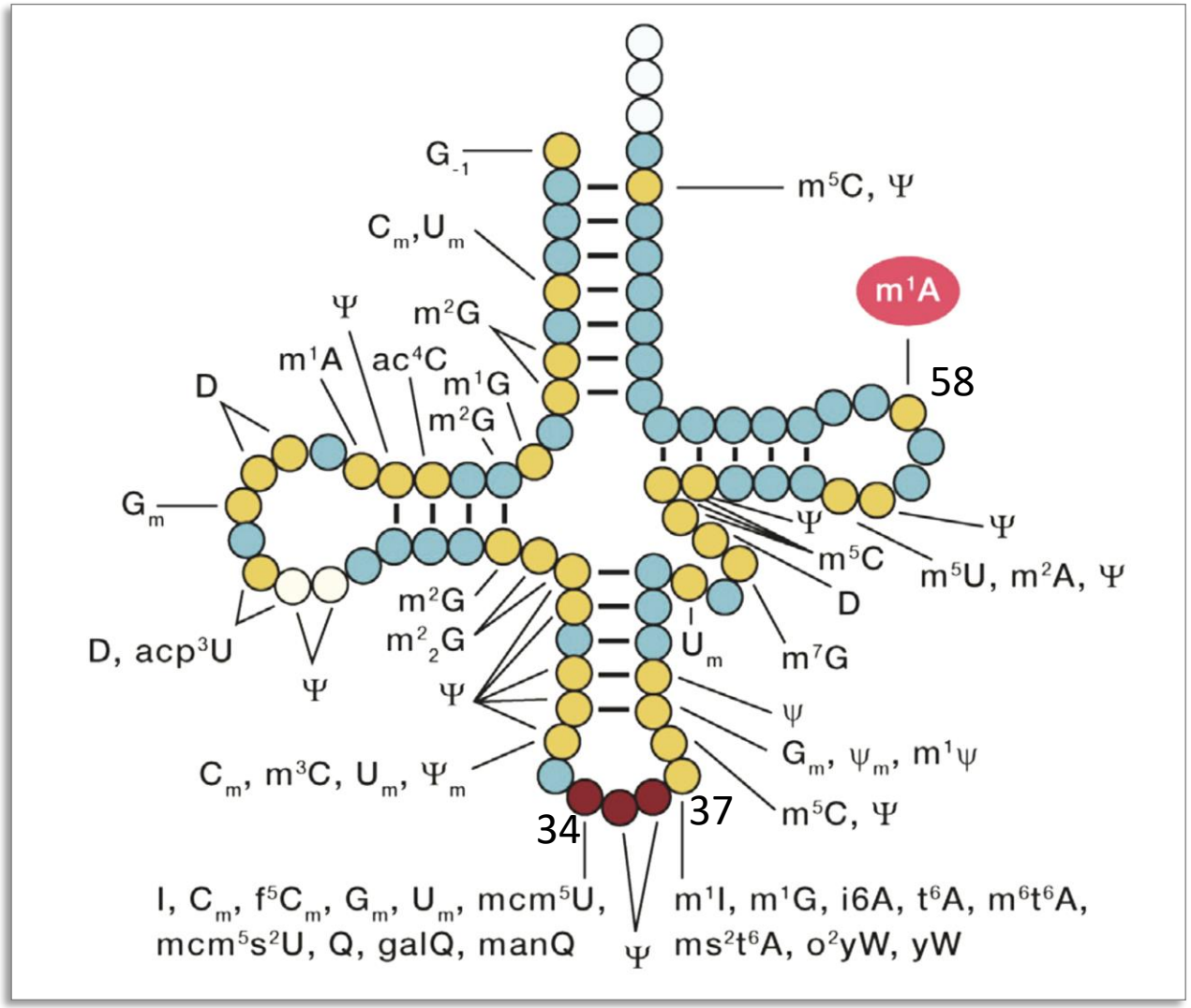
tRNAs *are decorated by dozens of marks,*

20% *of nucleotides are modified in mammalians tRNAs,*

the ***anticodon*** *loop is heavily modified with almost every tRNA carrying a mark at position 34 and/or 37,*

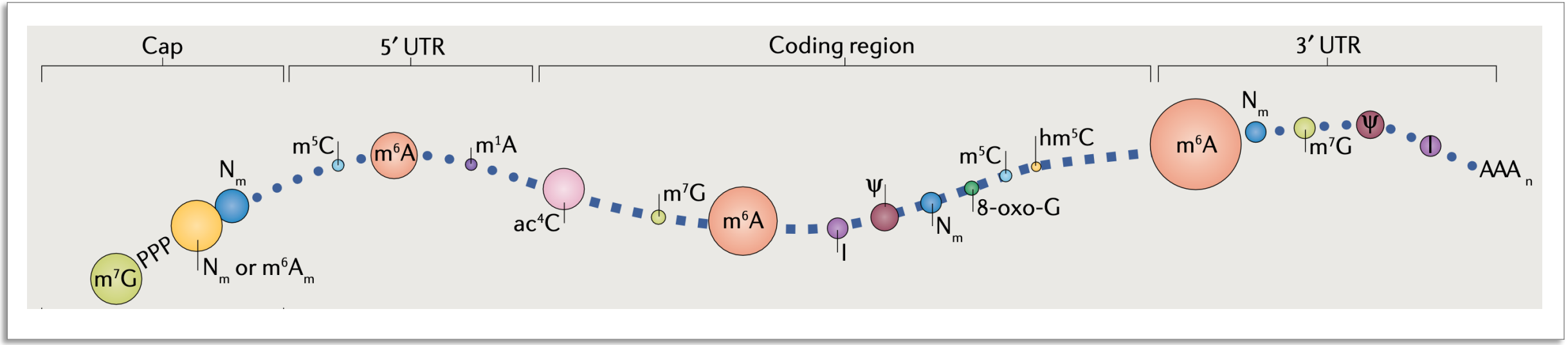
modifications are crucial for translation ***accuracy*** *and* ***efficiency****,*

tRNA modifications are ***dynamic.***



Adapted from: Roundtree I *et al*, *Cell* 2017

The mRNA epitranscriptome



*multiple marks decorate **mRNAs,***

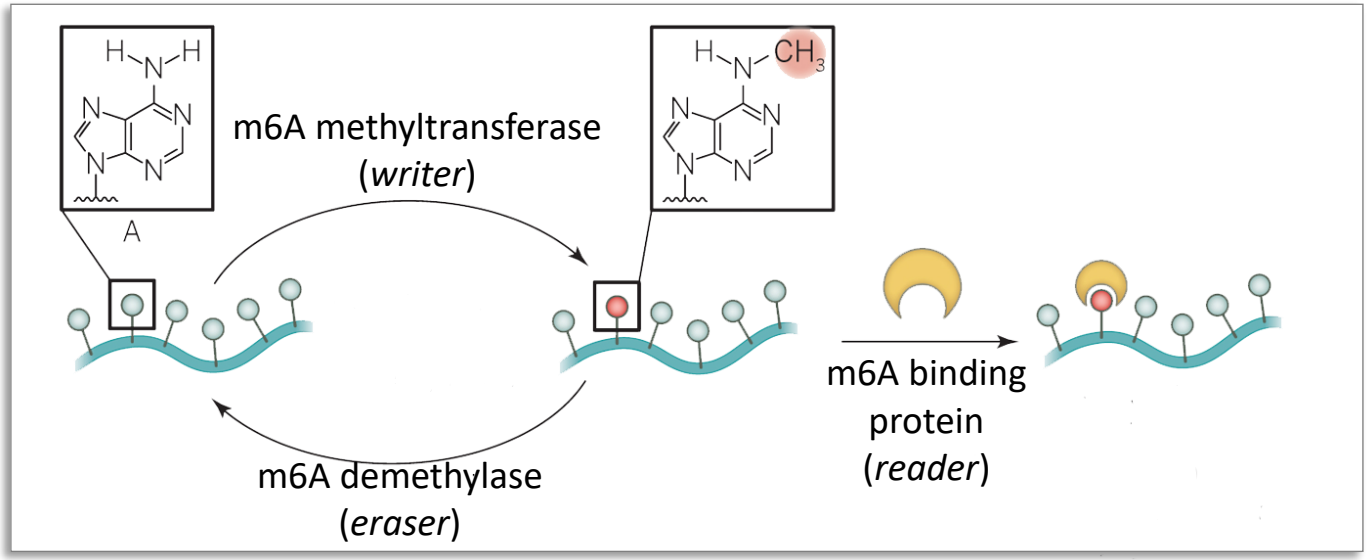
*marks are **heterogeneous** in **abundance** and **localization,***

*it is currently unclear if a **combinatorial code** exists.*

Adapted from: Zaccara S *et al*, *Nature Review Molecular Cell Biology* 2019

N6-Methyladenosine (**m6A**)

***m6A** are dynamic marks controlled by multiple effectors*



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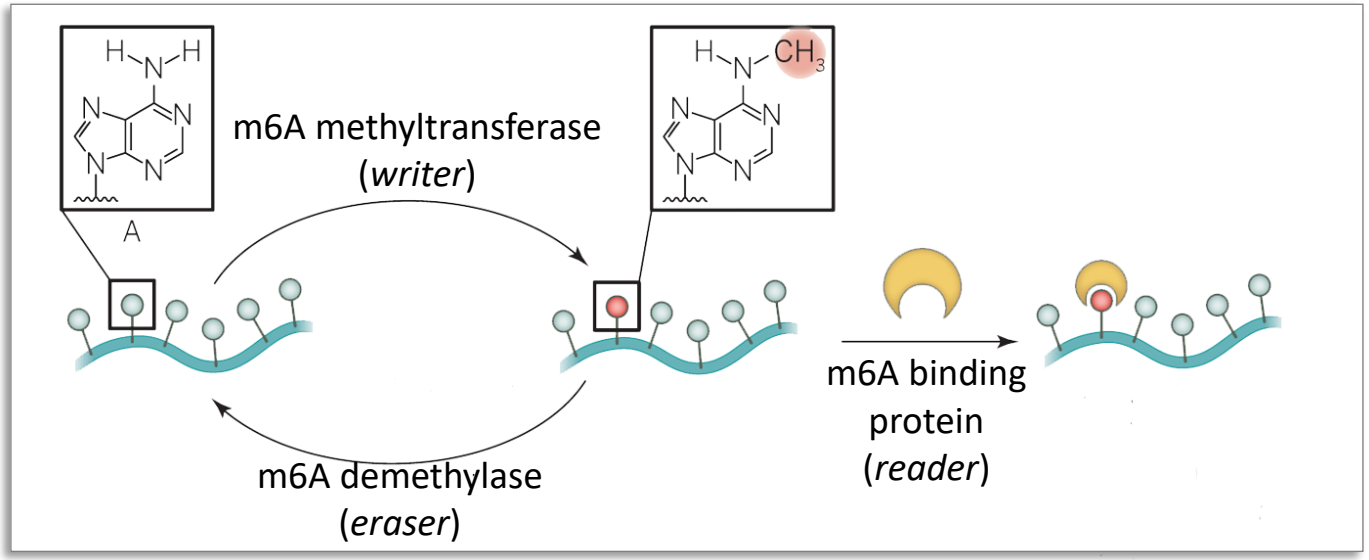

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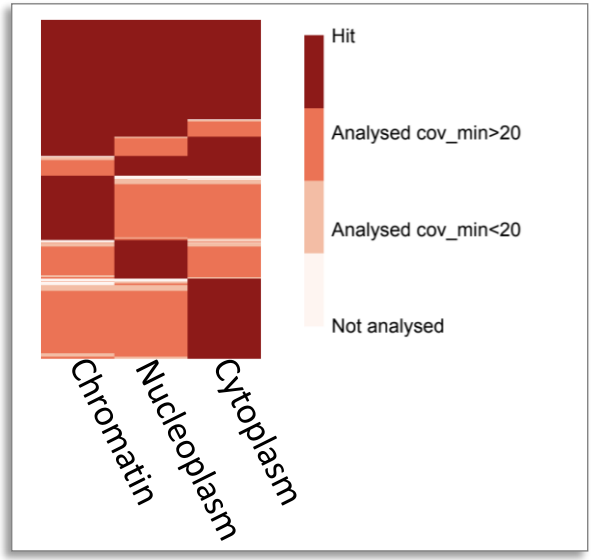

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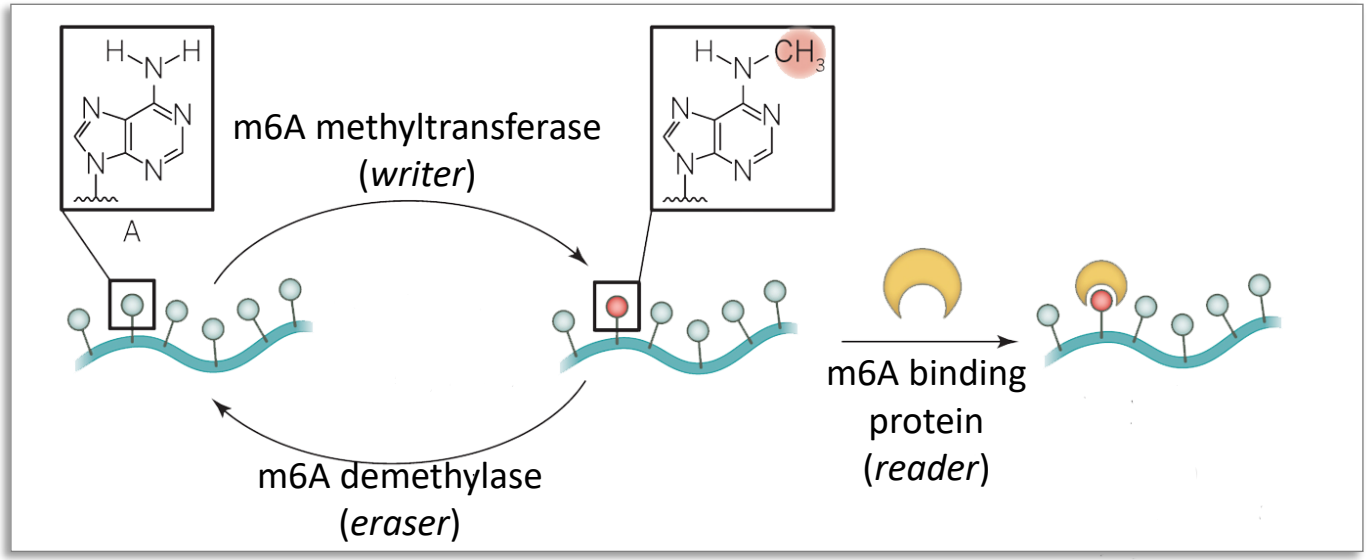

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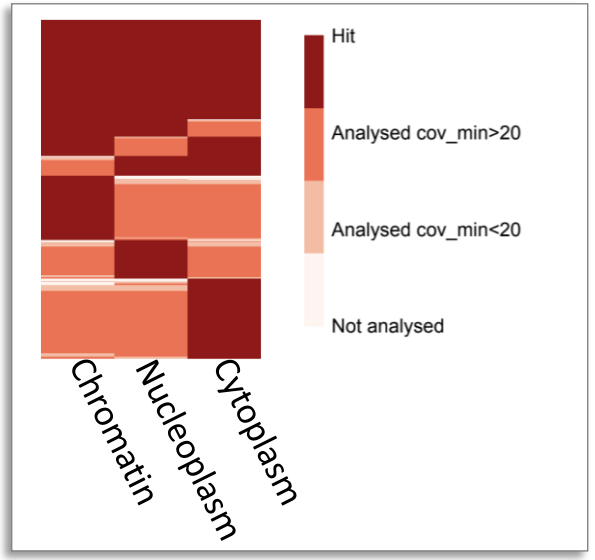
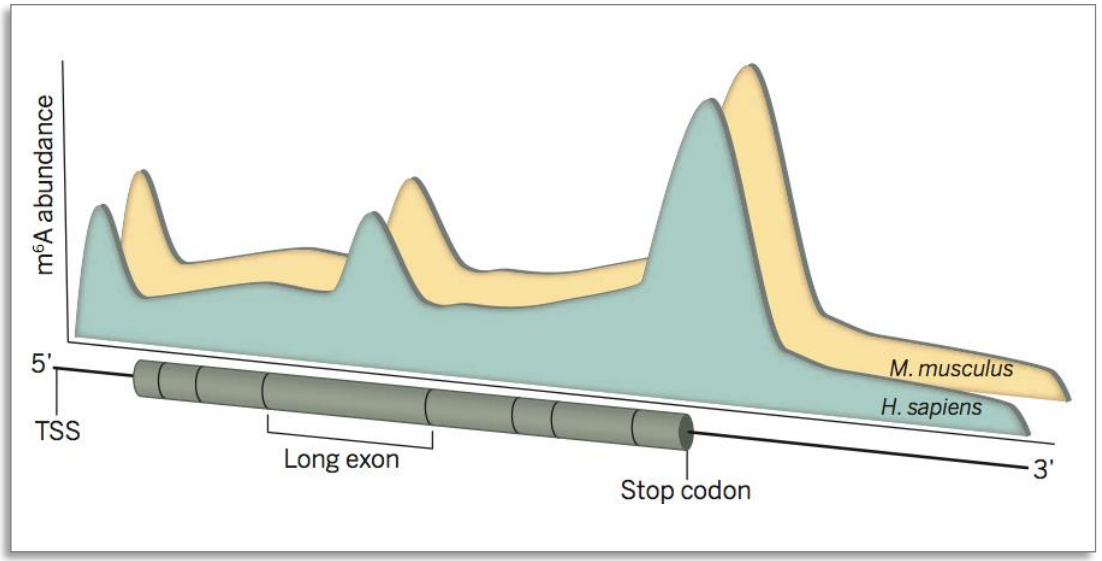


Adapted from: Coscujuela *et al*, Under Review

***m6A** marks are enriched at 3' ends and are conserved across species*



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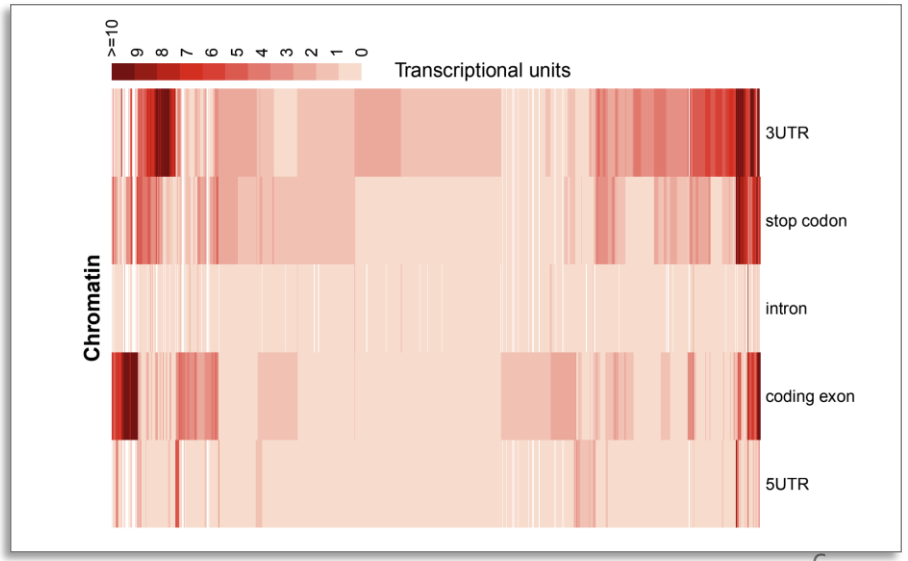


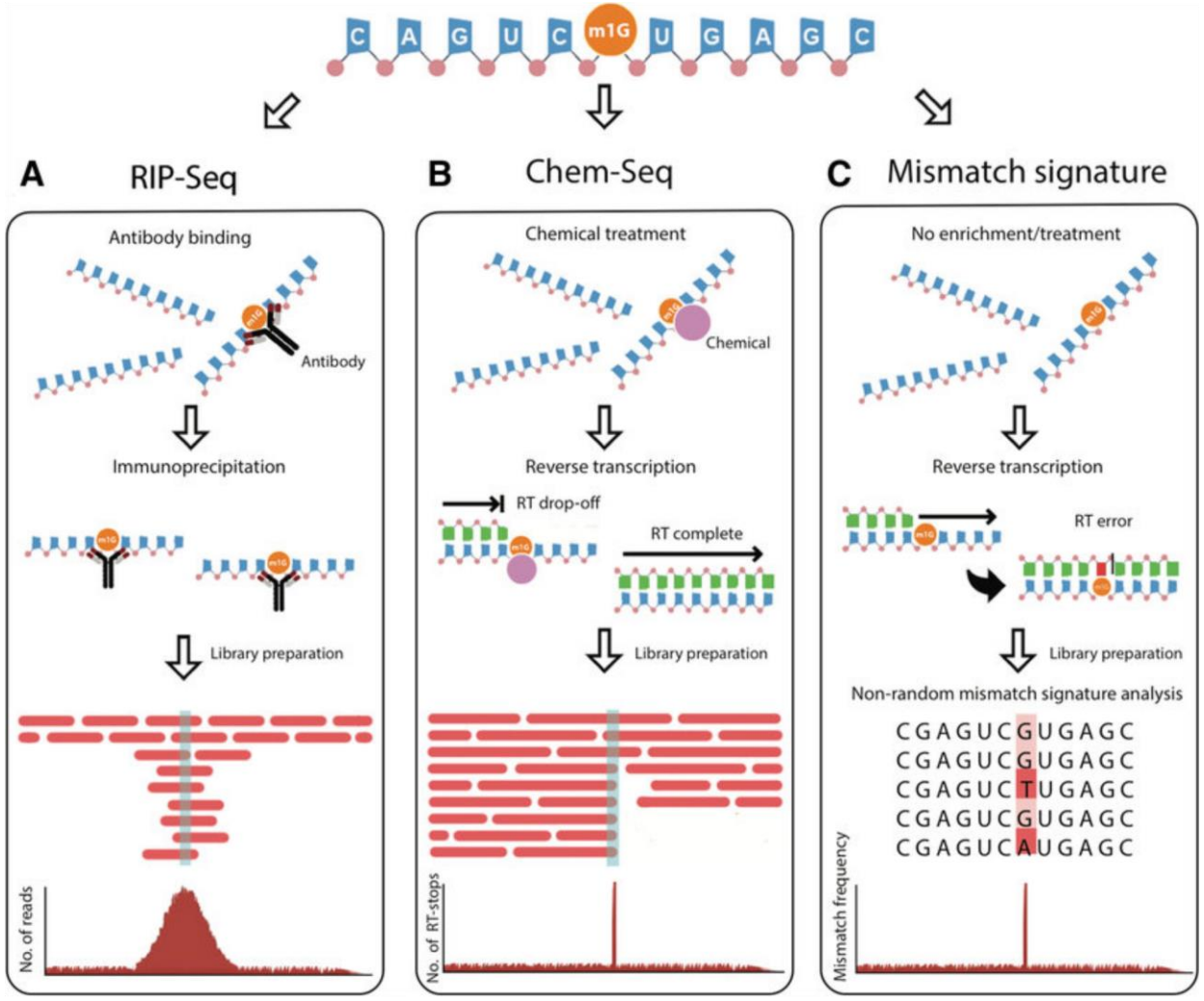
Adapted from: Coscujuela *et al*, Under Review

Established techniques for detecting RNA modifications

- **Biochemically**
- Inexpensive
- Quick
- Generally, only for bulk changes or simple detection

- **Mass Spectrometry**
- Highly quantitative
- Highly specific
- Hard to set up
- Low throughout
- Hard to obtain sequence specificity

- **NGS based methods**
- Transcriptome-wide
- Cross reactivity (antibody, chemical treatment)
- Need for specific assays/Ab for each mod
- Variable resolution



The RNA modification landscape in human disease, Jonkhout N et al, RNA. 2017 Dec

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Dot-blot

standard *lab equipment needed,*

quick *(~ 2 days) and* ***cheap*** *(~ 2 Euros per sample^) assay to profile m6A bulk levels,*

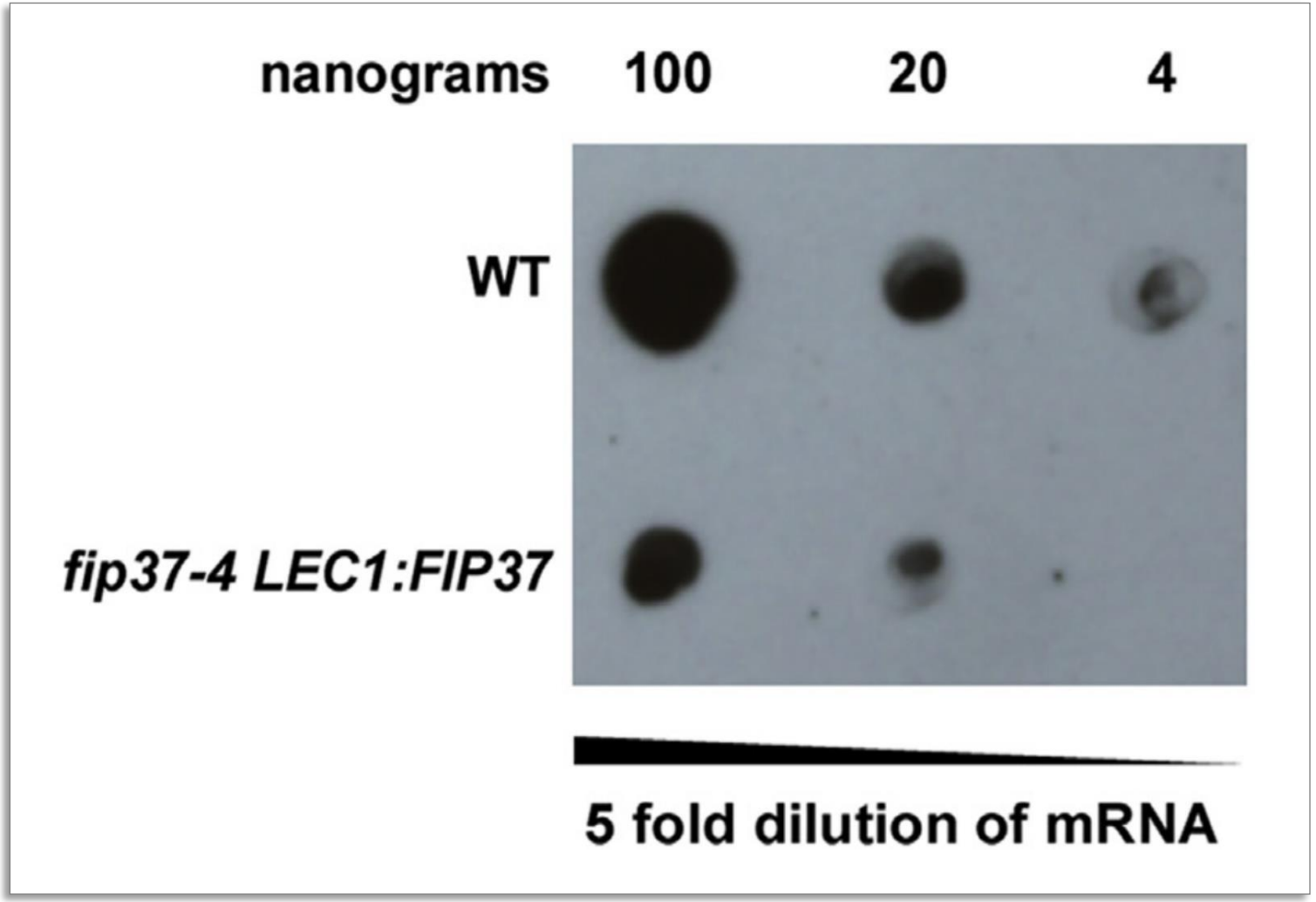
based on anti-m6A ***antibodies****,*

20 ug *of total RNA for mRNA purification,*

semi-quantitative* *due to the absence of an internal control.*

^ with a proficient experimental design.

* to improve quantification: methylene blue loading control and/or uniform dots.



Shen L et al, *Bio Protocols* 2017 Adapted from: Shen L et al, *Developmental Cell* 2016

ELISA

standard *lab equipment needed,*

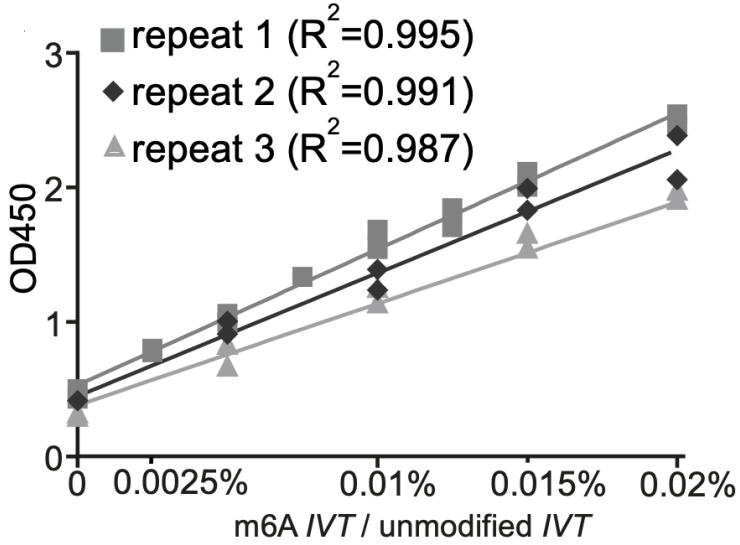
fast *(less than 1 day) and* ***cheap*** *(~ 10 Euro per sample) assay to profile m6A bulk levels,*

based on anti-m6A ***antibodies****,*

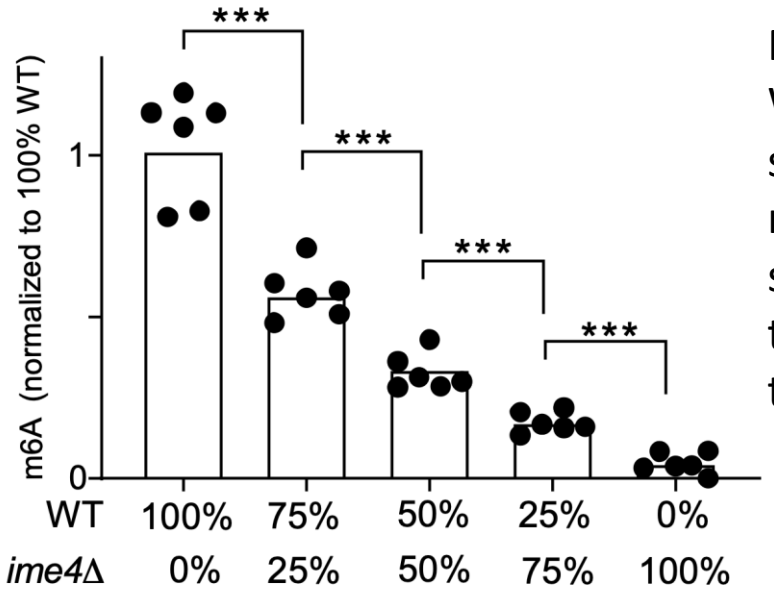
25 ng *of mRNA (5ug of total RNA),*

quantitative *for samples comparison,*

lower sensitivity *than dot-blot.*



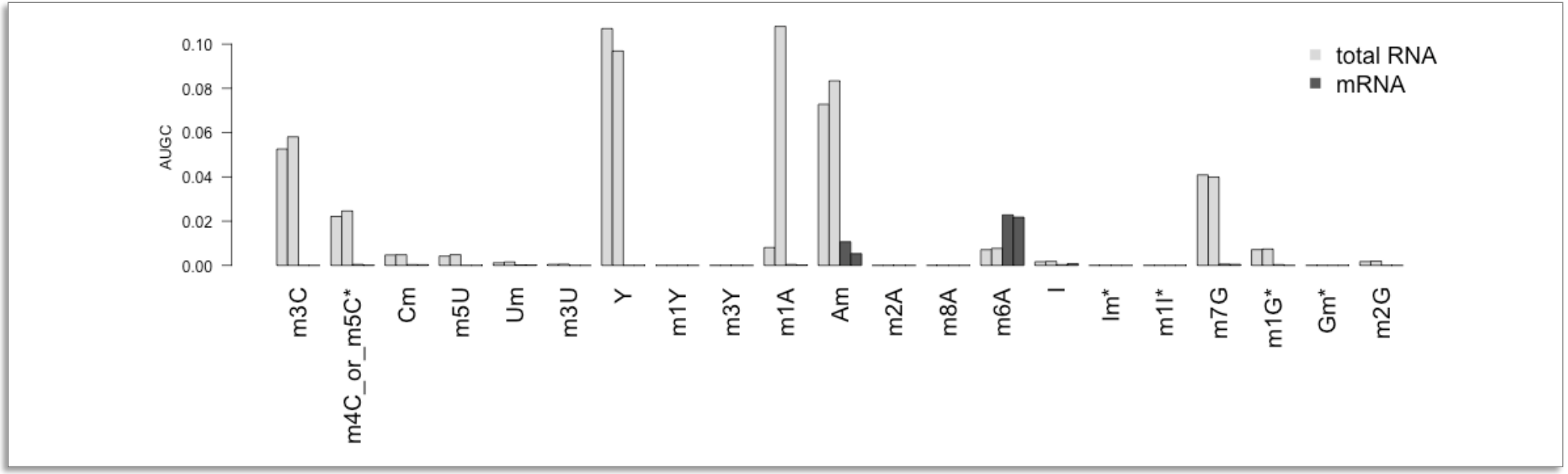
50 ng of unmodified IVT RNA with different quantities (0–10 pg) of m6A modified IVT RNA.



RNA levels in mixed WT and ime4Δ samples. Signals were normalized to standard curve, and the WT signal was set to 1. Unpaired t-test.

Ensinck I et al, *RNA* 2023

Mass Spectrometry



Unpublished

specific *lab equipment needed, therefore, often provided as service by facilities/companies with consequent inflation of* ***costs*** *and* ***time*** *(~150 Euro per sample),*

100 ng *of RNA (either total or messenger),*

many RNA modifications *profiling simultaneously,*

state of the art *in terms of sensitivity and precision.*

Espadas G et al, *Journal of Chromatography A* 2022

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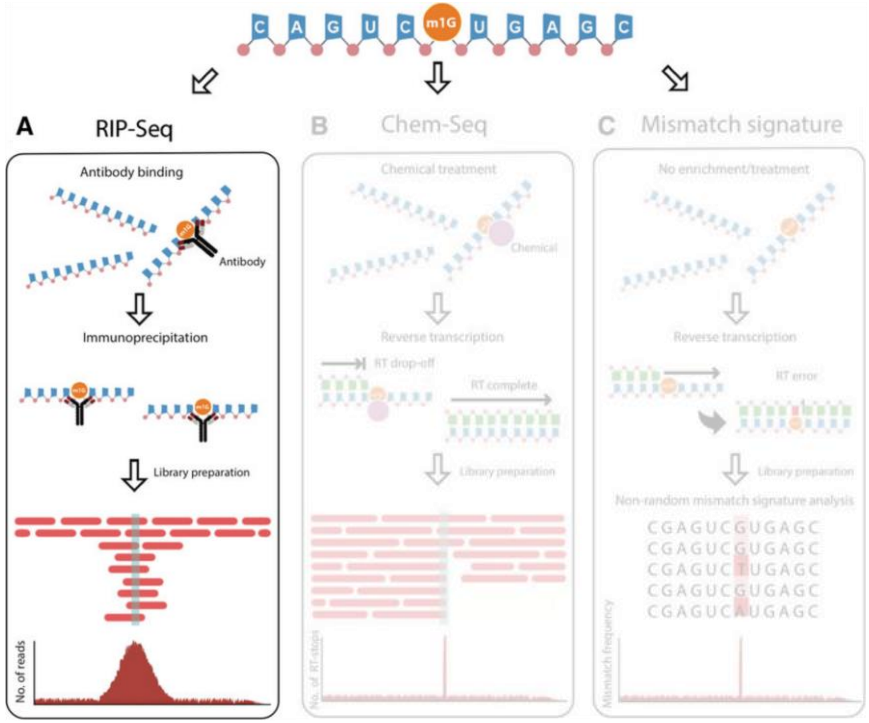


The RNA modification landscape in human disease, Jonkhout N et al, RNA. 2017 Dec

MeRIP-seq

assay to profile m6A localization in genomic space,

based on methylated fragments enrichment with anti-m6A ***antibodies****,*

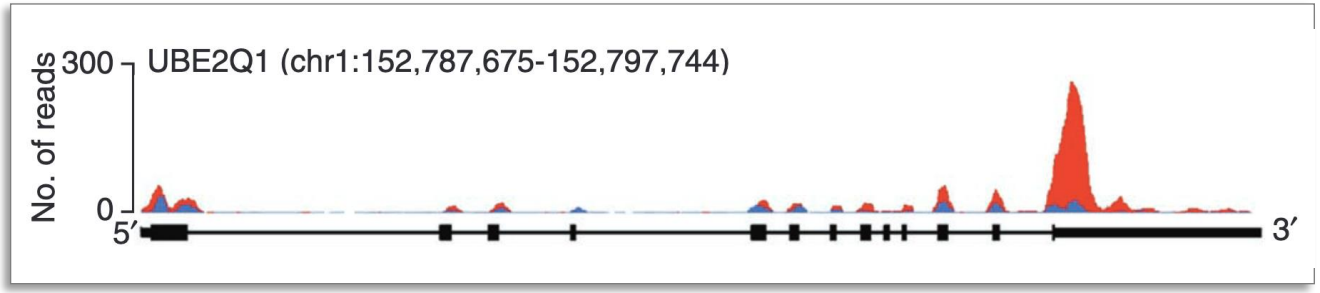
9 days for 4 samples,

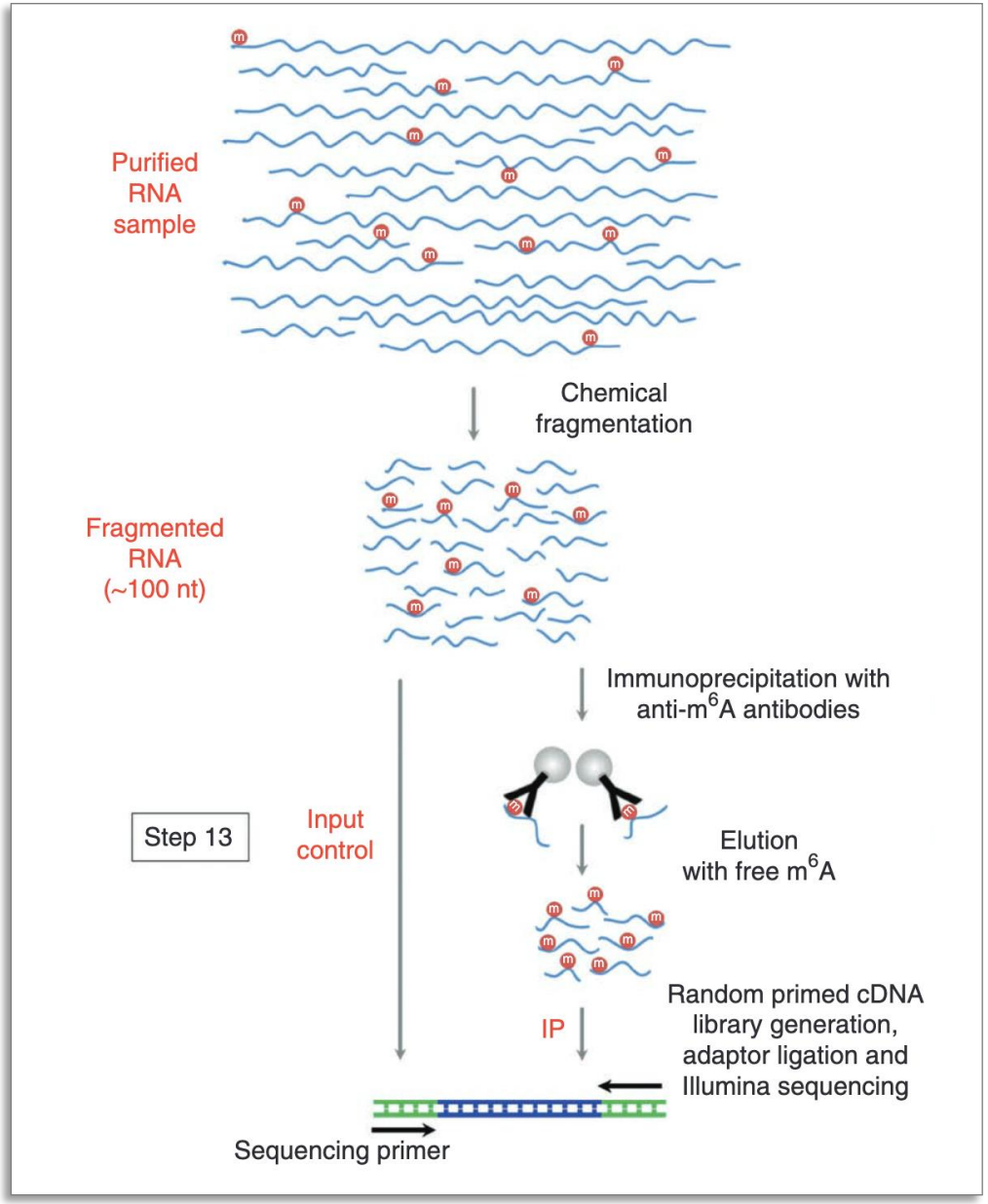
<u>hint about COST,</u>

300 ug *of total RNA or 5ug of mRNA,*

100nt resolution*,*

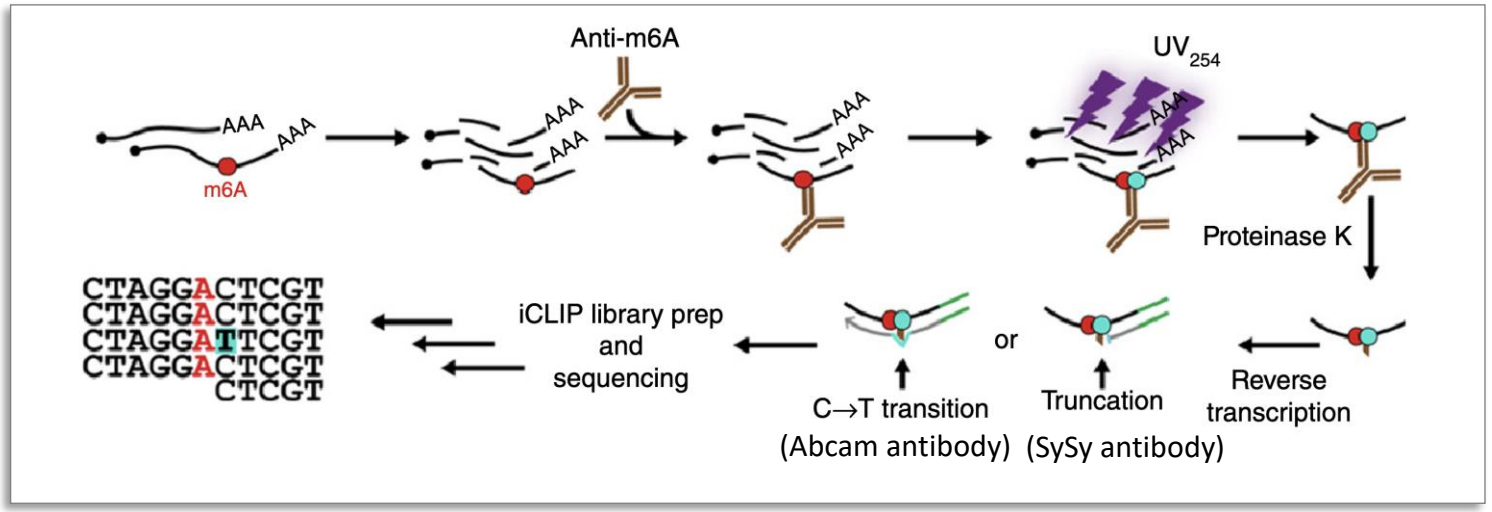
poorly informative *about* ***stoichiometry*** *and* ***abundance.***

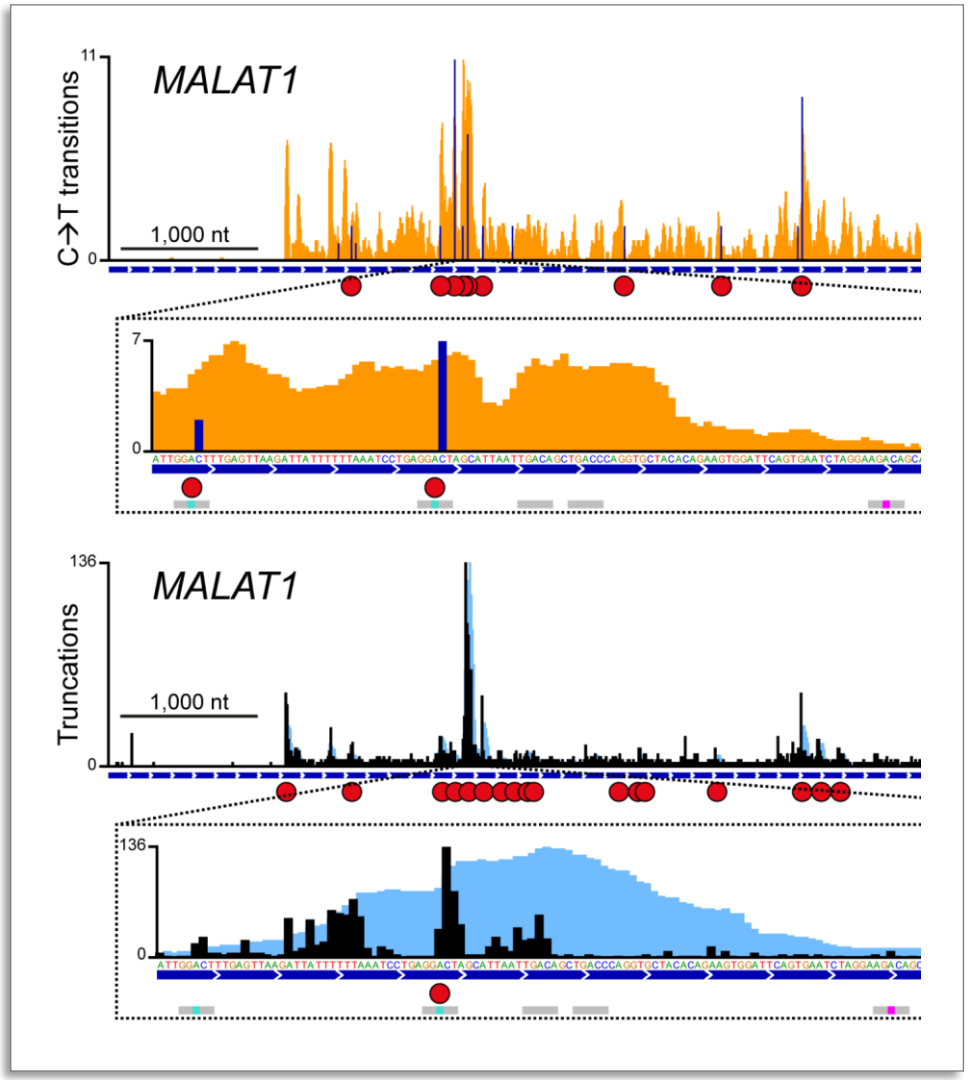




Dominissini D et al, *Nature Protocols* 2013

miCLIP





assay to profile m6A localization in genomic space,

based on anti-m6A ***antibodies UV crosslinking*** *and consequent*

induction *of* ***mutations*** *or* ***truncations****,*

hit about TIME,

hint about COST,

20 ug *of fragmented RNA,*

single base resolution*,*

poorly informative *about* ***stoichiometry*** *and* ***abundance.***

Adapted from: Linder B et al, *Nature Methods* 2015

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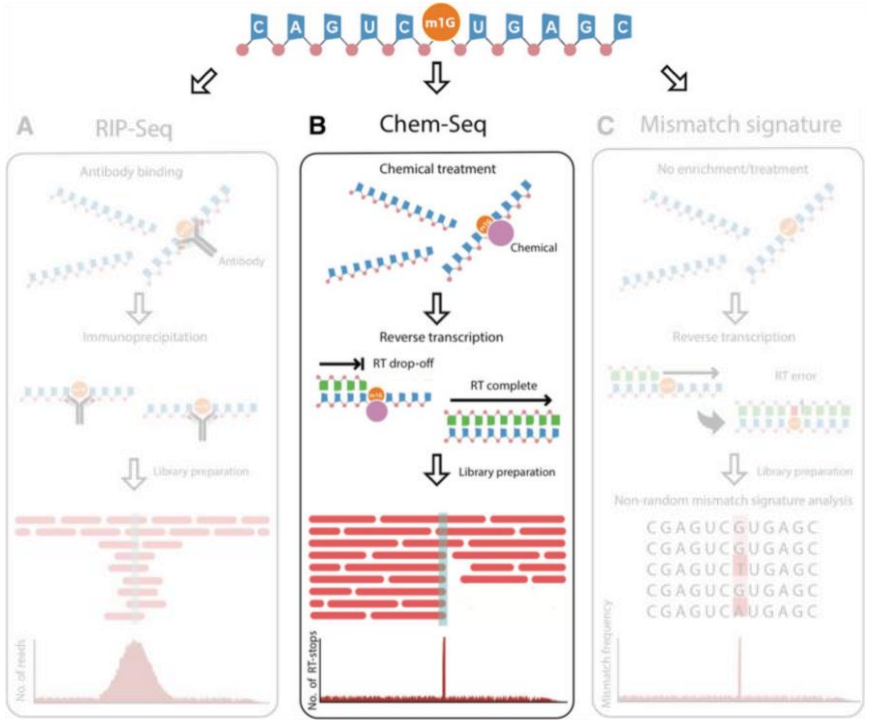

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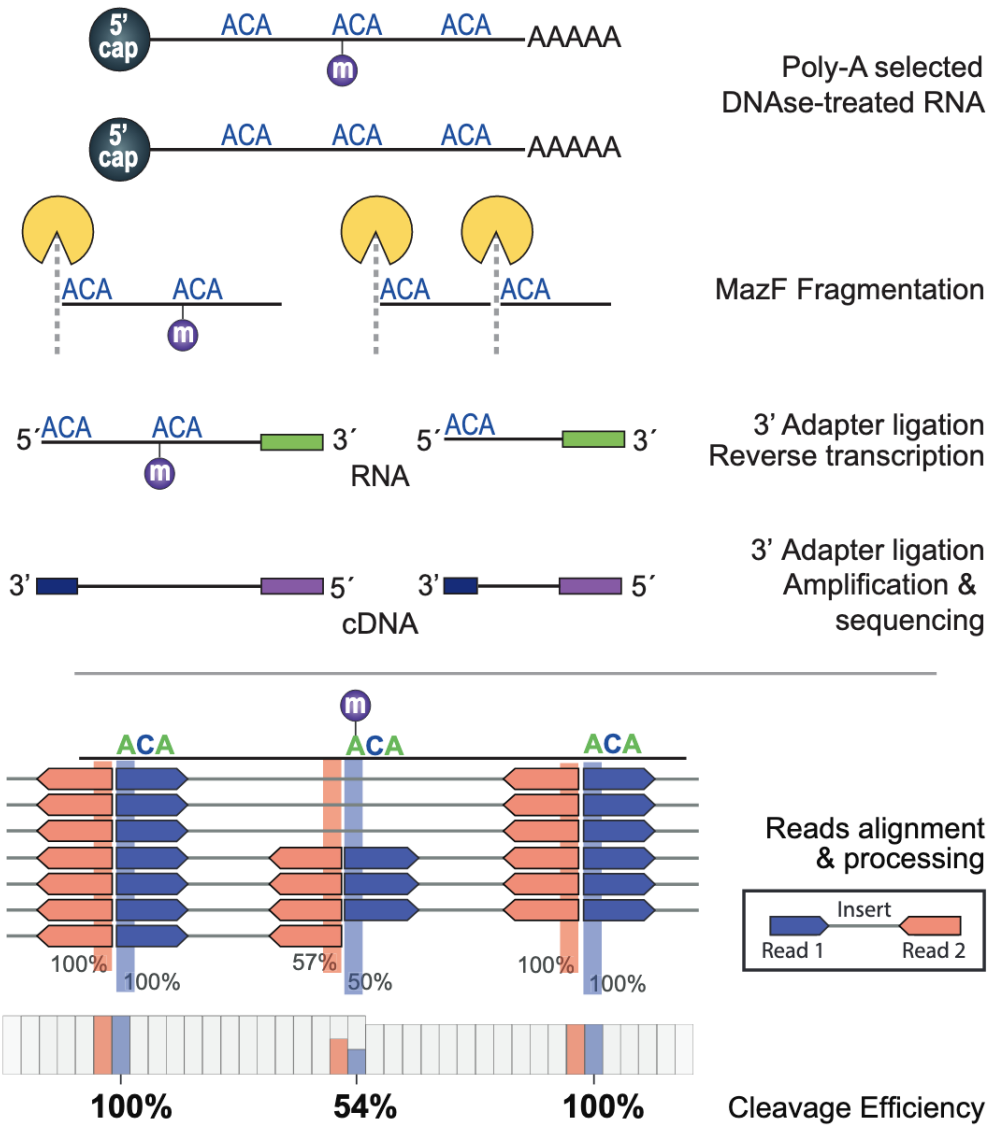
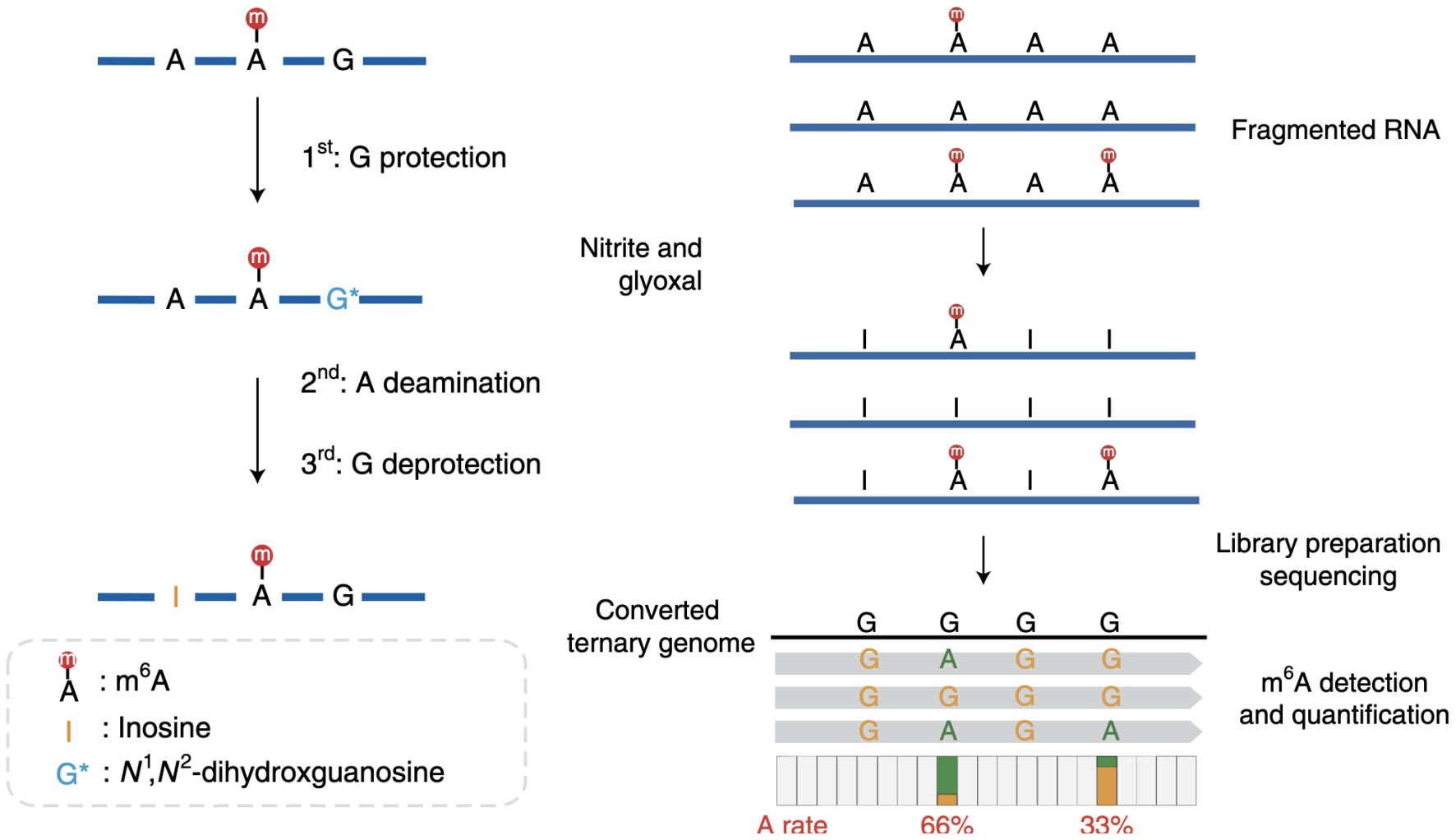
MAZTER-seq

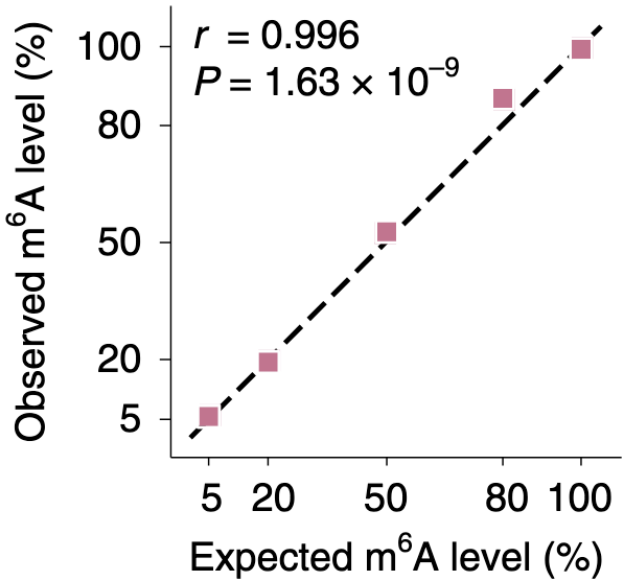


- m6A-Ab independent
- Relies on cleavage of unmethylated ACA motifs by MazF RNAse
- Base resolution detection of 16-25% m6A sites
- Requires 100ng polyA+ RNA

Garcia-Campos MA, *Cell* 2019

GLORI





- Relies on glyoxal and nitrite-mediated deamination of unmethylated As (GLORI) while keeping m6A intact
- Provides m6A stoichiometry
- Requires 100ng pA+ RNA

Liu C, *Nat Biotech* 2022

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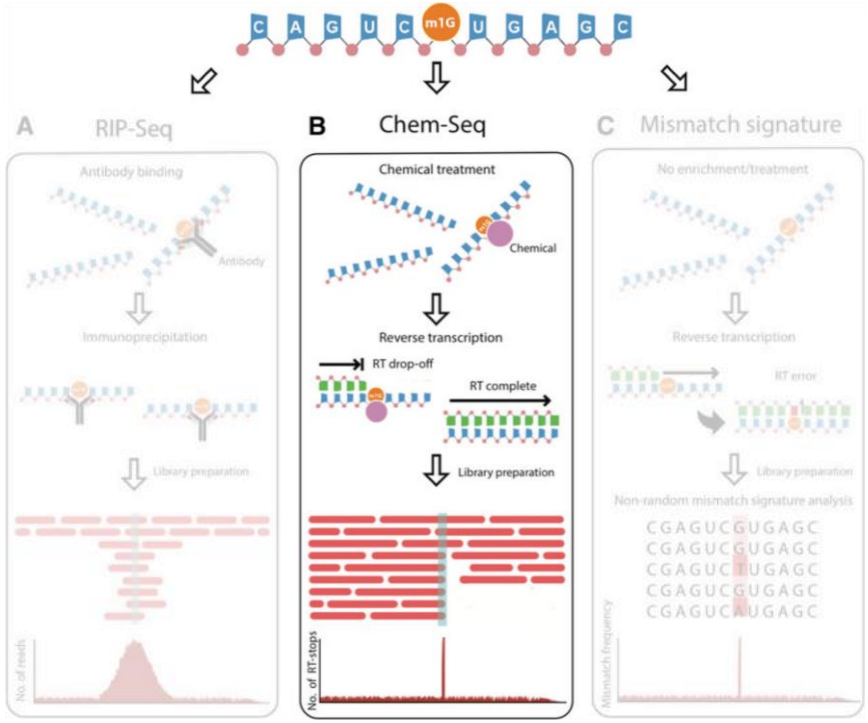
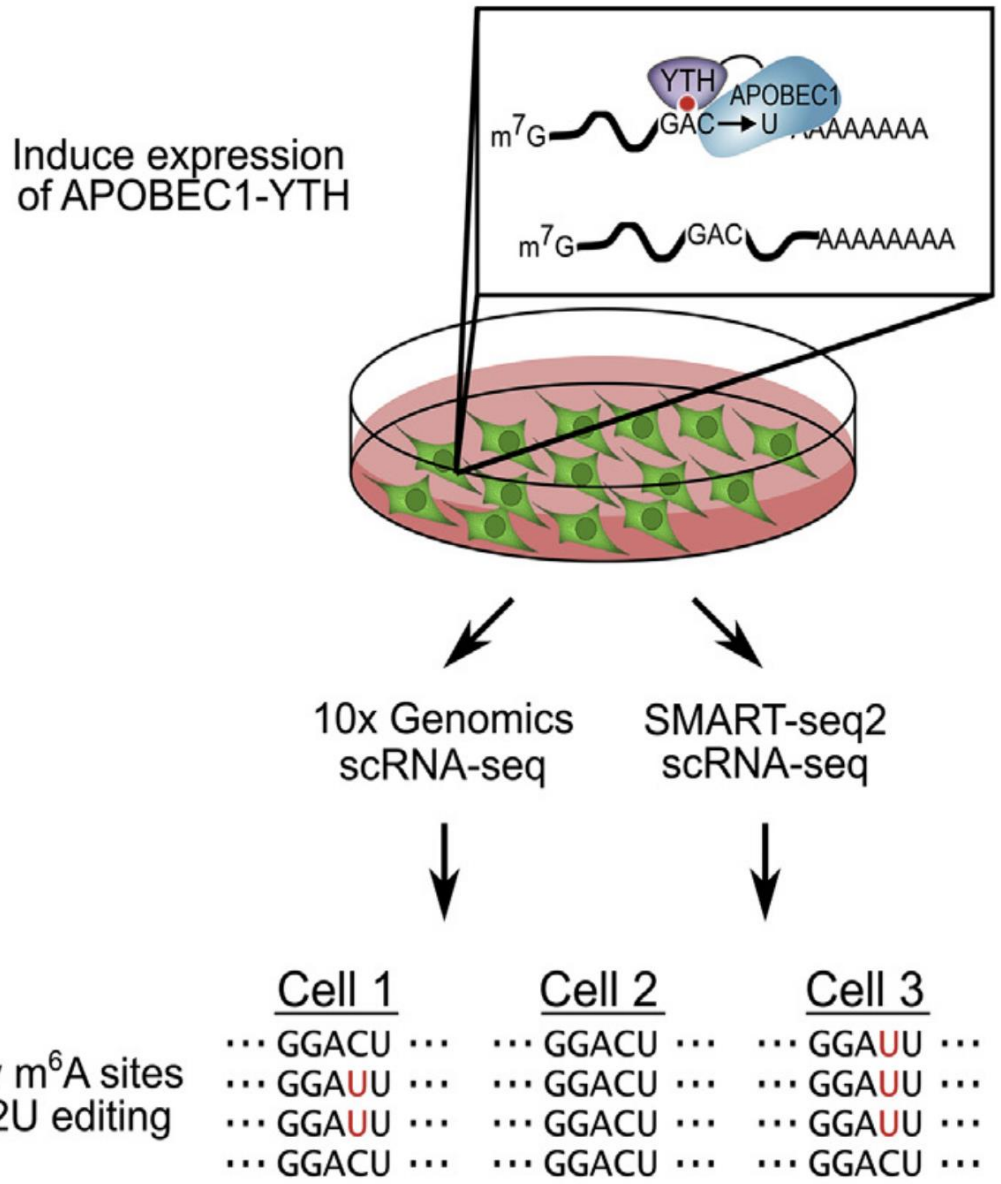
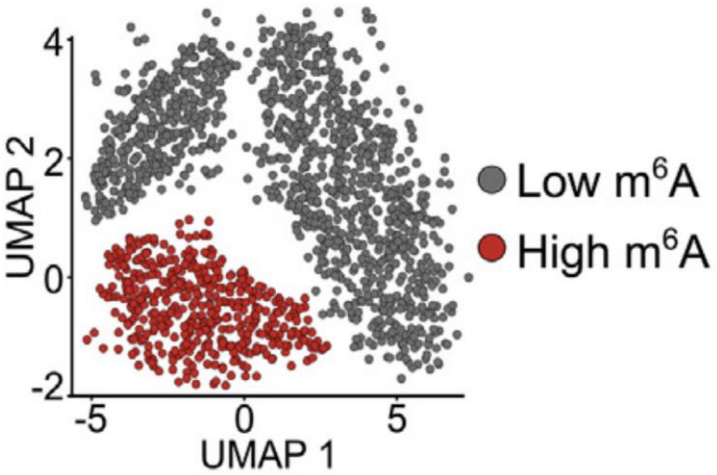
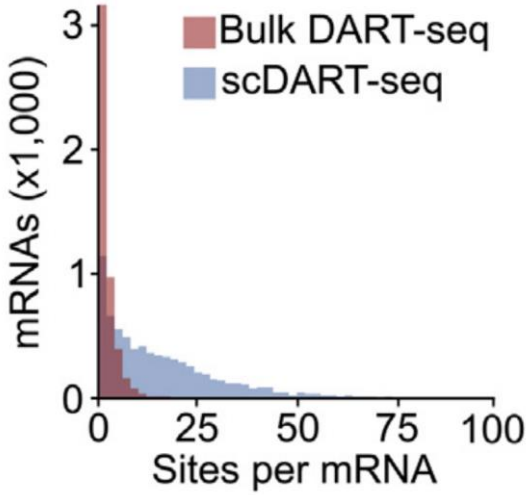


The RNA modification landscape in human disease, Jonkhout N et al, RNA. 2017 Dec

scDART-seq







- Single cell m6A mapping
- APOBEC1-YTH mediated deamination (C-to-U) adjacent to m6A
- C-to-U detected via standard RNA-seq
- Requires APOBEC1 fused to m6A-binding YTH domain and APOBEC1-YTH expression
- Requires 10ng total RNA
- Distinguishes m6A from m6Am

Tegowski M, *Mol Cell* 2022

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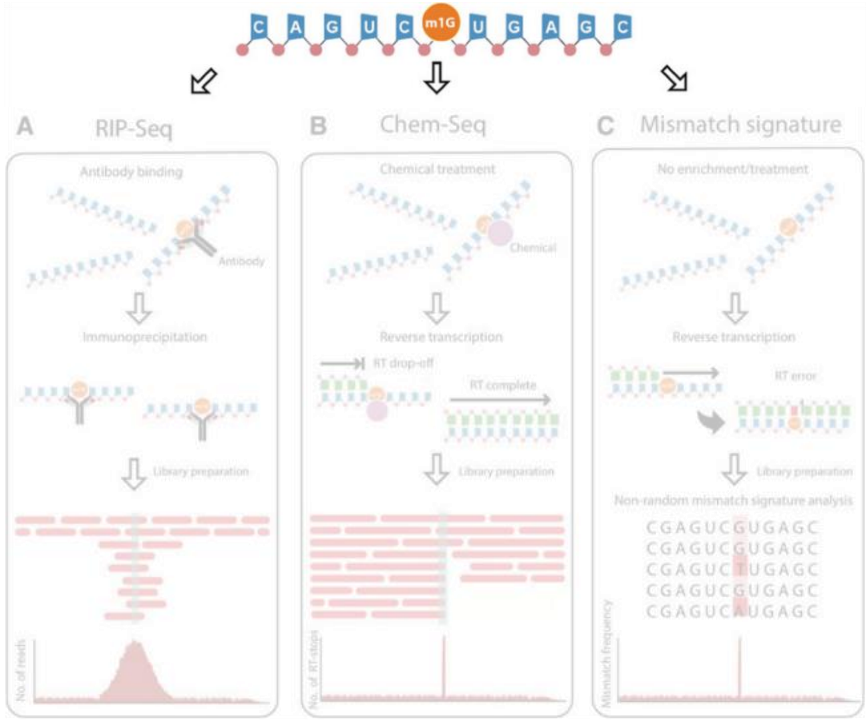
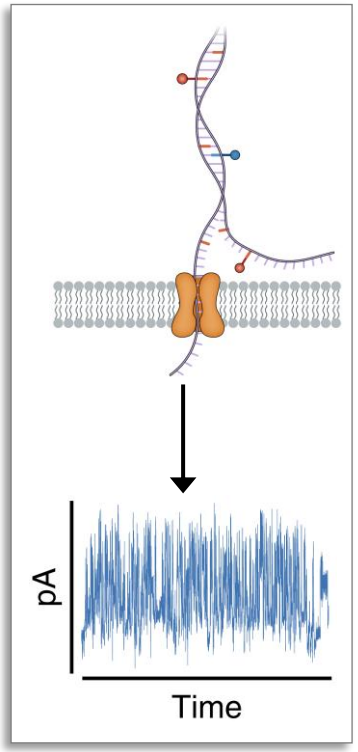
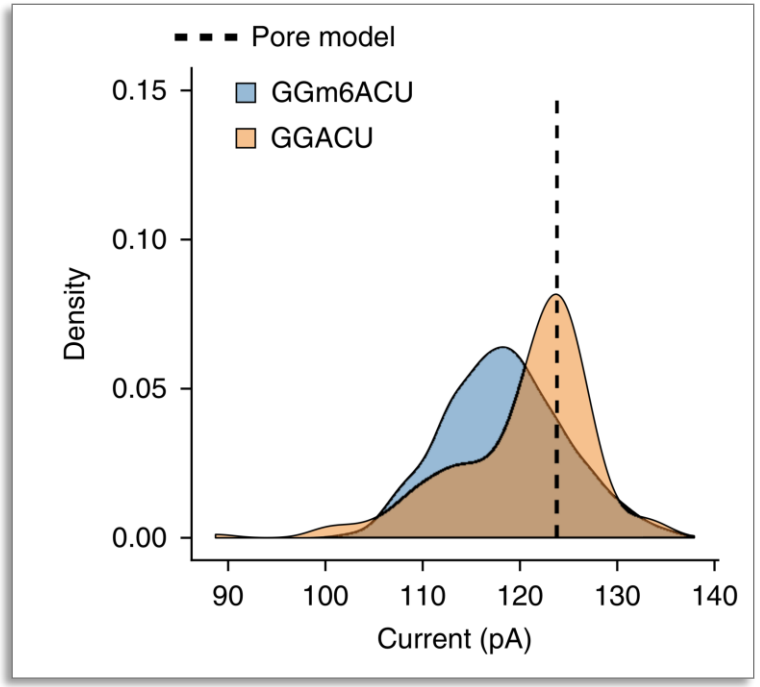


The RNA modification landscape in human disease, Jonkhout N et al, RNA. 2017 Dec

Nanopore native RNA-seq

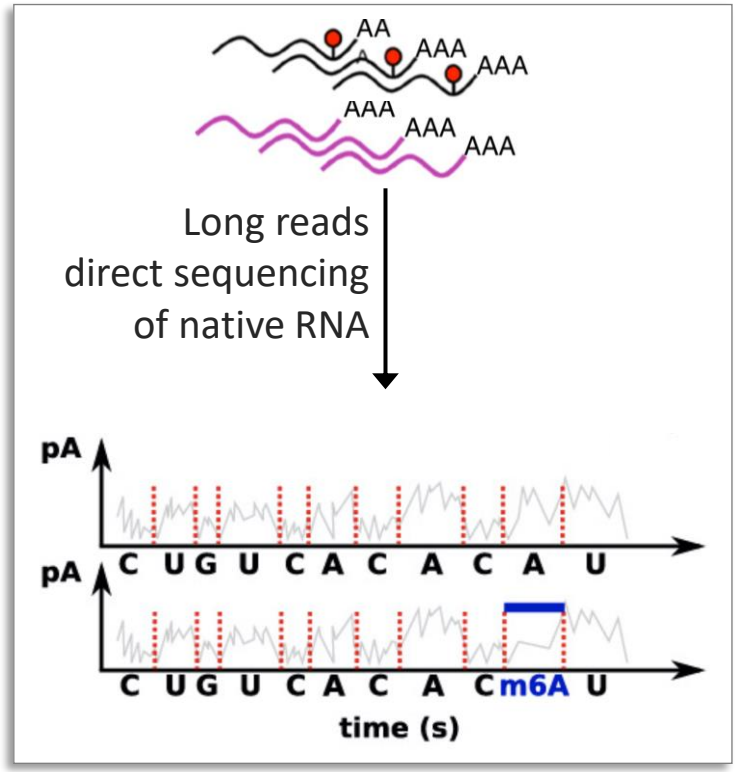


The signal is obtained in form of current over time



The signal is influenced by the presence of RNA modifications

Positional and quantitative



Adapted from Furlan M *Front Genet* 2020

Adapted From
Sedlazeck *et al*, Nat Rev Genet 2018
and Workman *et al*, Nat Meth 2019

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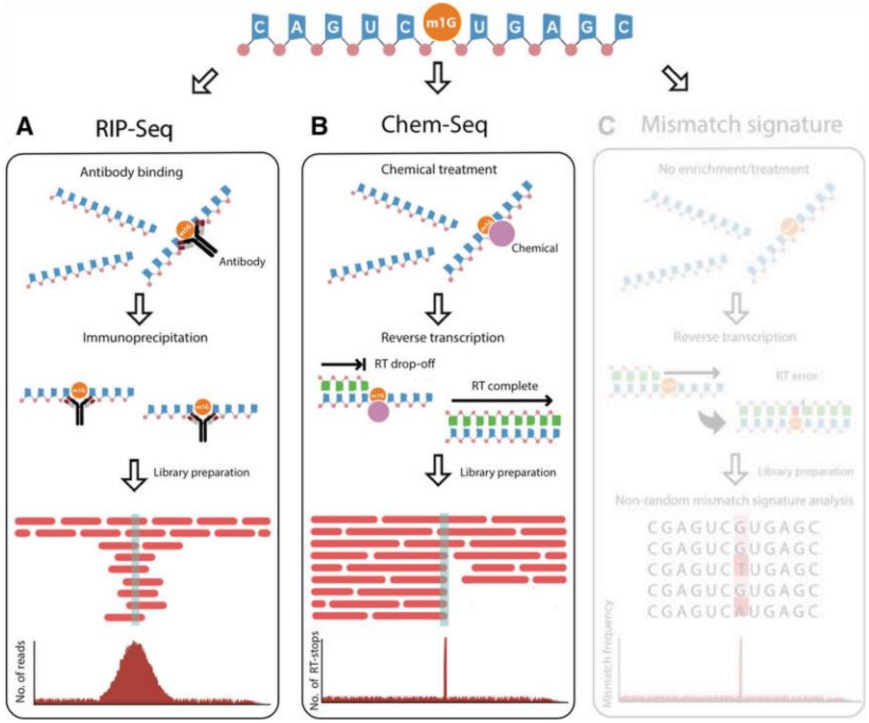
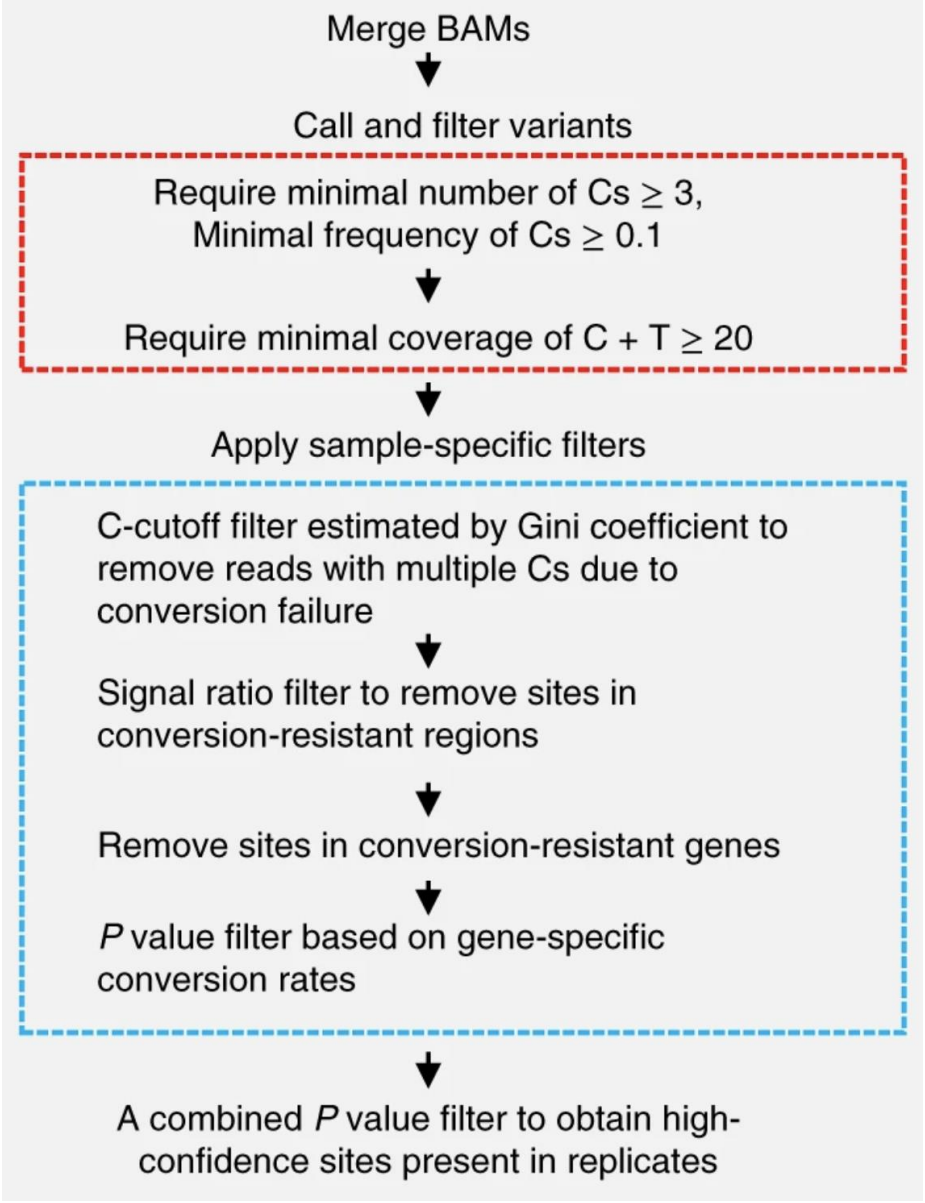
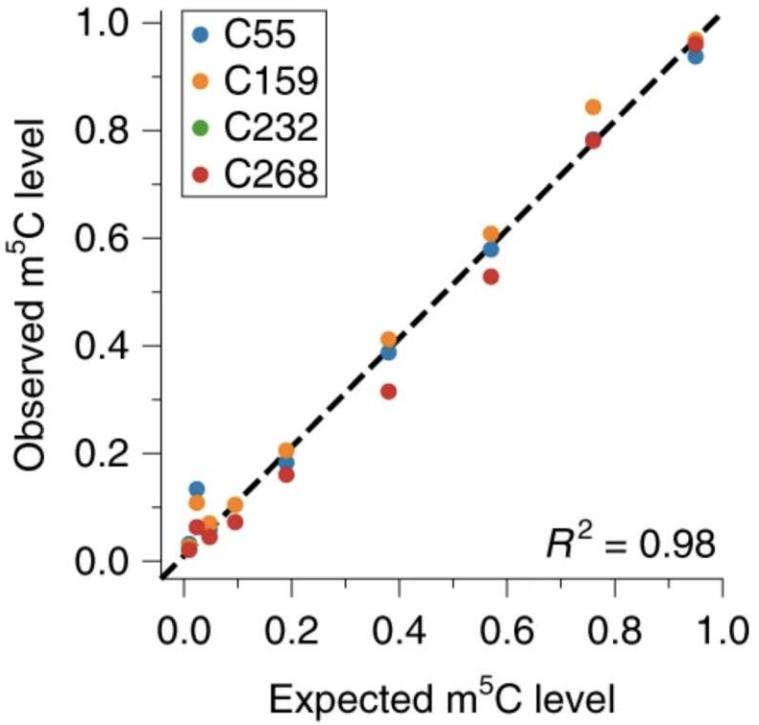


The RNA modification landscape in human disease, Jonkhout N et al, RNA. 2017 Dec

m5C

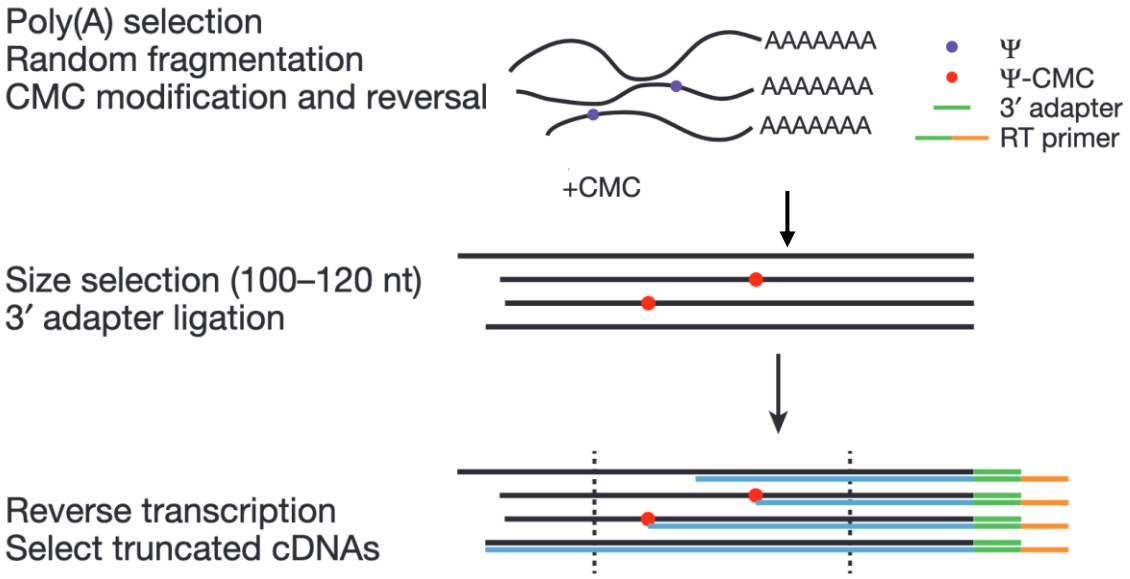


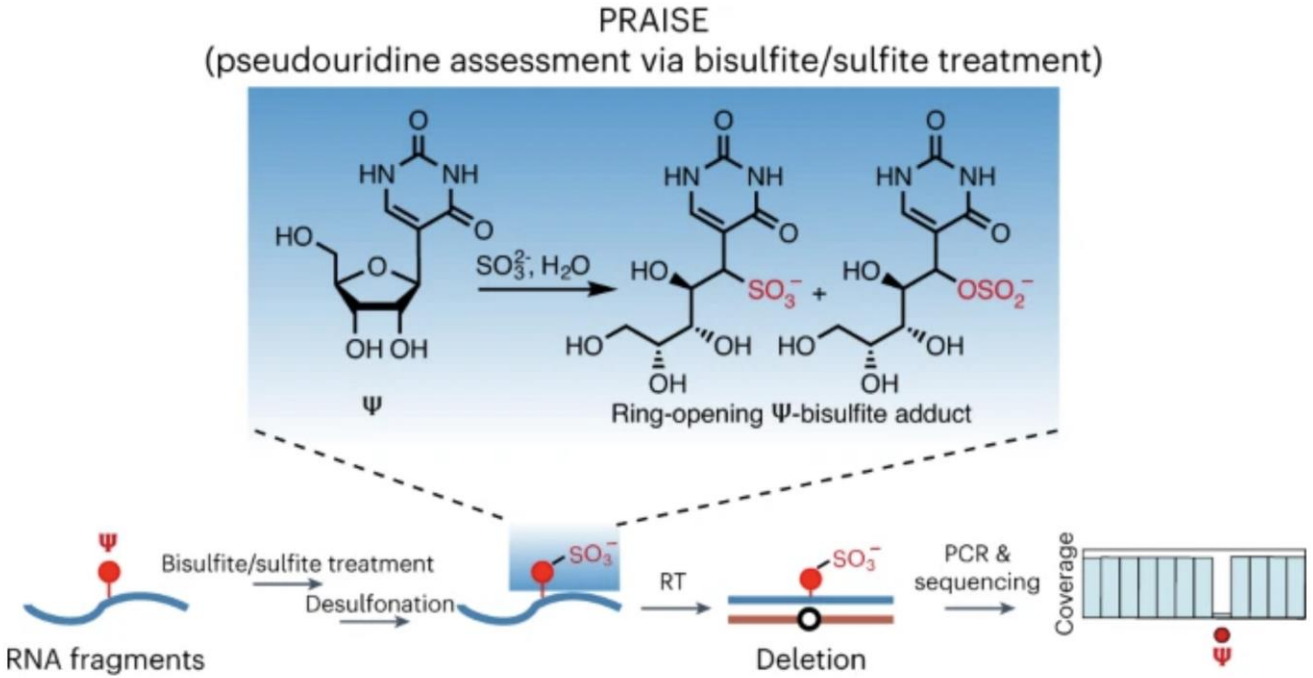


- Based on bisulfite sequencing
- several hundred exonic m5C sites found
- 62–70% of the sites had <20% methylation)
- 8–10% of the sites had >40% methylation

Huang T, *Nat Struct Mol Biol* 2019

Pseudouridine





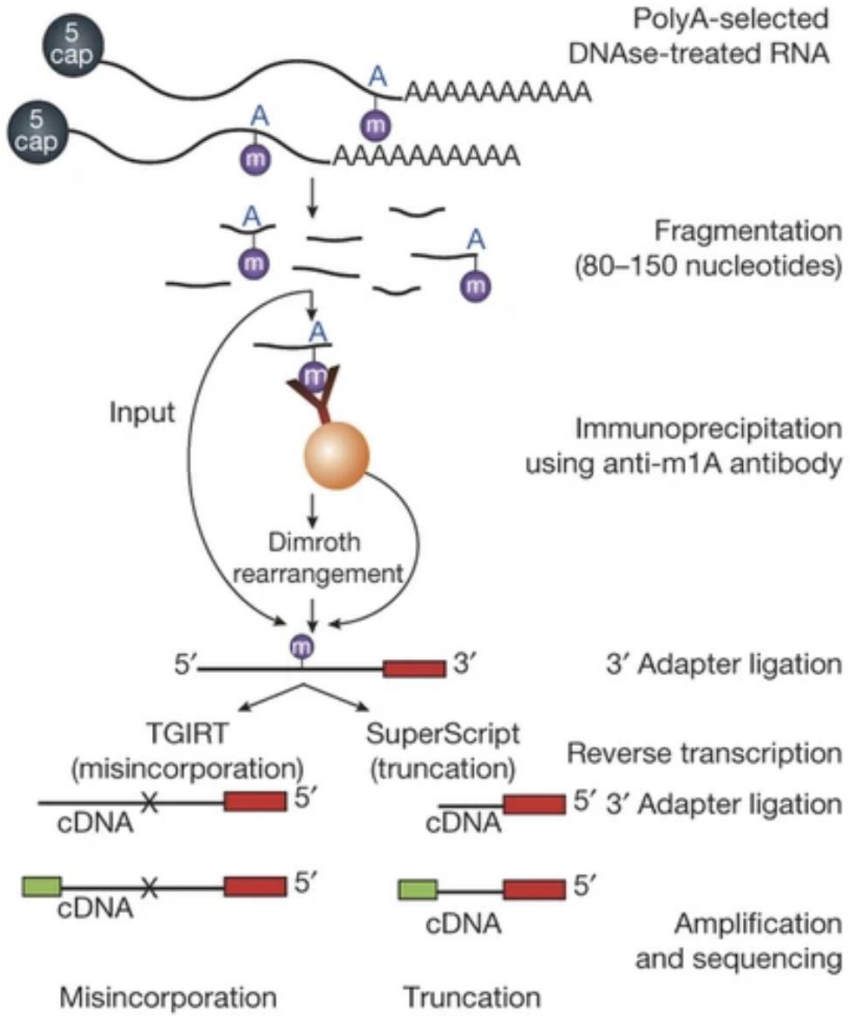
- Ψ is selectively modified by CMC leading to a block during RT

Carlile TM, *Nature* 2014

- Ψ is labeled by bisulfite and leads to nucleotide deletions during RT
- Ab independent
- Provides stoichiometry

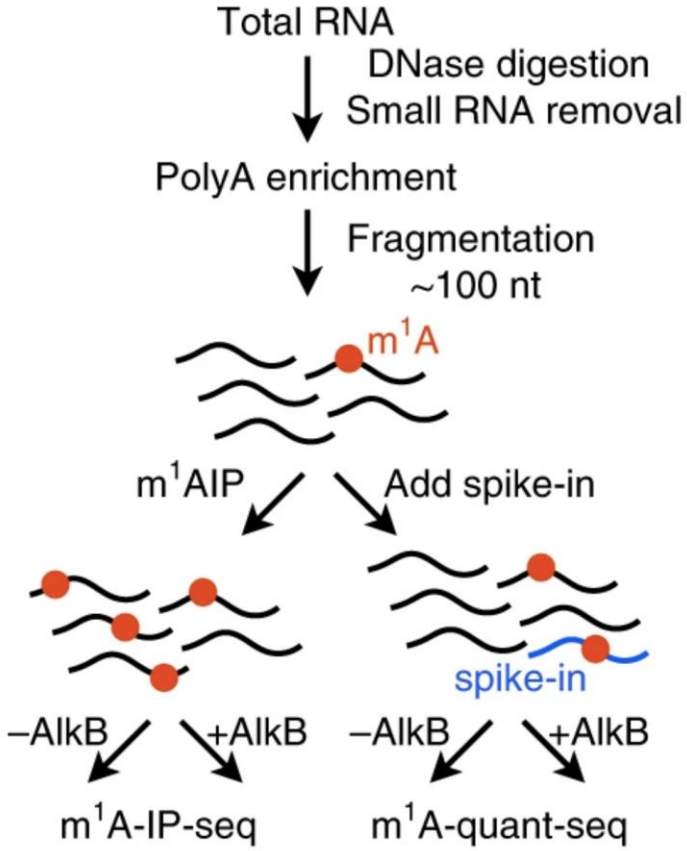
Zhang M, *Nat Cell Biol* 2023

m1A



- Relies on m1A-Ab
- Generates misincorporations or truncations

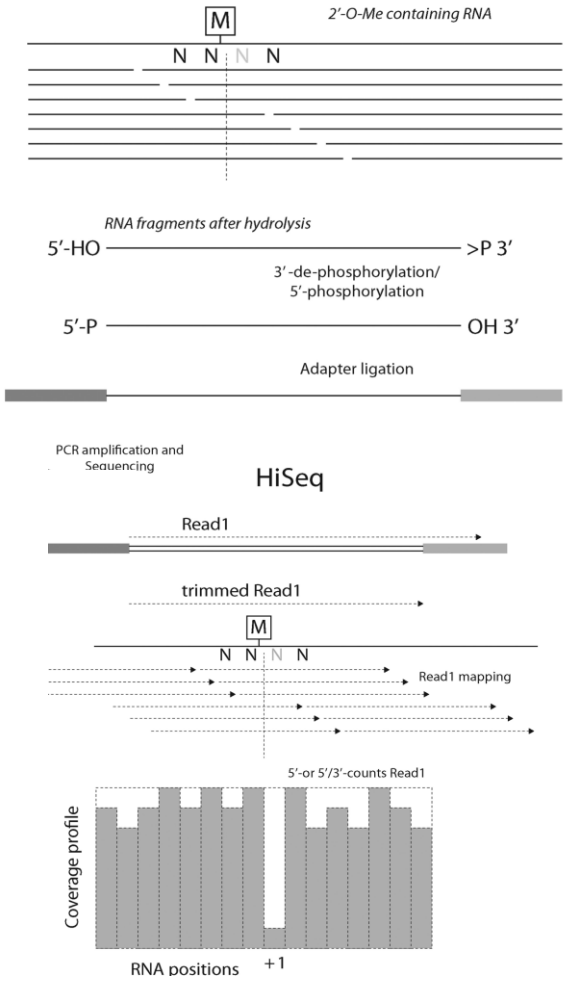
Safra M, *Nature* 2017



- Combines IP via m1A-Ab via RT mutational signatures
- Directed-evolution platform to evolve RTs for efficient read through m1A and mutation signatures
- AlkB used for demethylating m1A as control sample
- m1A-quant-seq avoids IP and relies on spike-ins

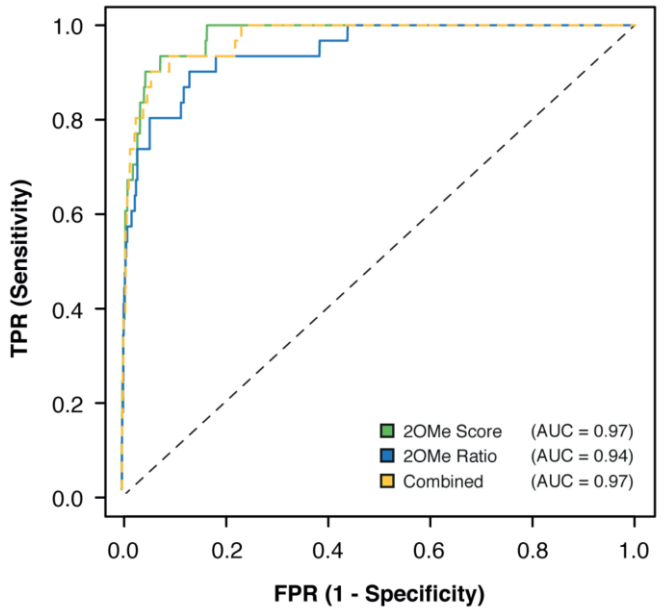
Zhou H, *Nat Meth* 2019

2’-O-methylation (Nm)



- alkaline fragmentation of total RNA coupled to a commonly used ligation approach
- 2’-O-Me residues protect the 3’-adjacent phosphodiester bond from cleavage, generating a typical gap

Marchand V, *NAR* 2016

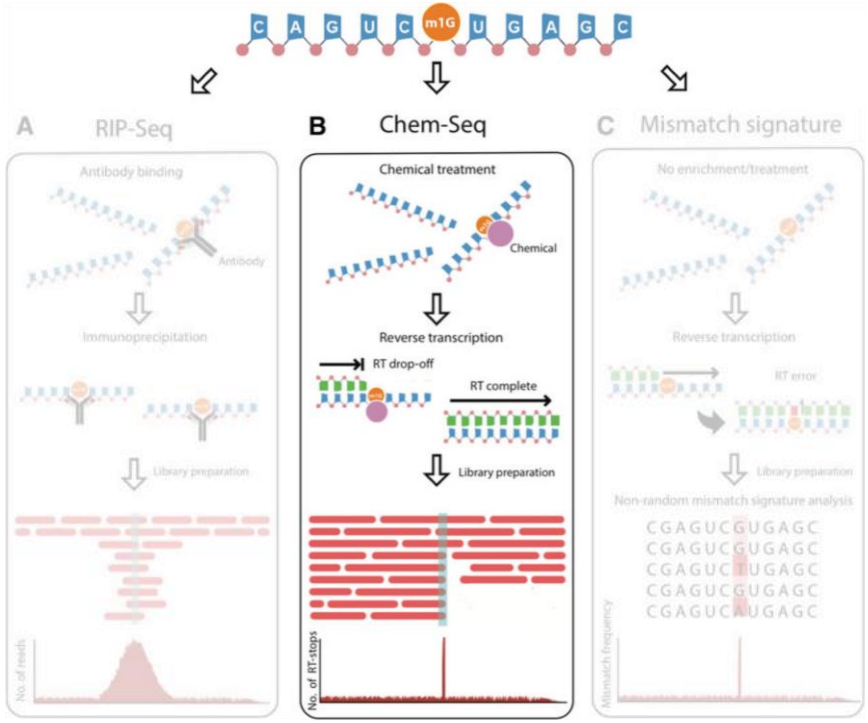


- Nm induce, under limiting [dNTP], a specific RT stop one nt downstream of the methylated site
- Total RNA from cells is subjected to RT under either high or low [dNTP], using random hexamers coupled to the 3’ seq. adapter

Incarnato D, *NAR* 2016

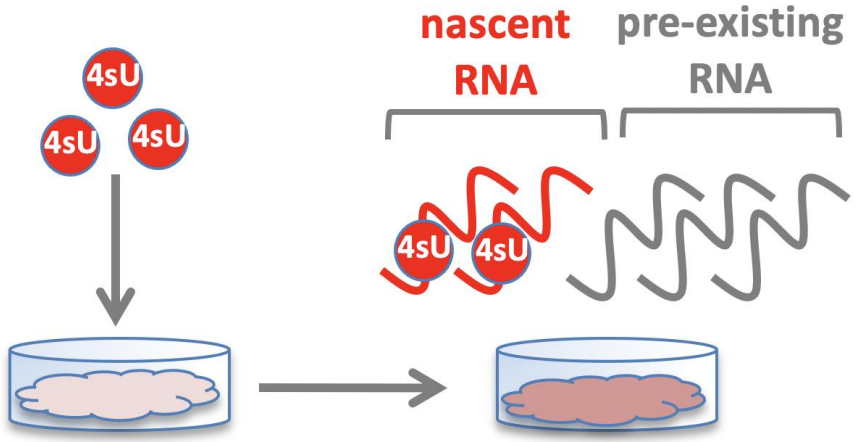
Outline

- Background on RNA modifications
- Methods to profile m6A
  - Bulk levels – Dot-blot, ELISA/colorimetric, MassSpec
  - Genome-wide, Antibody-based – MeRIP-seq, miCLIP
  - Genome-wide, not Antibody-based – MAZTER-seq, GLORI
  - Single cell – scDART-seq
  - Single molecule – Nanopore native RNA-seq
- Other endogenous modifications – m5C, Ψ, m1A, Nm
- **Exogenous modifications** – 4sU-seq, SLAM-seq, nano-ID

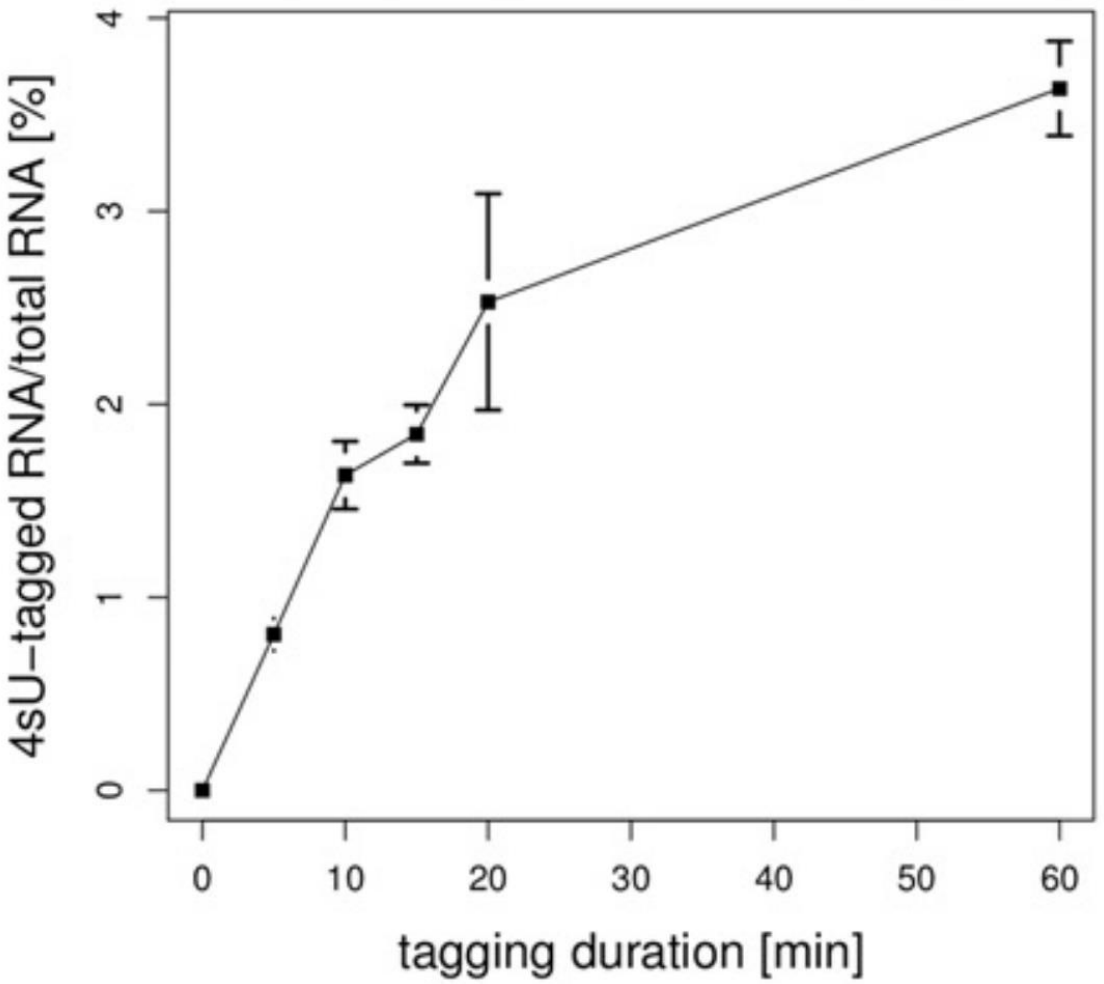


The RNA modification landscape in human disease, Jonkhout N et al, RNA. 2017 Dec

4sU-seq

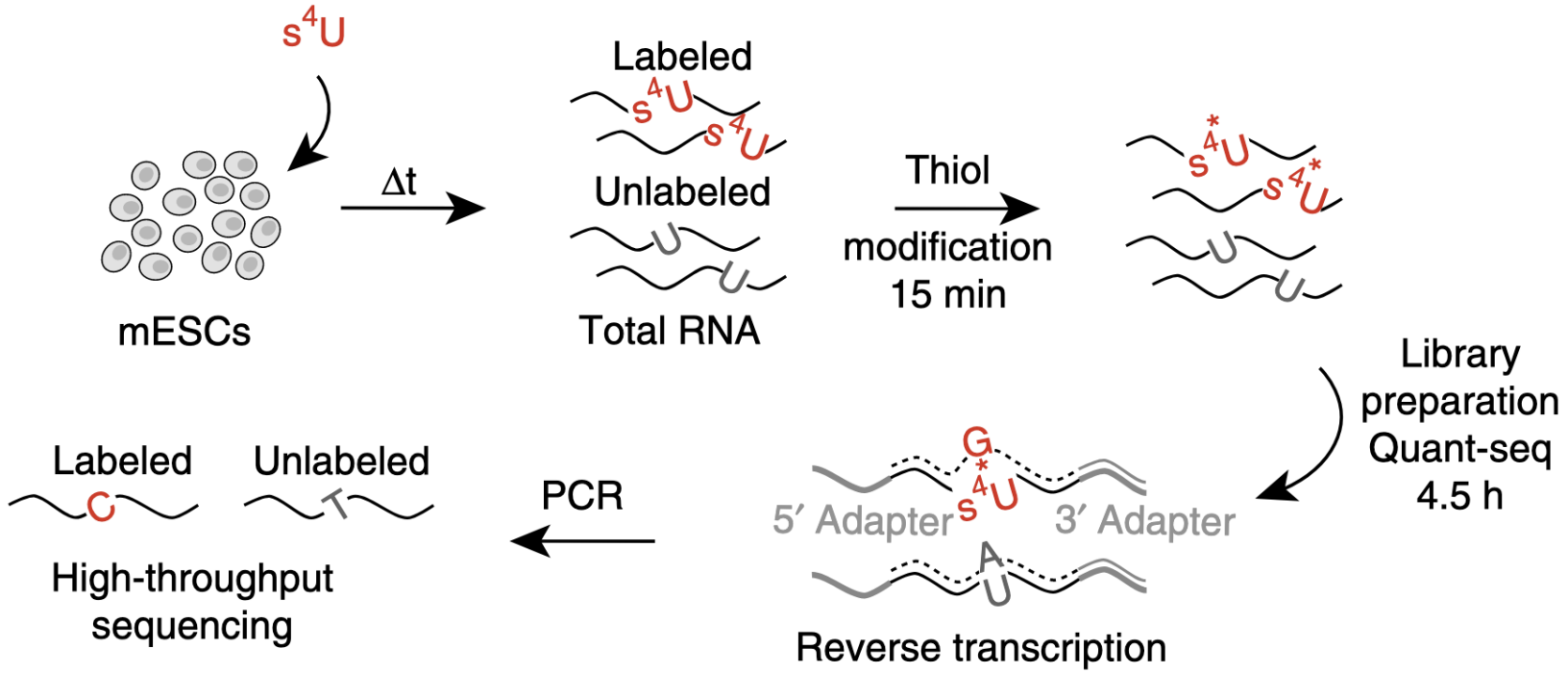


- 4sU gets incorpoted in nascent RNA
- 4sU does not interfere with cell metabolism
- Thiol-specific biotinylation leads into tagged (newly transcribed)
- and untagged (preexisting) RNA
- Biotynilated RNA can be purified using streptavidin-coated magnetic beads



Windhager L, *Genome Res 2012*

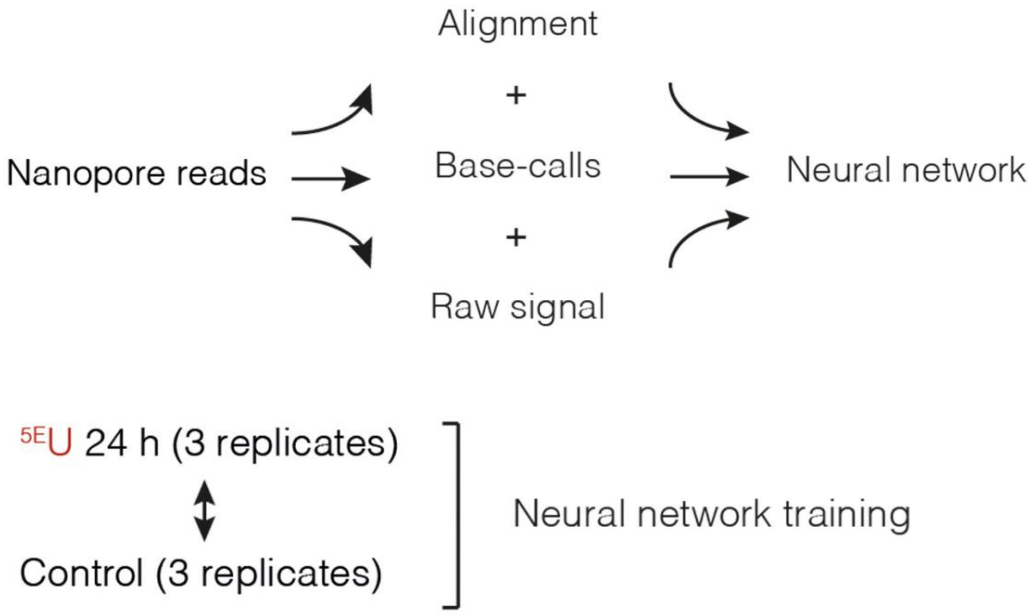
SLAM-seq

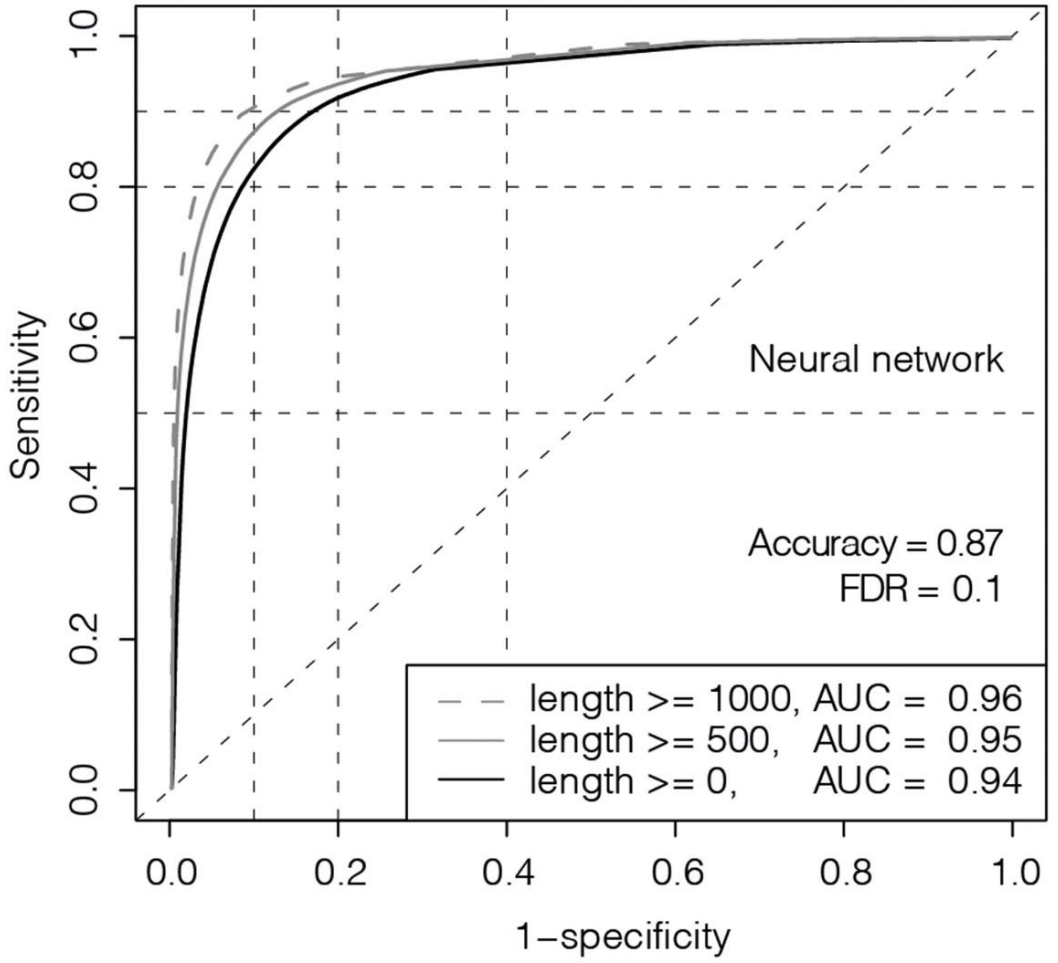


- Thiol-assisted conversion of 4sU incorporated in nascent RNA
- Allows detecting nascent RNA *in silico*
- Does not require physical separation of 4sU+ RNA
- Not fully compatible with short pulses
- Medium / low sensitivity

Herzog VA, *Nat Meth 2017*

nano-ID





- Detection of 5EU+ reads with Nanopore dRNA-seq
- Does not require chemical treatments
- Does not require physical separation of labelled RNA
- Allows quantifying synthesis and degradation rates

Maier, KC *Genome Res 2020*

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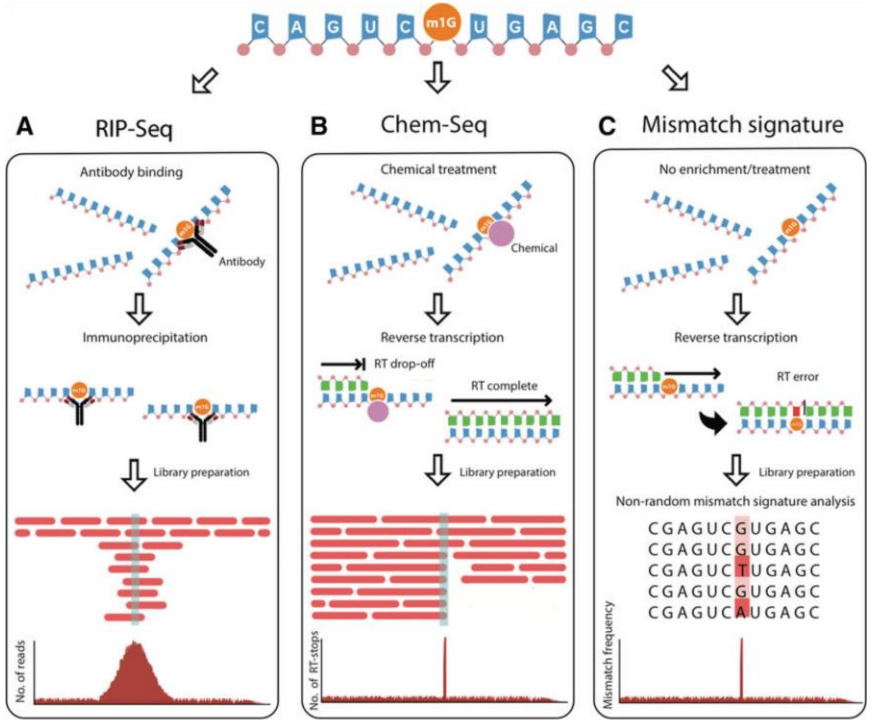
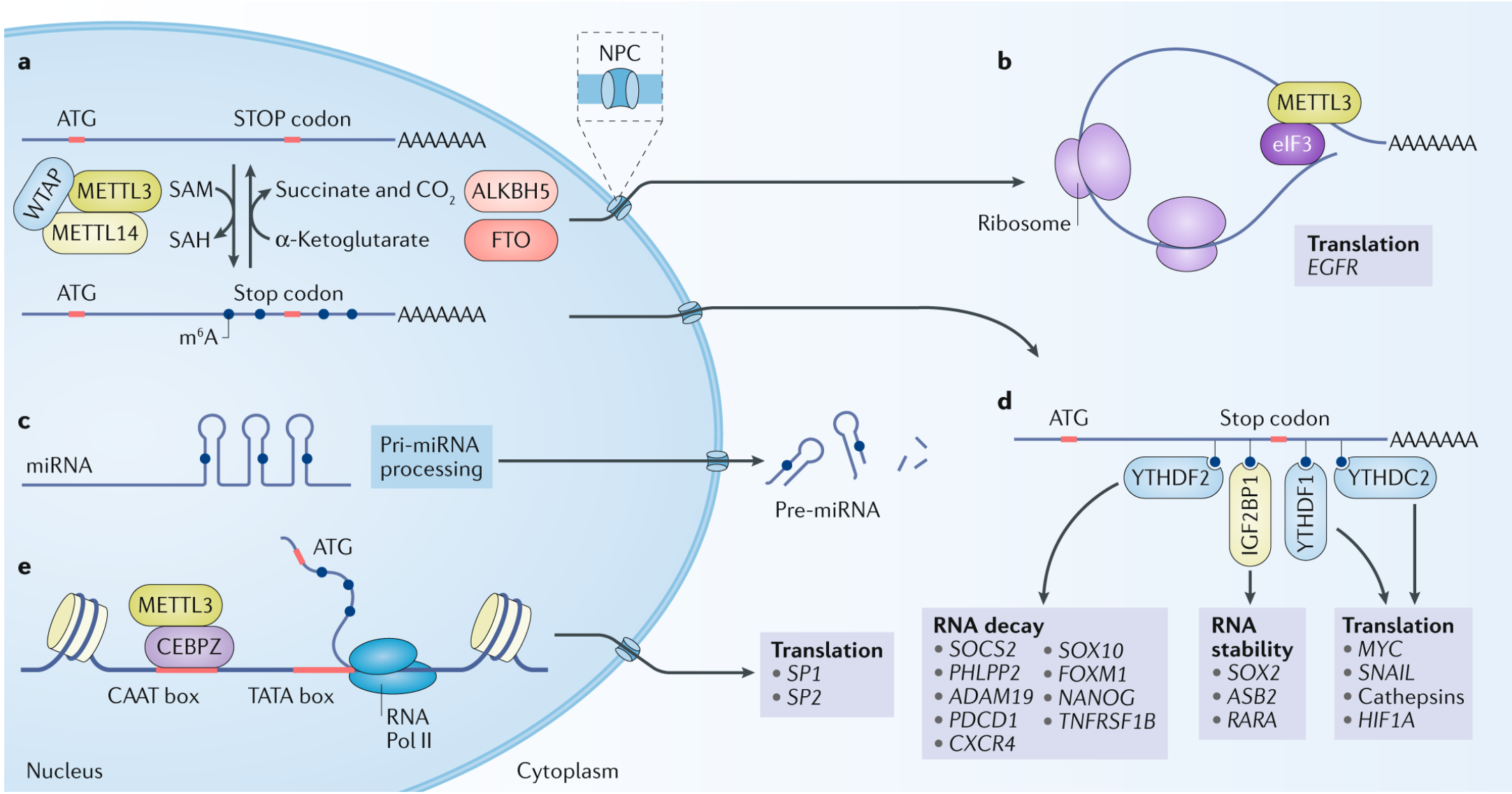


The RNA modification landscape in human disease, Jonkhout N et al, RNA. 2017 Dec

The goal for all of these techniques is to detect RNA modifications to better understand their functional role in biology



Barbieri, Kouzarides, *Nature Reviews* 2020