

Methods for RNA modifications profiling

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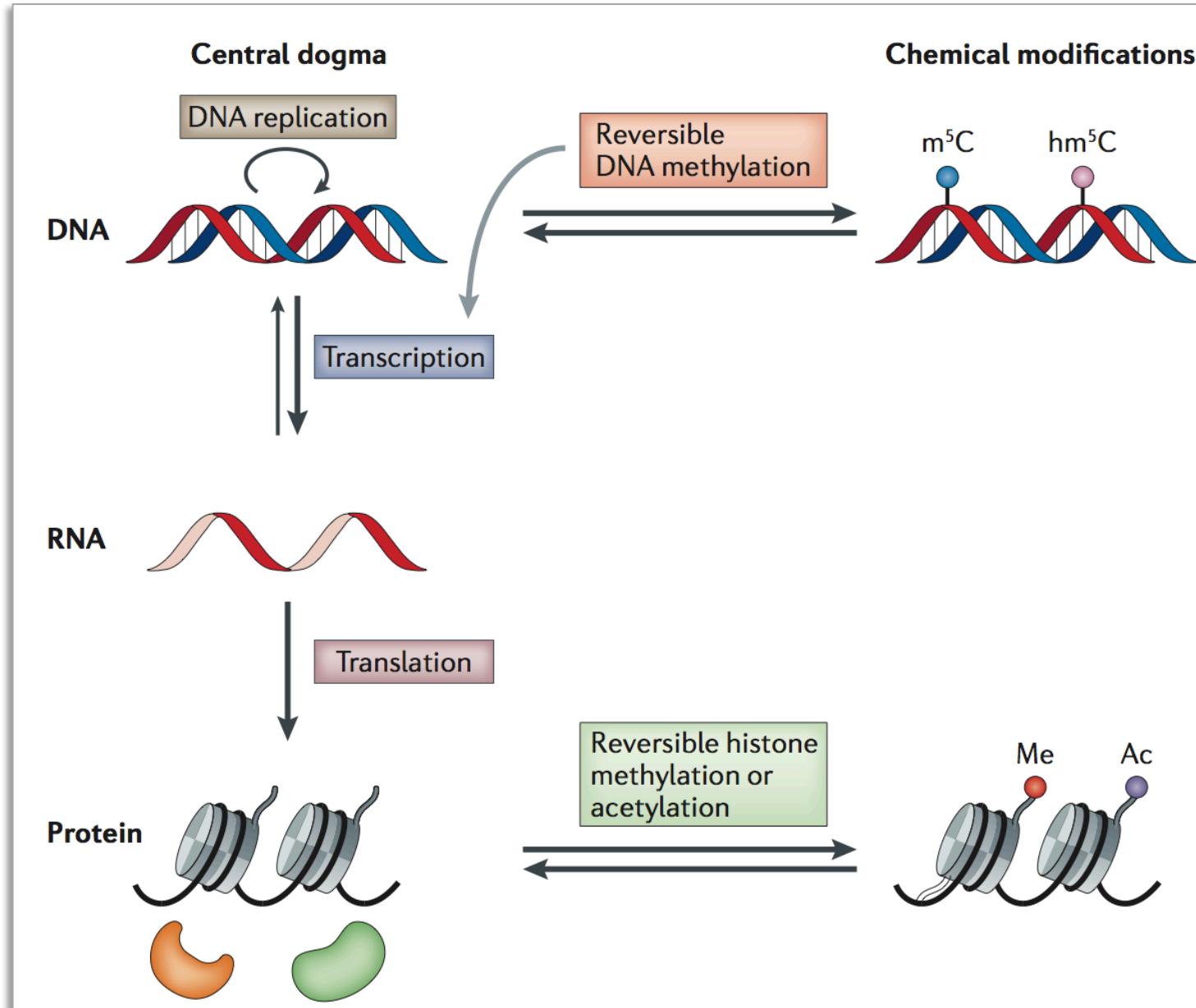


methylation
chromatin
decoding
sequencing
polymerase
transcription
nascent RNA
epigenomics

Outline

- Background on RNA modifications
- Methods to profile m6A
 - Bulk levels - Dot-blot, ELISA/colorimetric, MassSpec
 - Genome-wide, Ab-based - MeRIP-seq, miCLIP, m6A-LAIC-seq, m6A-seq2
 - Genome-wide, not Ab-based - MAZTER-seq, m6A-sac-seq, GLORI, DART-seq
 - Single locus - SCARLET, MazF-PCR
 - Single cell and molecule – scDART-seq, Nanopore native RNA-seq
- Other mods – m5C, Ψ , m1A, Nm, RNA editing
- Nascent RNA – 4sU-seq, TT-seq, SLAM-seq, nano-ID
- Combinations of mods - IVT
- Computational methods – m6A absolute, Differential, Nanopore
- Benchmarking of methods – Short and long reads

The epitranscriptome

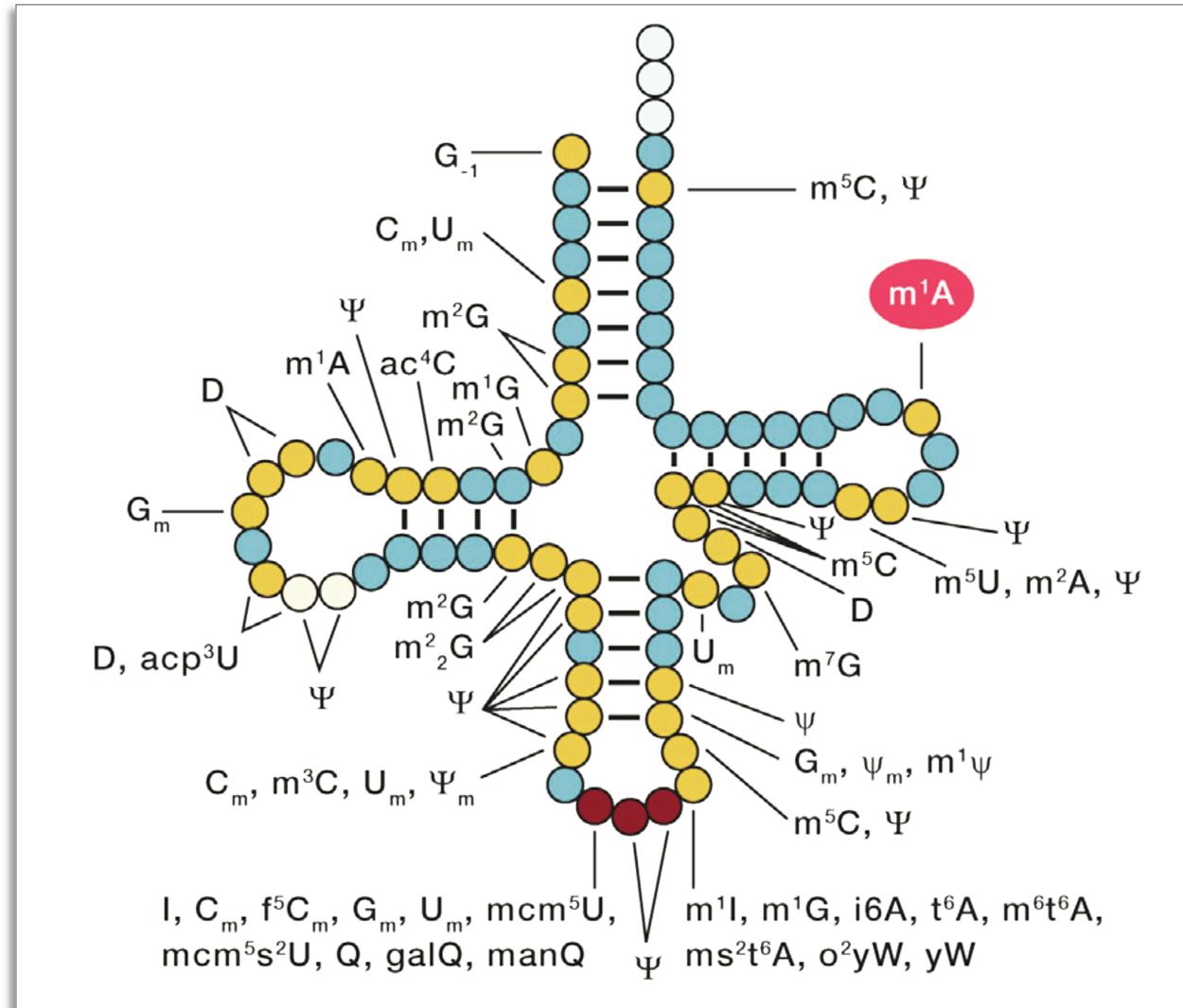


Adapted from: Fu Y et al, *Nature Review Genetics* 2014

The tRNA epitranscriptome

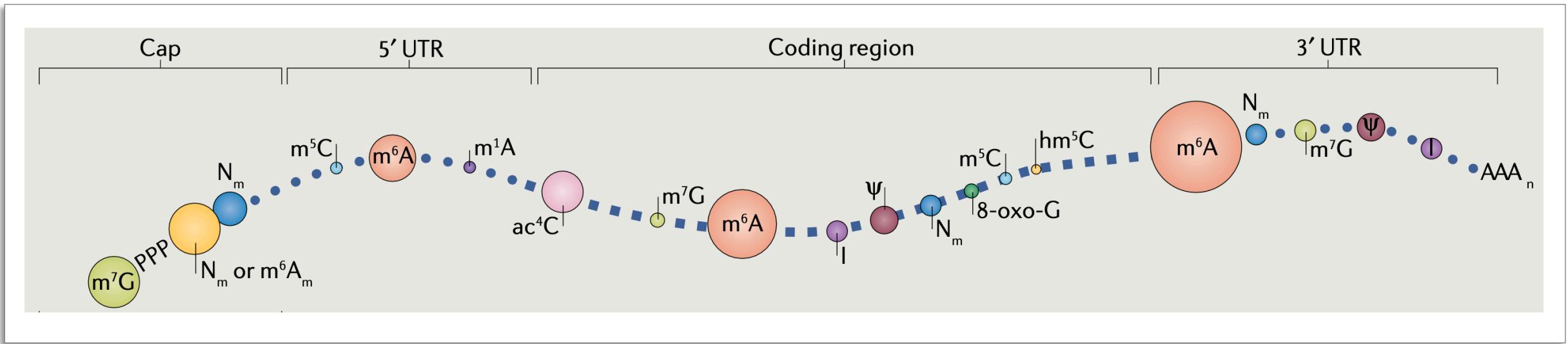
Dozens of RNA modifications exist

tRNAs are decorated by many of them



Adapted from Roundtree I et al, Cell 2017

The mRNA epitranscriptome



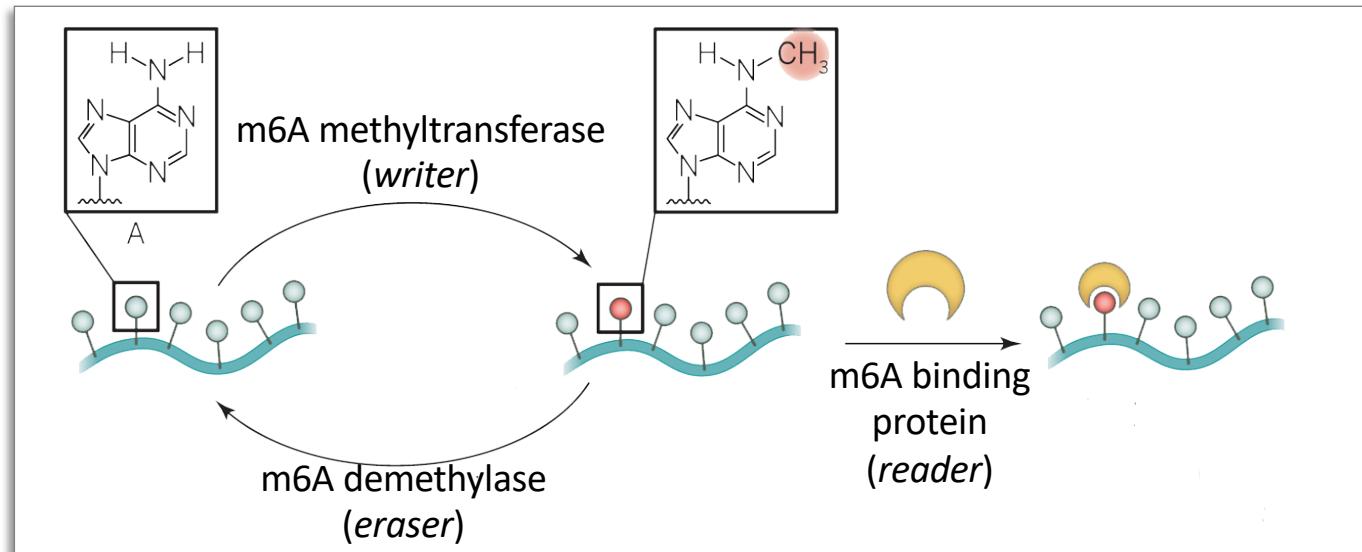
Adapted from Zaccara S, *Nat Rev Mol Cell Biol* 2019

Multiple marks decorate mRNAs

*It is currently unclear if a **combinatorial code** exists*

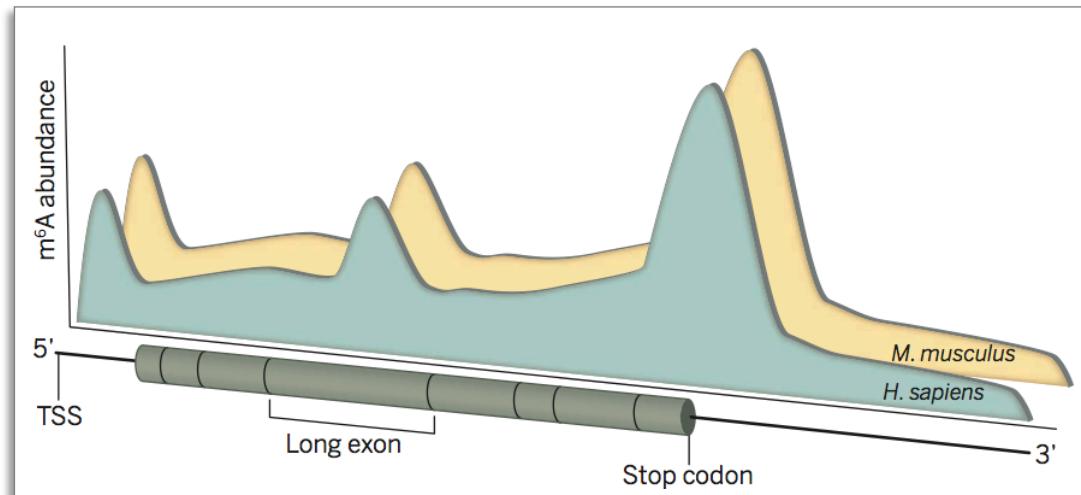
N6-Methyladenosine (m6A)

m6A are dynamic marks controlled by multiple effectors

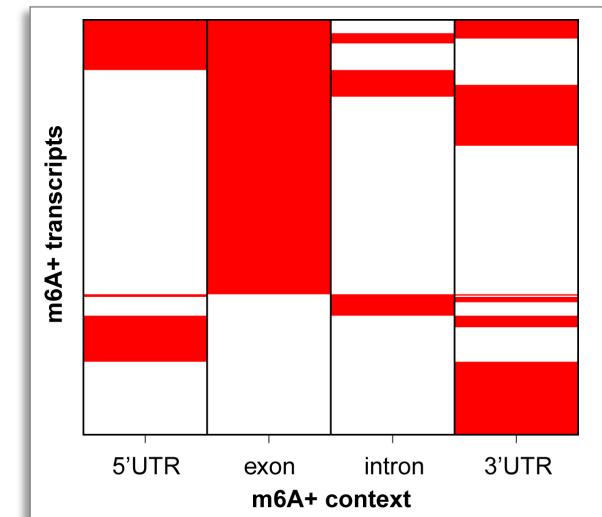


Adapted from Dominissini D et al, Science 2014

Are enriched at 3' ends and are conserved across species



Adapted from Dominissini D et al, Science 2014



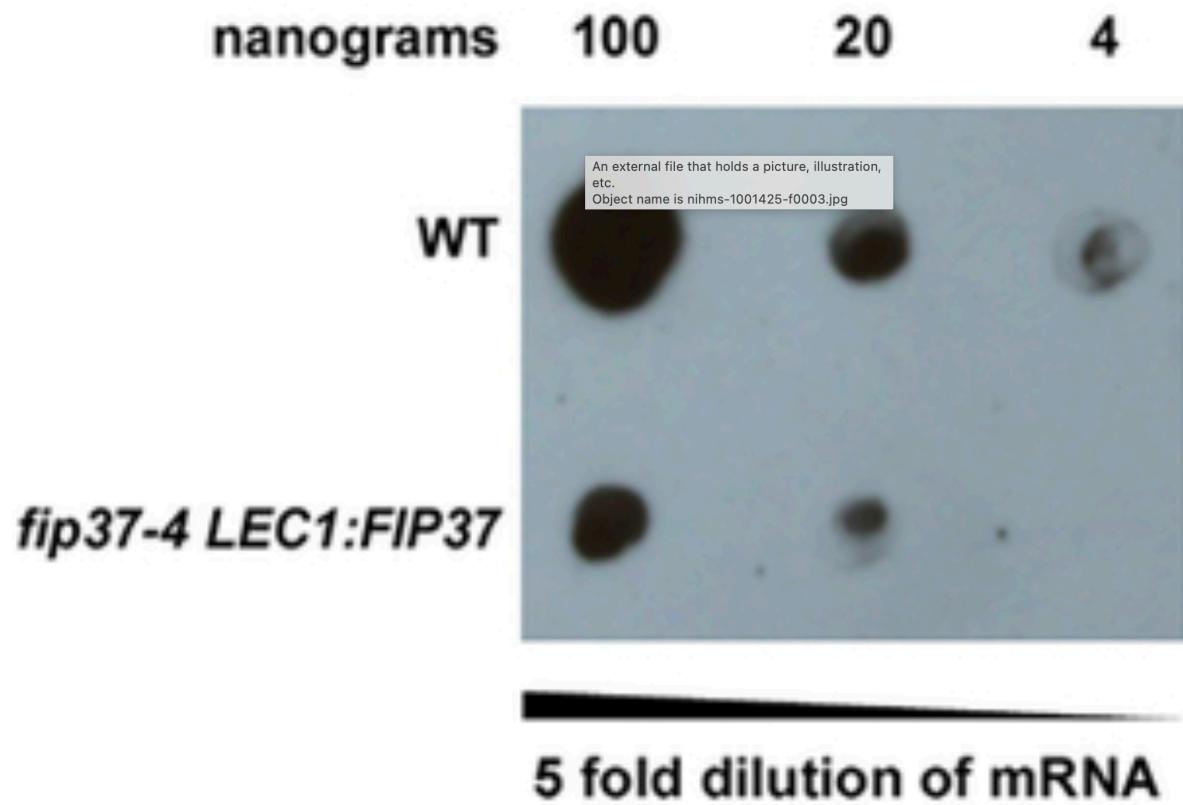
unpublished

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Dot-blot

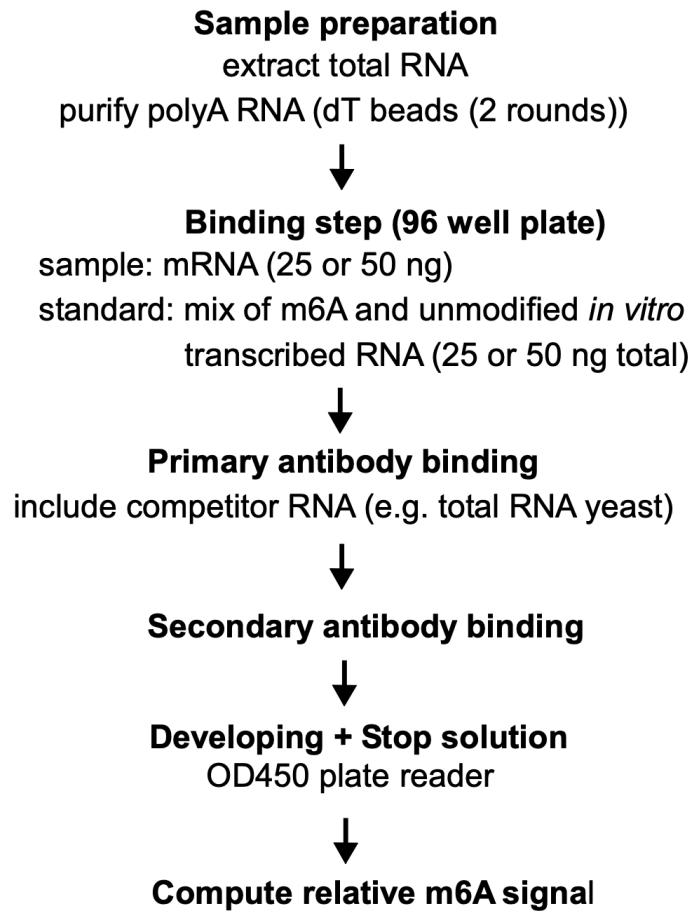
- Fast and cheap assessment of bulk levels
- Based on Ab anti-m6A
- Qualitative, poorly quantitative
- 20 ug total RNA
- Can be applied to mRNA

Shen L, *Bio Protoc* 2017

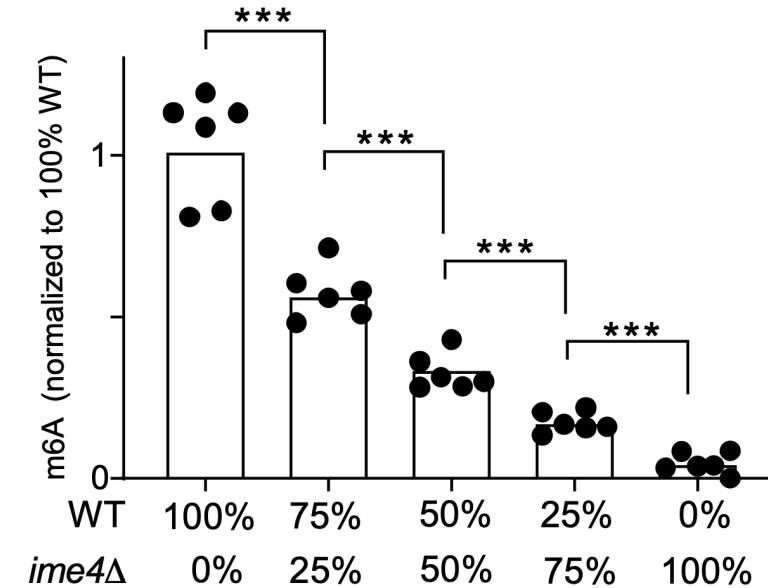
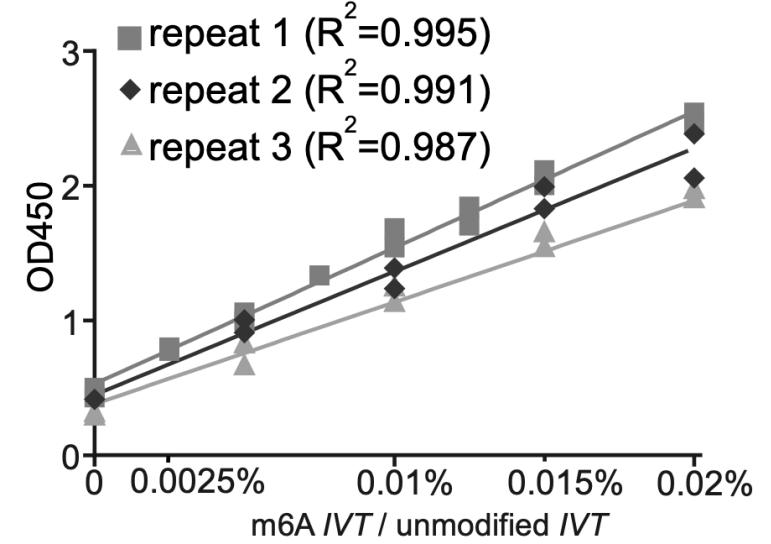


Shen L, *Dev Cell* 2016

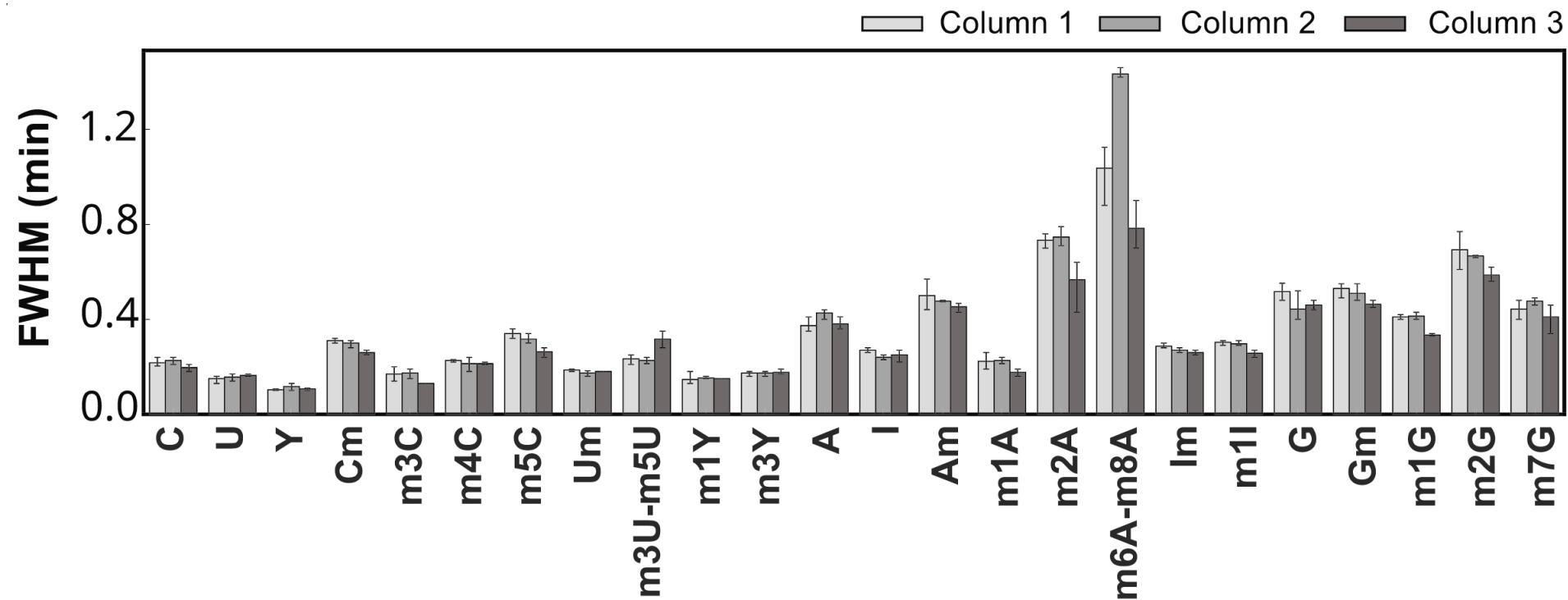
ELISA



- Fast, less than a day
- It can be scaled down to 25 ng of mRNA



Mass Spec

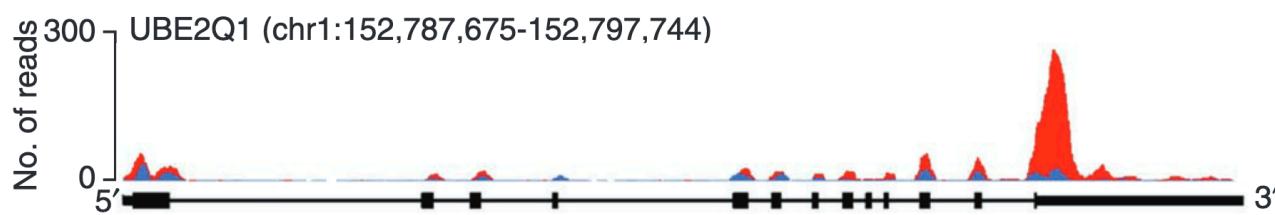


- Liquid chromatography-tandem mass spectrometry
- It is considered the gold standard for RNA mods quantification

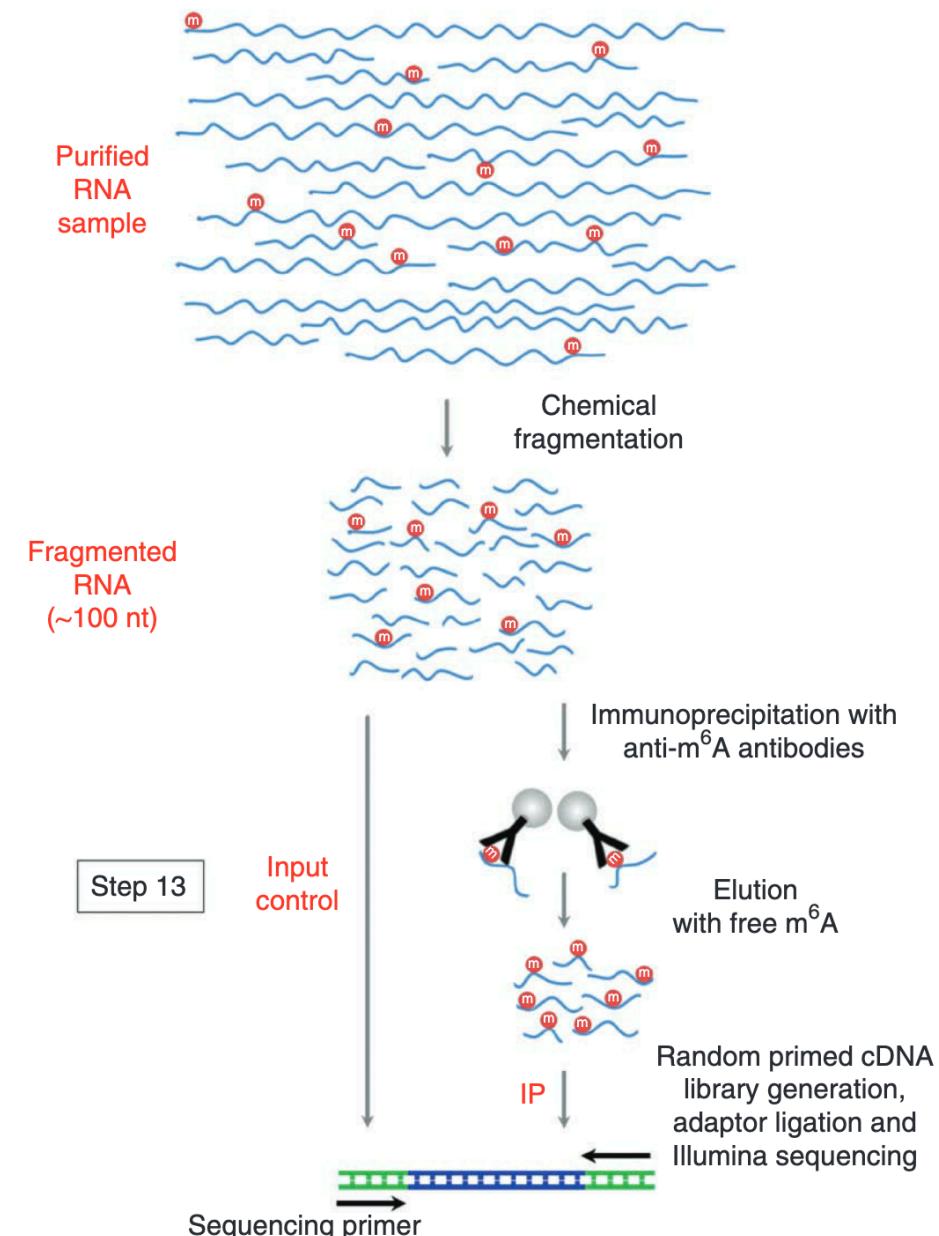
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MeRIP-seq

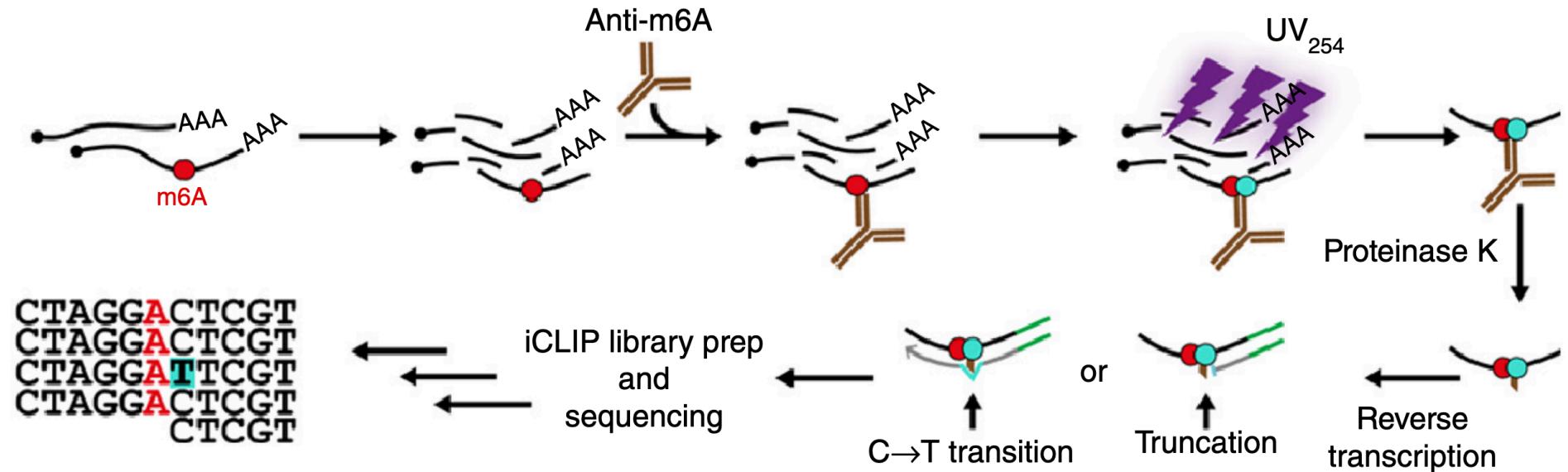
- Based on anti-m⁶A Ab
- 9 days for 4 conditions (IP + input)
- 300ug total RNA or 5ug polyA+
- 100nt fragmentation size



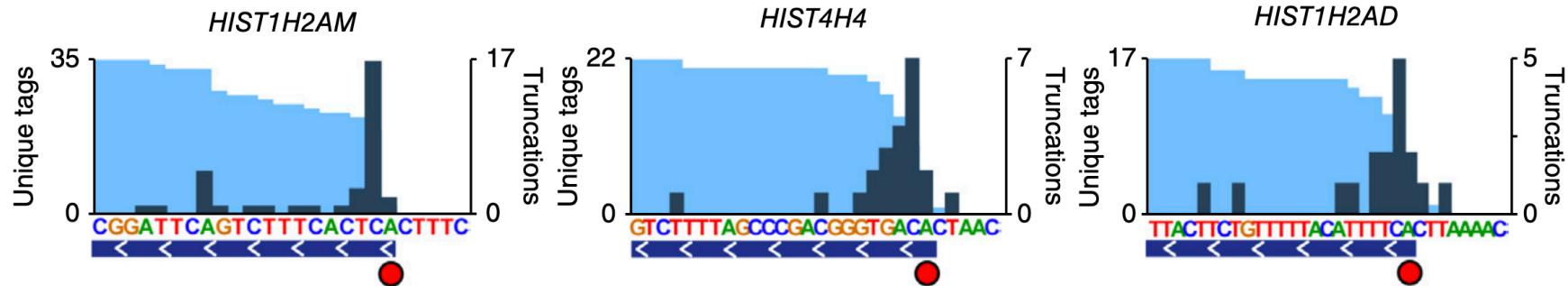
Dominissini D, *Nat Prot* 2013



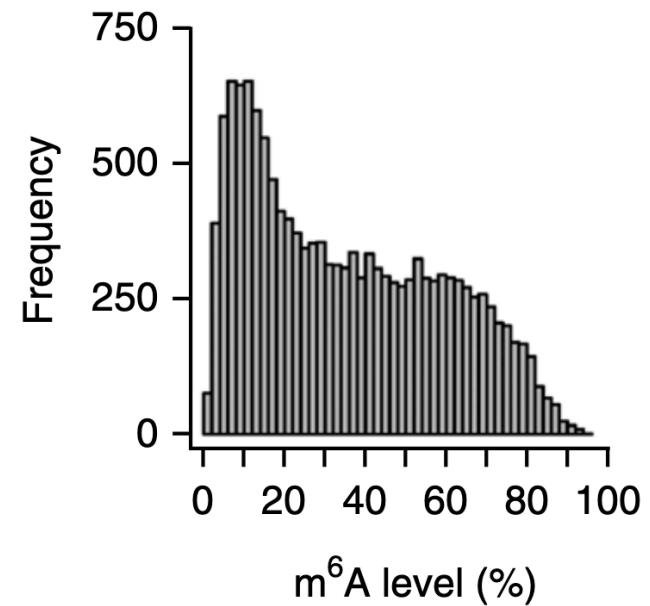
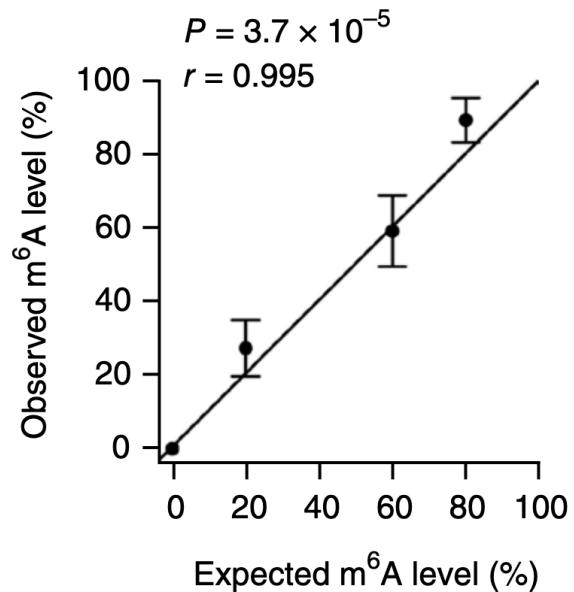
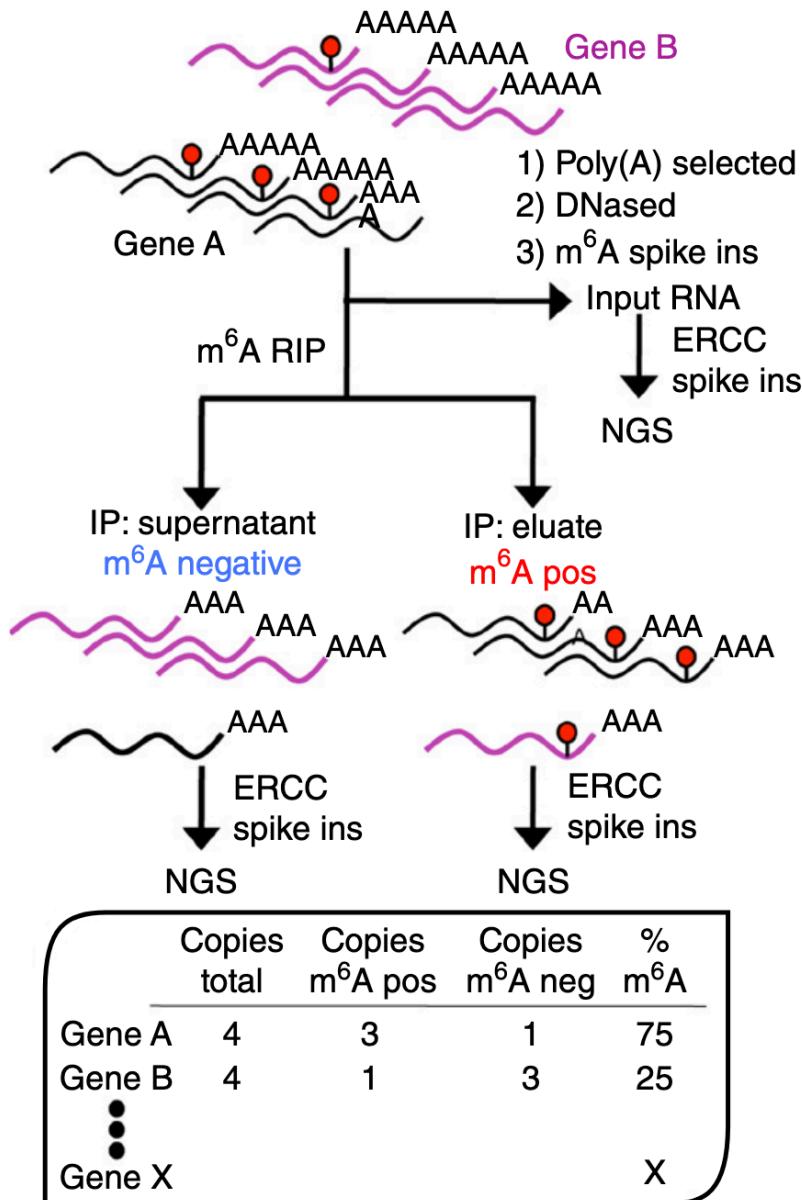
miCLIP



- Relies on m6A Ab and UV crosslinking
- Relies on Ab-specific mutation signatures
- 20ug fragmented RNA
- Base resolution m6A calling



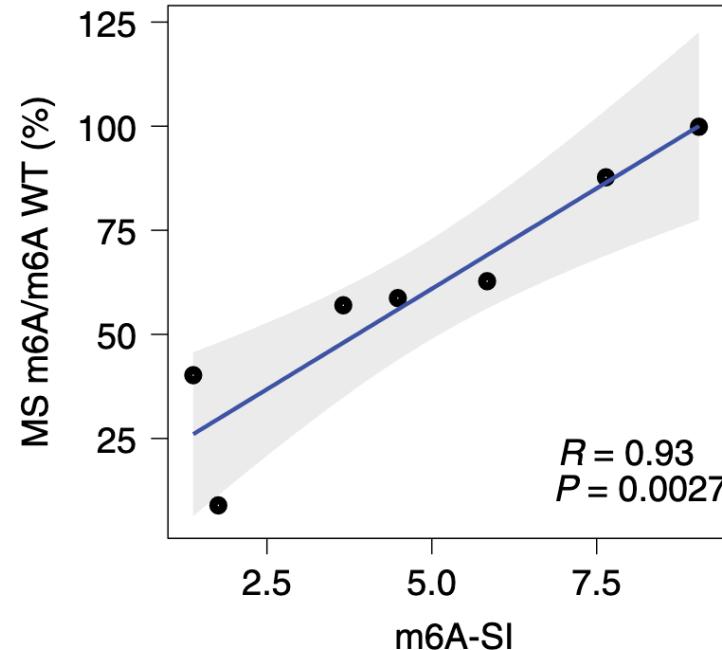
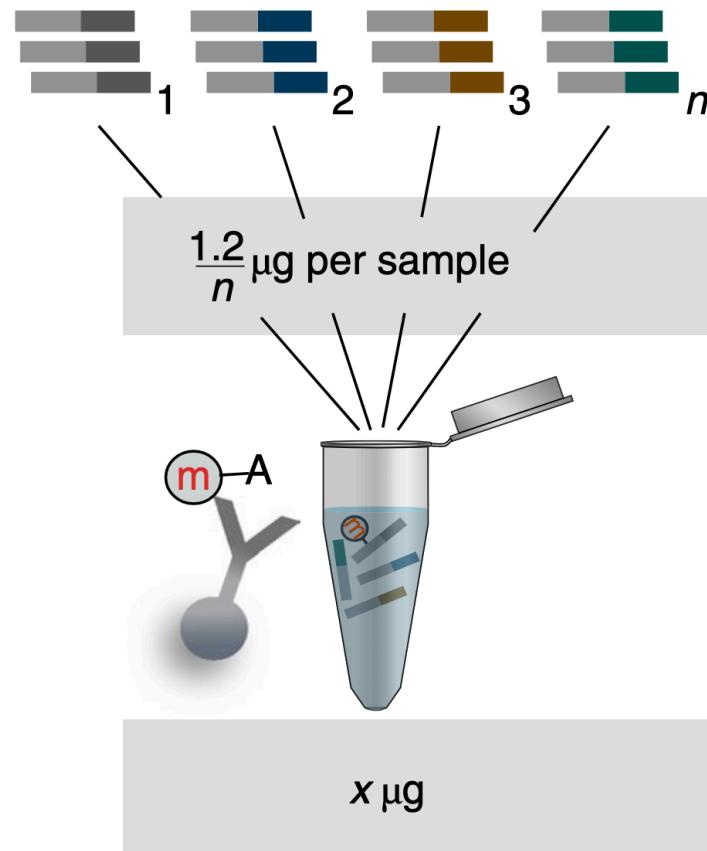
m6A-LAIC-seq



- Relies on m6A-Ab
- Provides m6A stoichiometry
- It is not positional

Molinie B, *Nat Meth* 2016

m6A-seq2

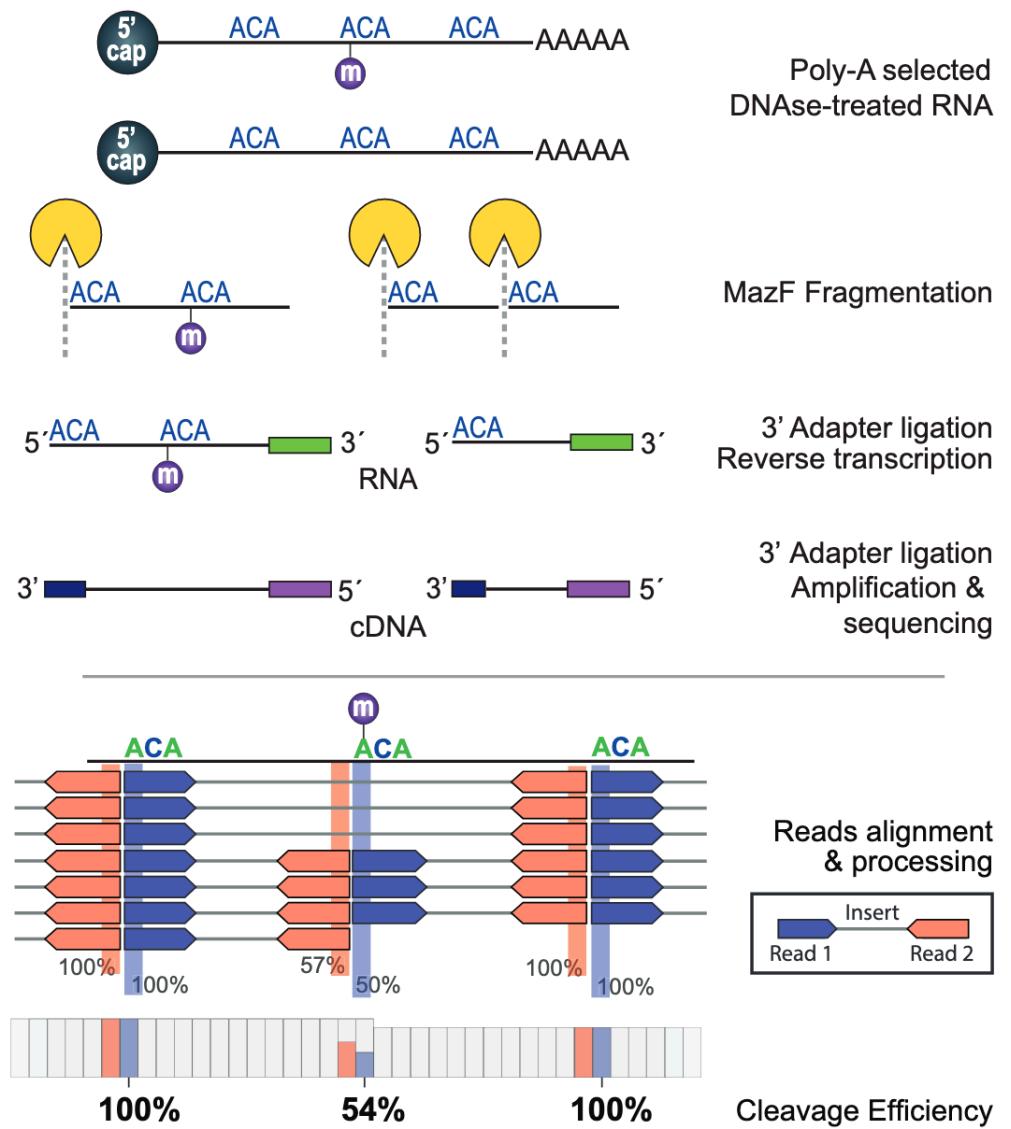


- Reduced RNA amount (100ng)
- Competitive m6A-Ab IP following barcoding up to 12 samples
- Improved signal-to-noise
- Allows comparison between conditions
- Provides estimate of bulk levels

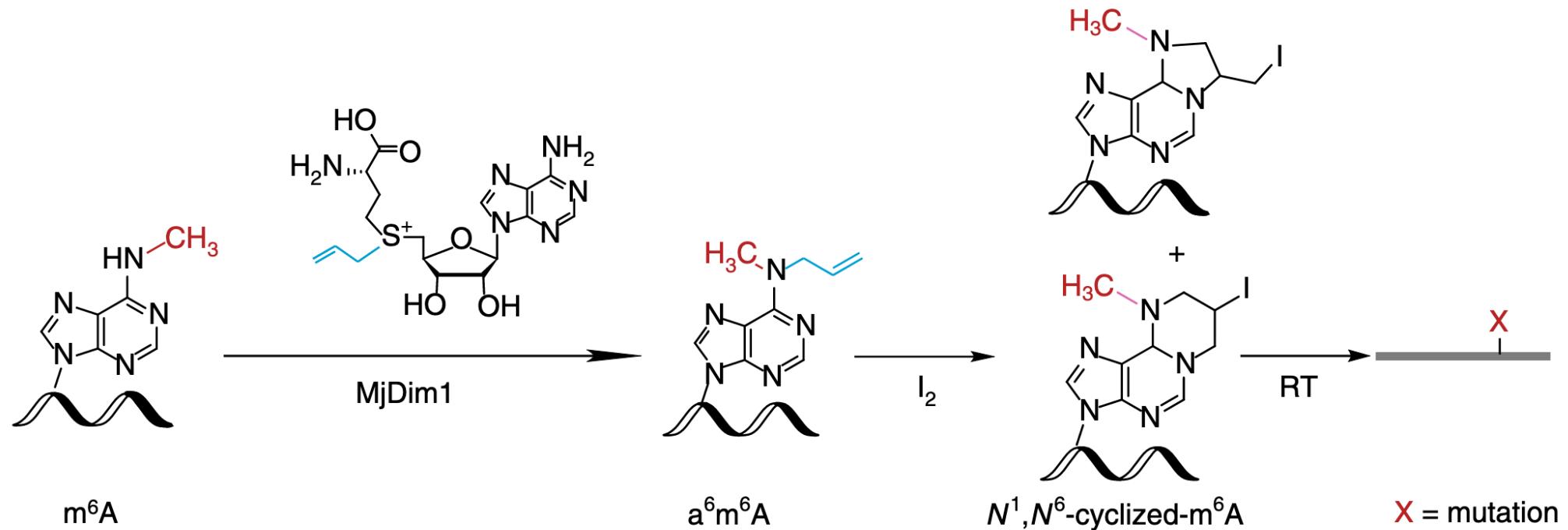
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MAZTER-seq

- 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

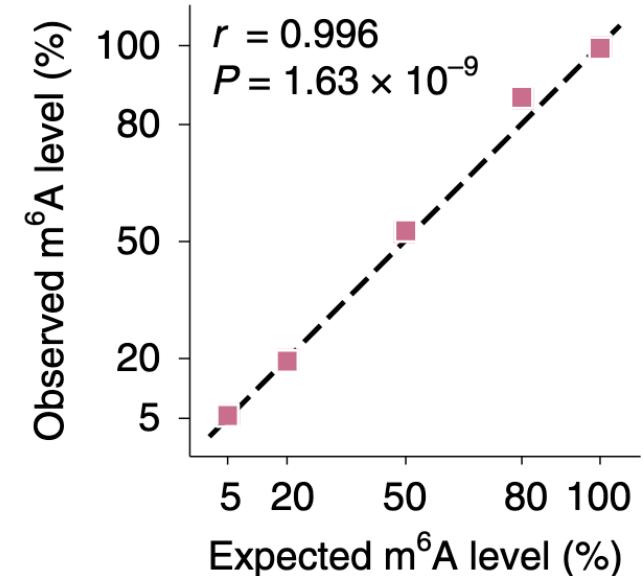
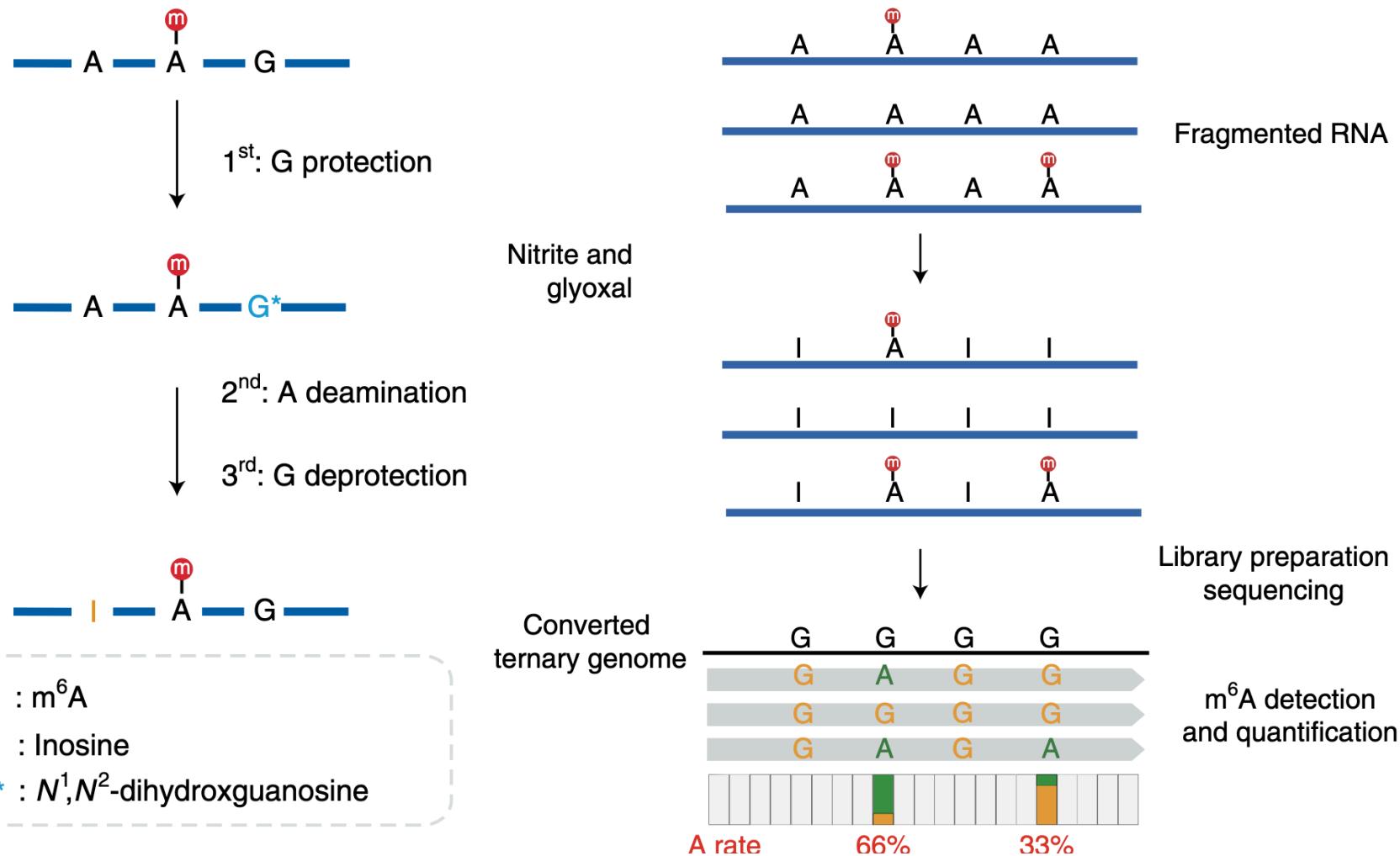


m6A-sac-seq



- Single base resolution
- Requires 30ng of pA+ or ribo- RNA
- Provides stoichiometry
- Requires spikeins to characterize the mutation for different m6A sequence contexts

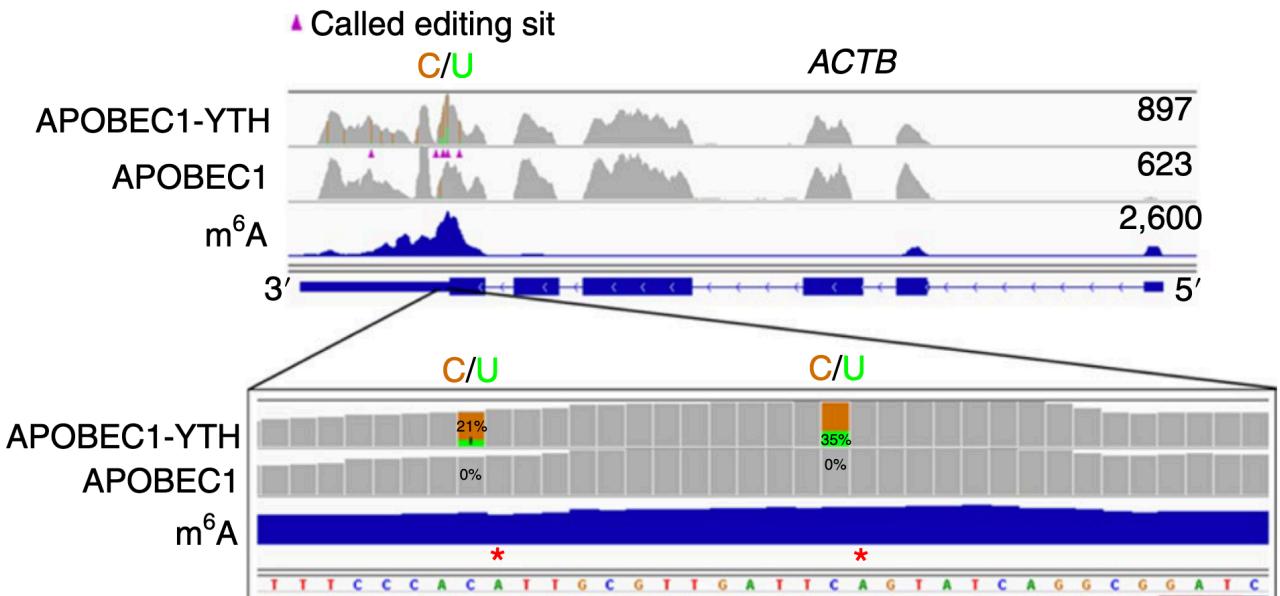
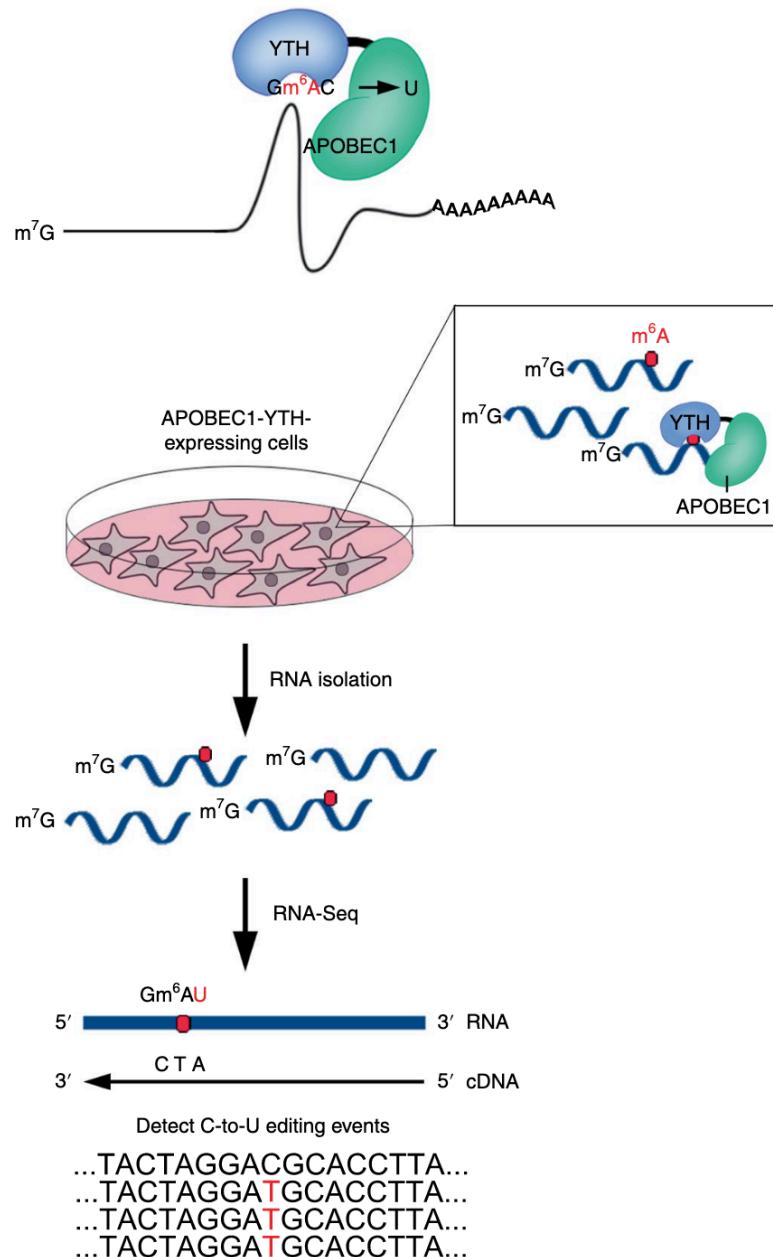
GLORI



- Relies on glyoxal and nitrite-mediated deamination of unmethylated As (GLORI) while keeping m^6A intact
- Provides m^6A stoichiometry
- Requires 100ng pA+ RNA

Liu C, *Nat Biotech* 2022

DART-seq

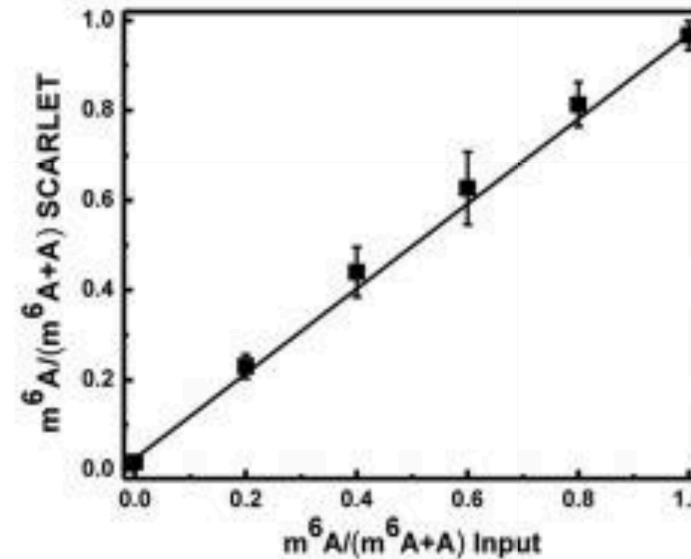
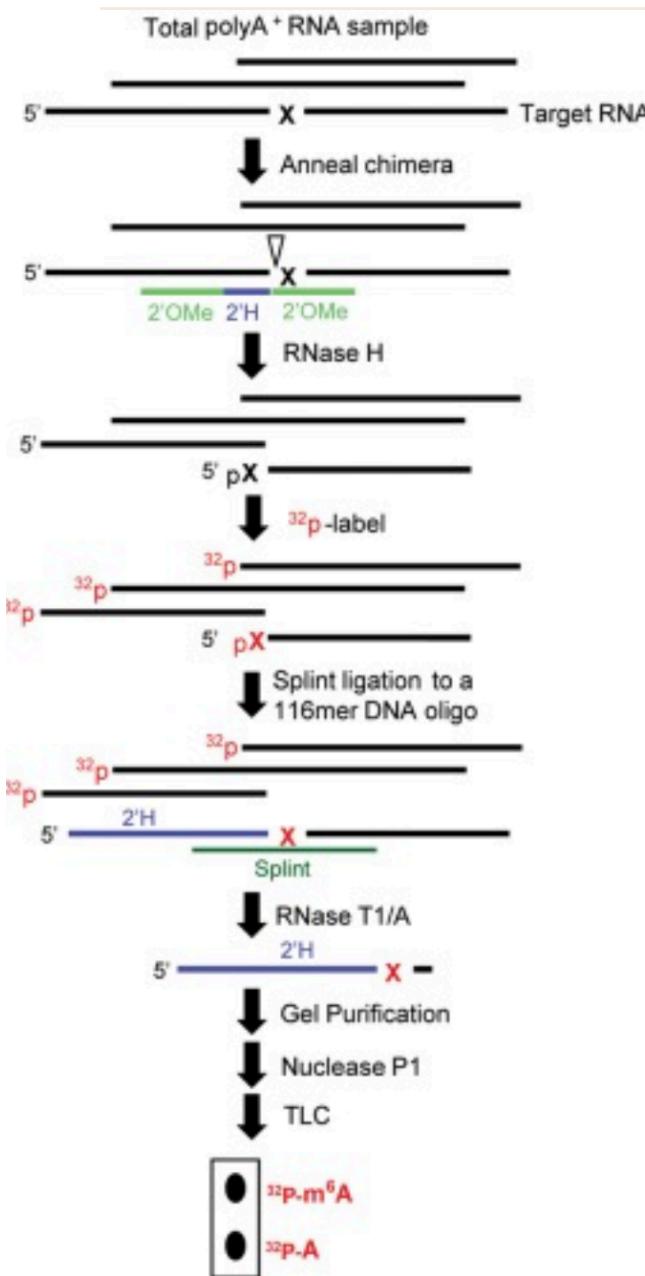


- APOBEC1-YTH mediated deamination (C-to-U) adjacent to m⁶A
- C-to-U detected via standard RNA-seq
- Requires APOBEC1 fused to m⁶A-binding YTH domain and APOBEC1-YTH expression
- Requires 10ng total RNA
- Distinguishes m⁶A from m⁶Am

Meyer KD, *Nat Meth* 2019

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SCARLET



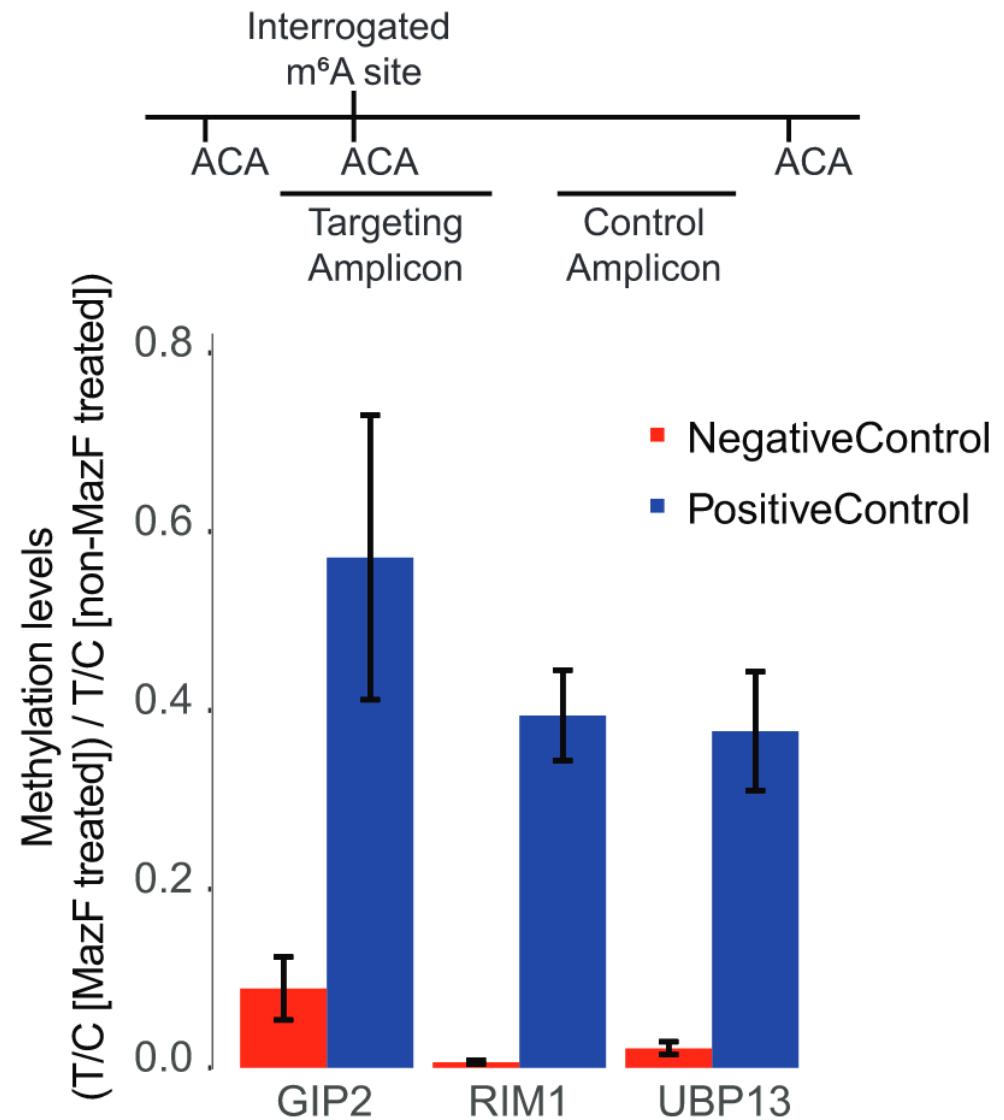
- site-specific cleavage and radioactive-labeling followed by ligation-assisted extraction and thin-layer chromatography (SCARLET)
- still sensitive when as little as ~1 fmol RNA template was present in 1 μg polyA⁺ RNA

Liu N, *RNA* 2013

MazF-PCR

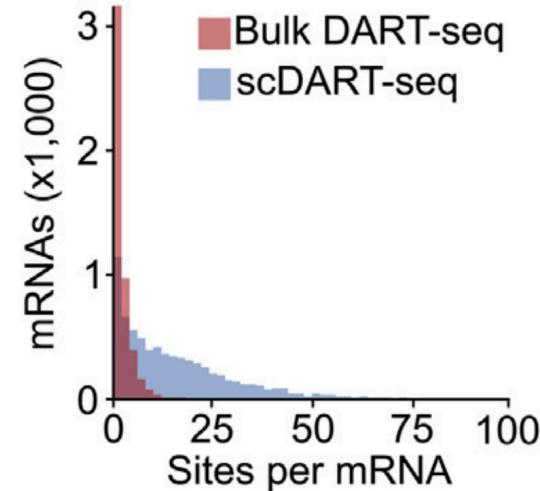
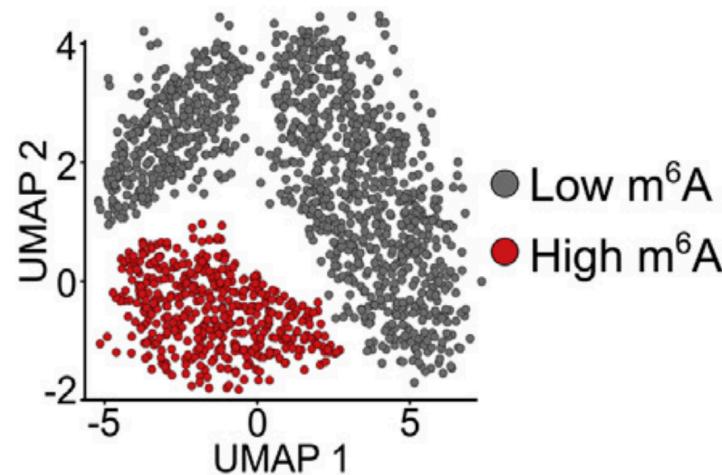
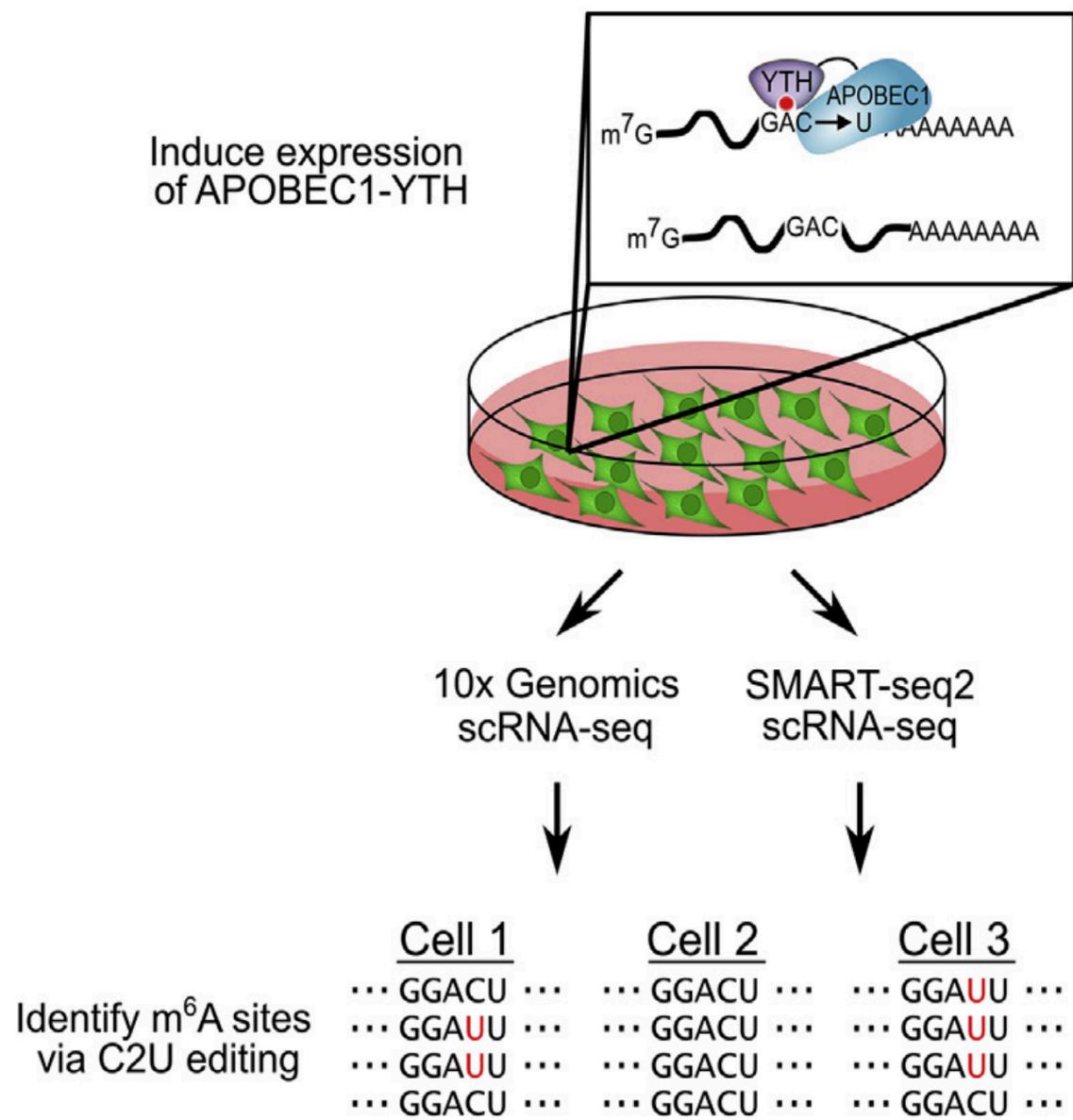
- Relies on MAZTER-seq approach
- Requires 25-50ng polyA+ RNA

Garcia-Campos MA, *Cell* 2019



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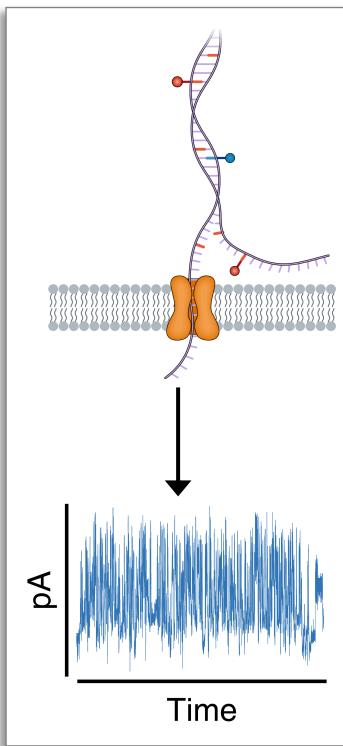
scDART-seq



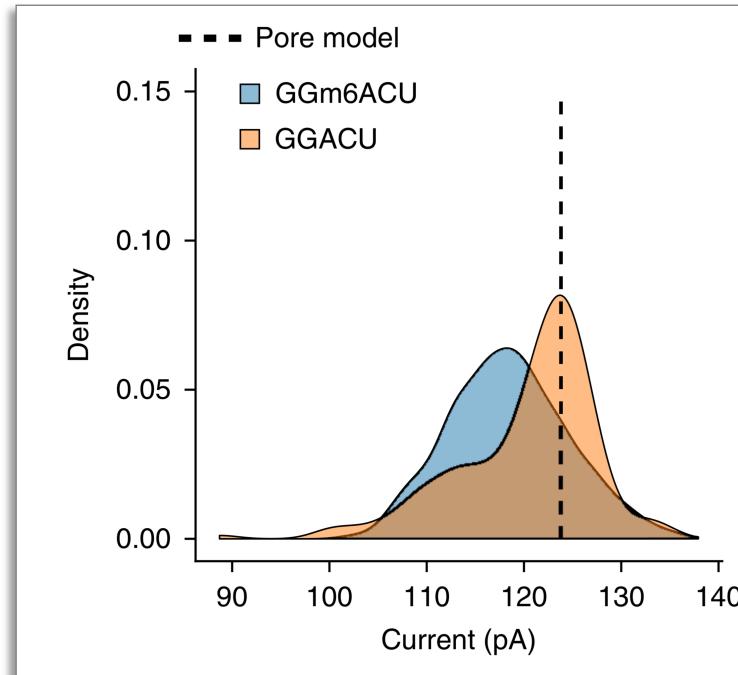
- Single cell m₆A mapping
- Based on DART-seq

Tegowski M, *Mol Cell* 2022

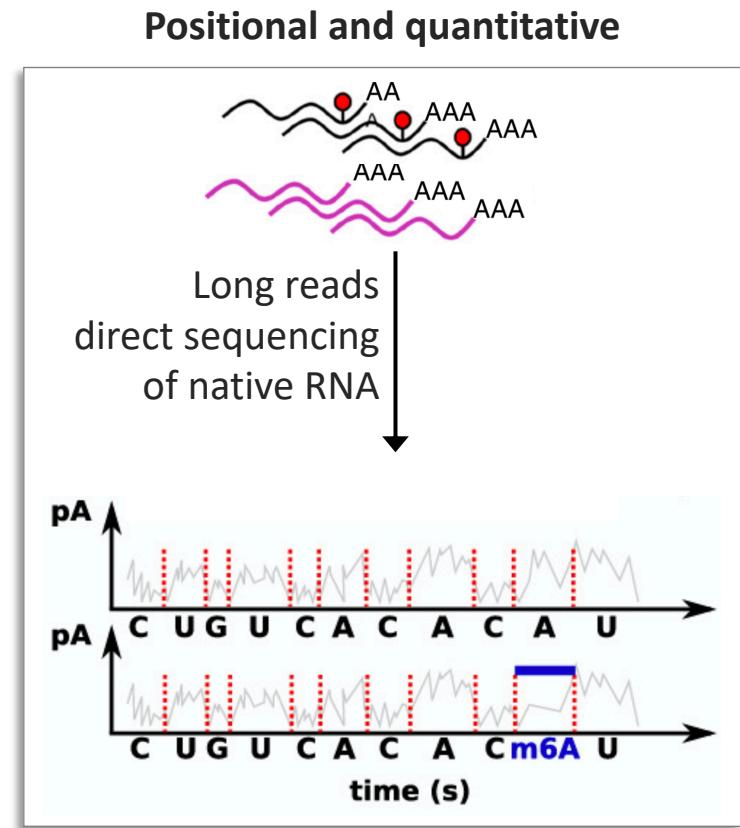
Nanopore native RNA-seq



The signal is obtained in form of current over time

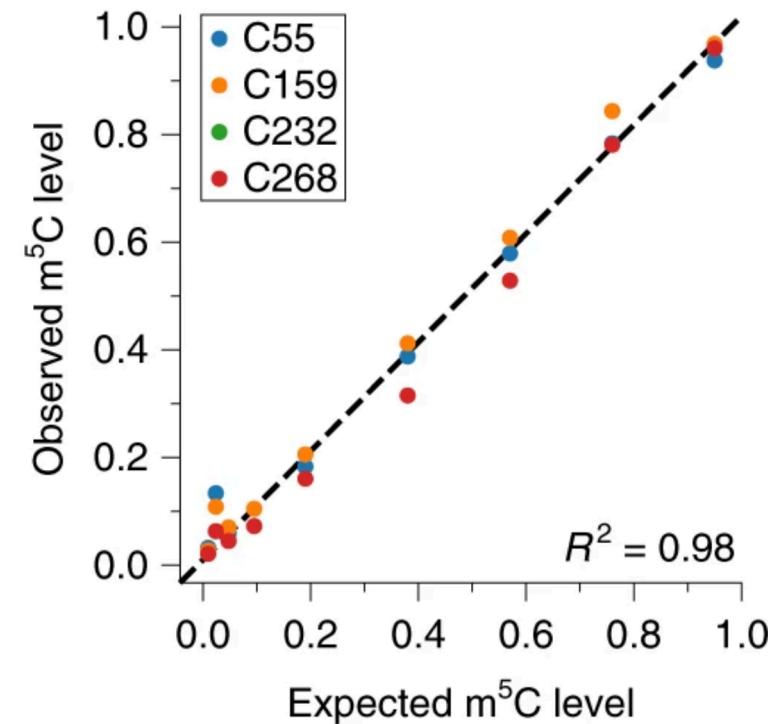
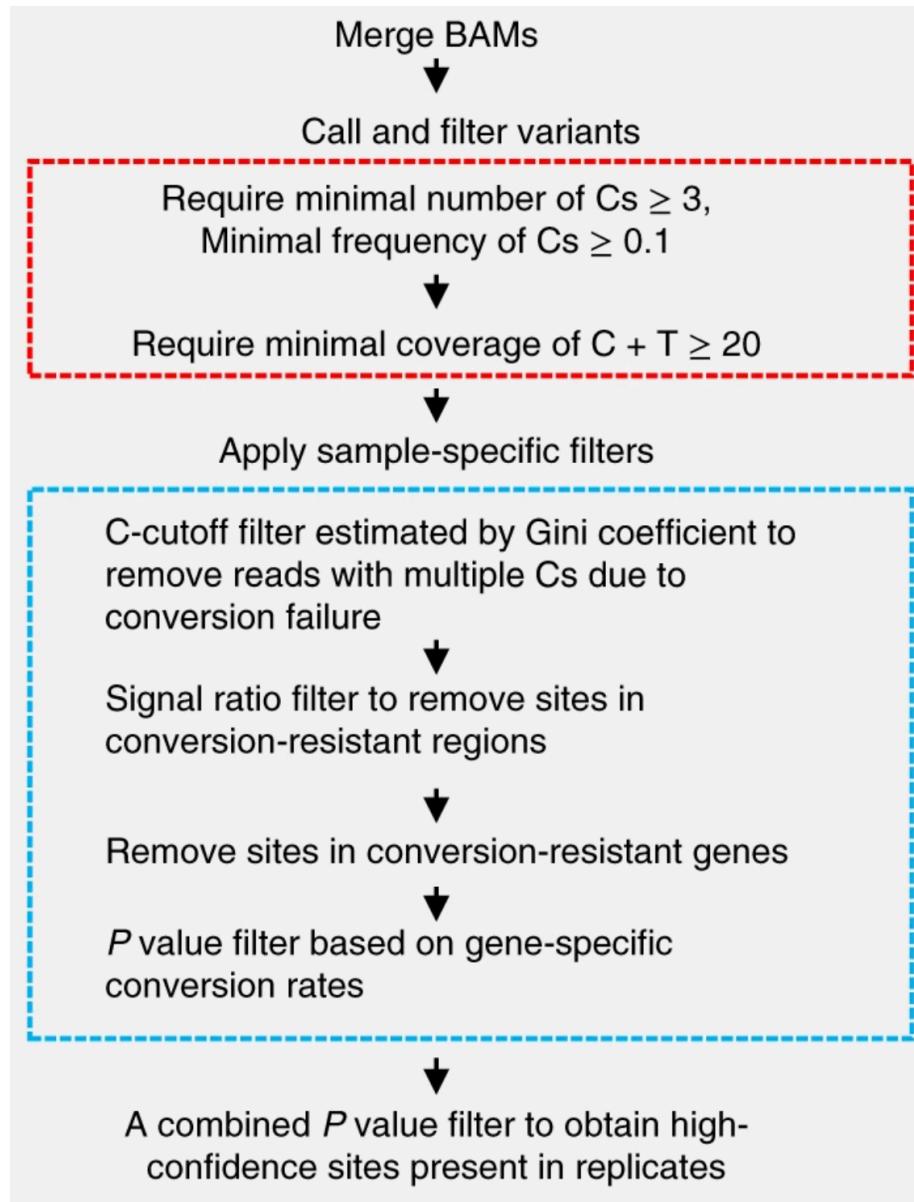


The signal is influenced by the presence of RNA modifications



Adapted from Furlan M *Front Genet* 2020

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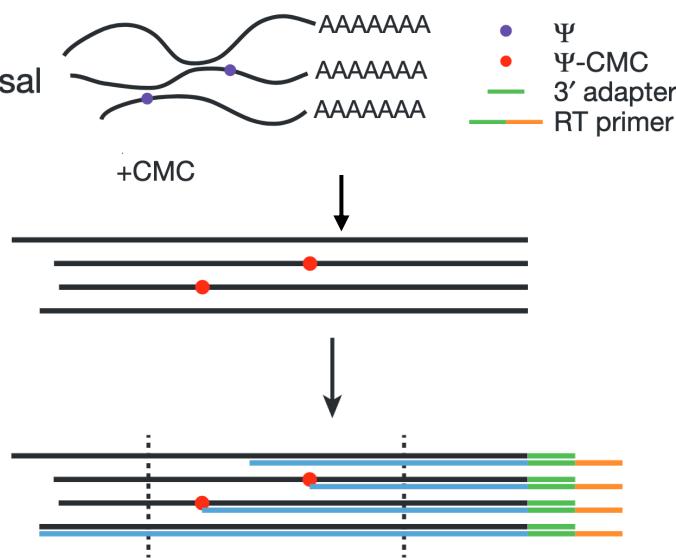
- Based on bisulfite sequencing
- several hundred exonic m5C sites found
- 62–70% of the sites had <20% methylation
- 8–10% of the sites had >40% methylation

Pseudouridine

Poly(A) selection

Random fragmentation

CMC modification and reversal



Size selection (100–120 nt)

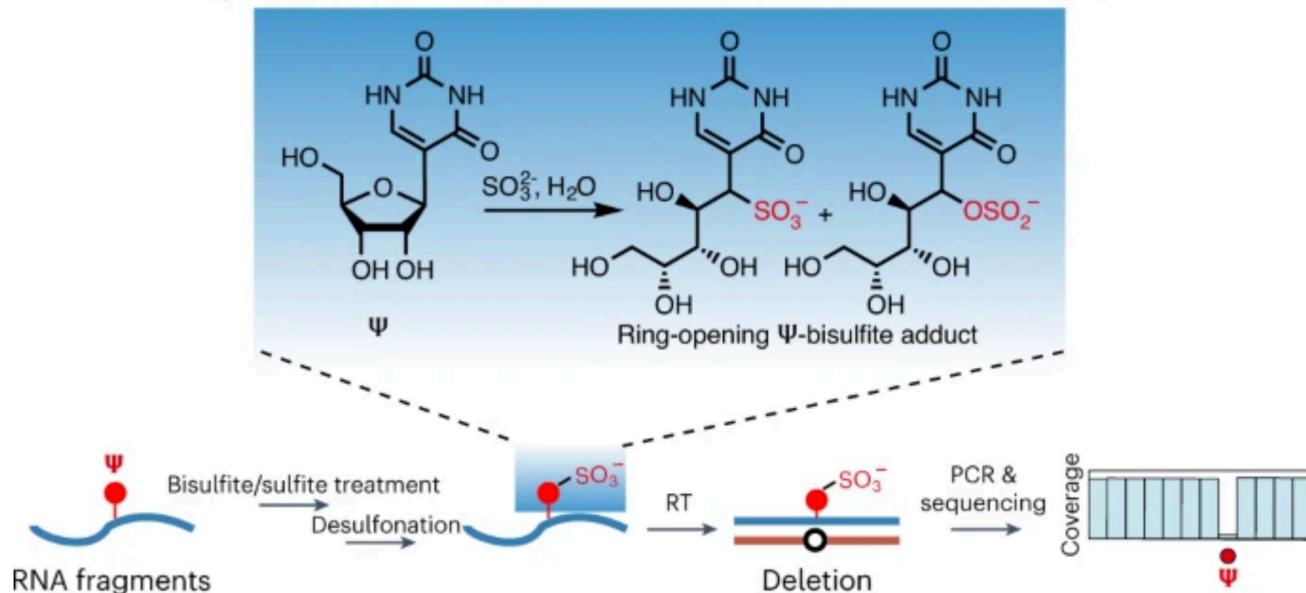
3' adapter ligation

Reverse transcription
Select truncated cDNAs

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

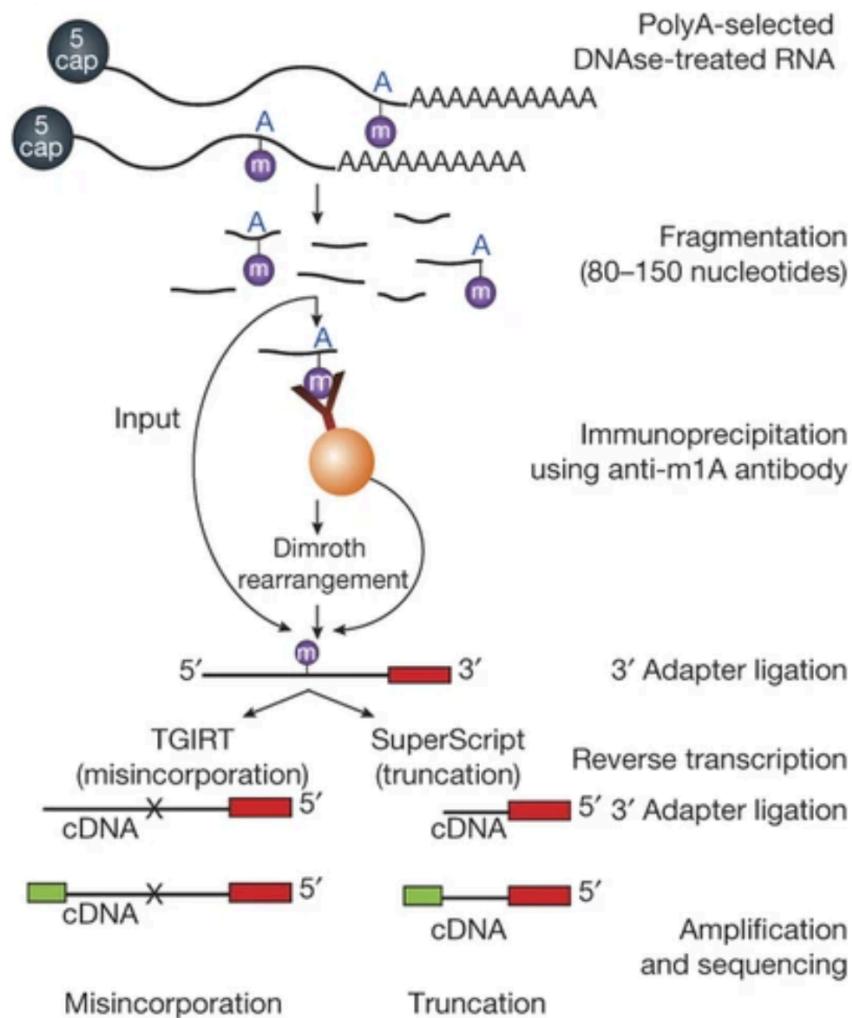
Carlile TM, *Nature* 2014

PRAISE
(pseudouridine assessment via bisulfite/sulfite treatment)



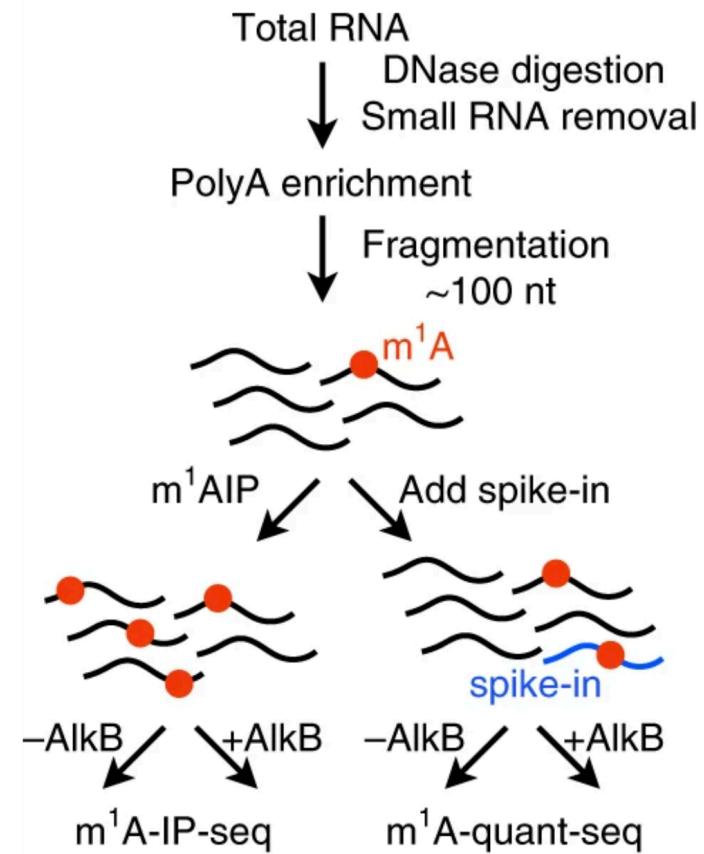
- Ψ is labeled by bisulfite and leads to nucleotide deletions during RT
- Ab independent
- Provides stoichiometry

Zhang M, *Nat Cell Biol* 2023



- Relies on m1A-Ab
- Generates misincorporations or truncations

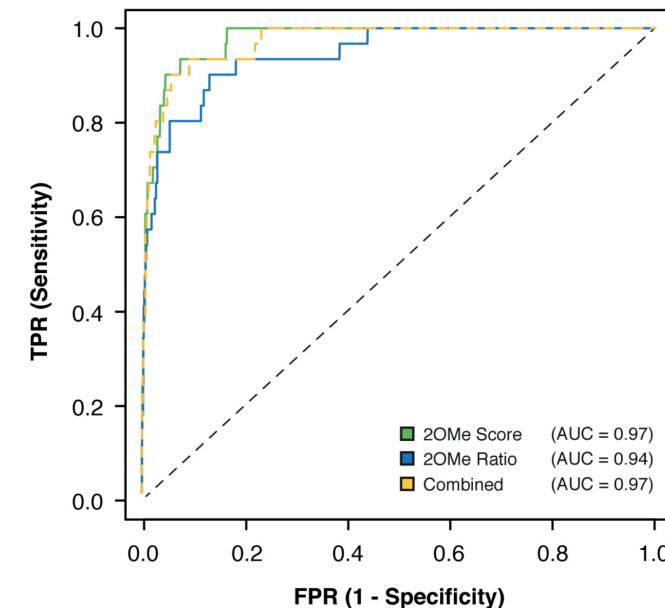
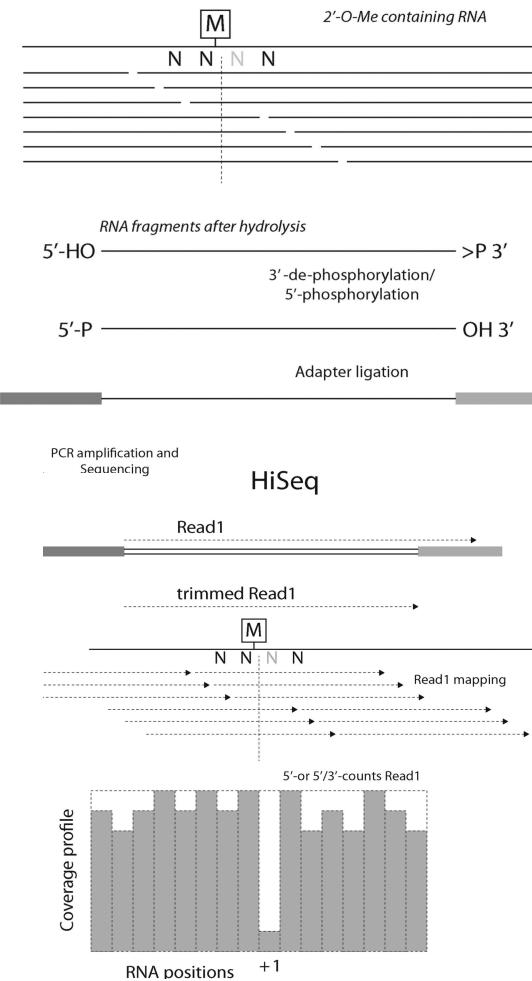
Safra M, *Nature* 2017



- 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

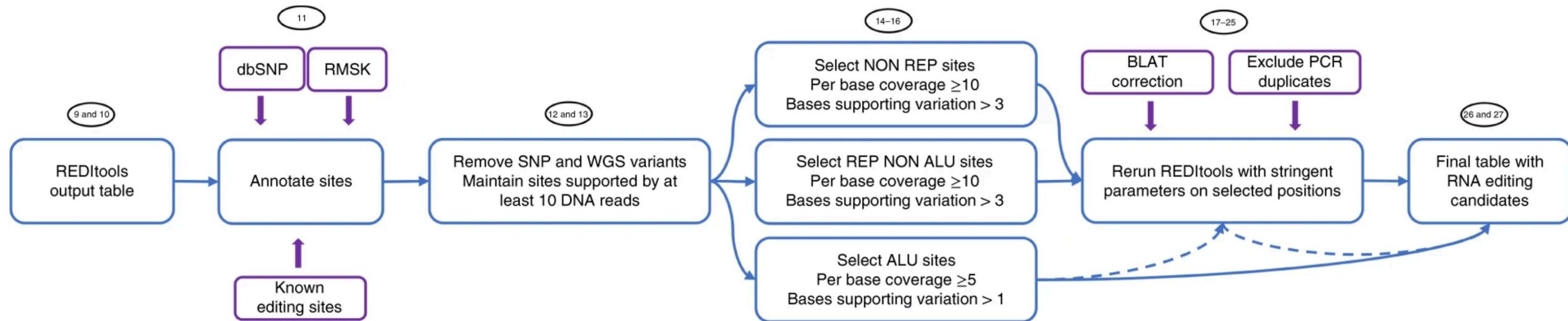
Zhou H, *Nat Meth* 2019

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
- Incarnato D, *NAR* 2016

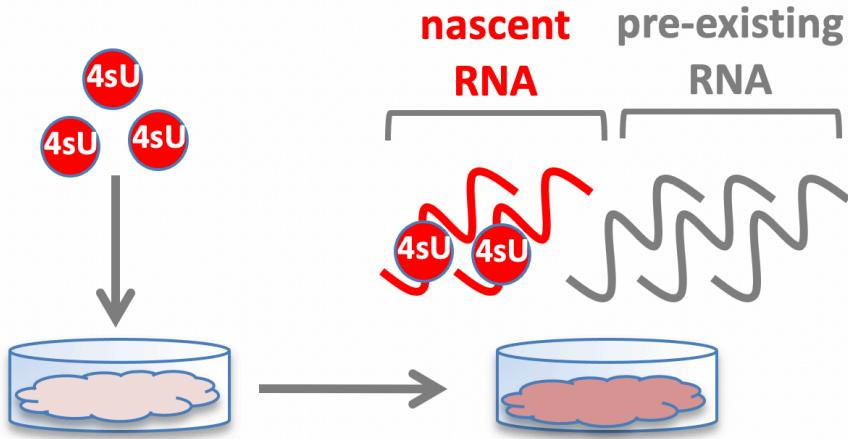
RNA editing



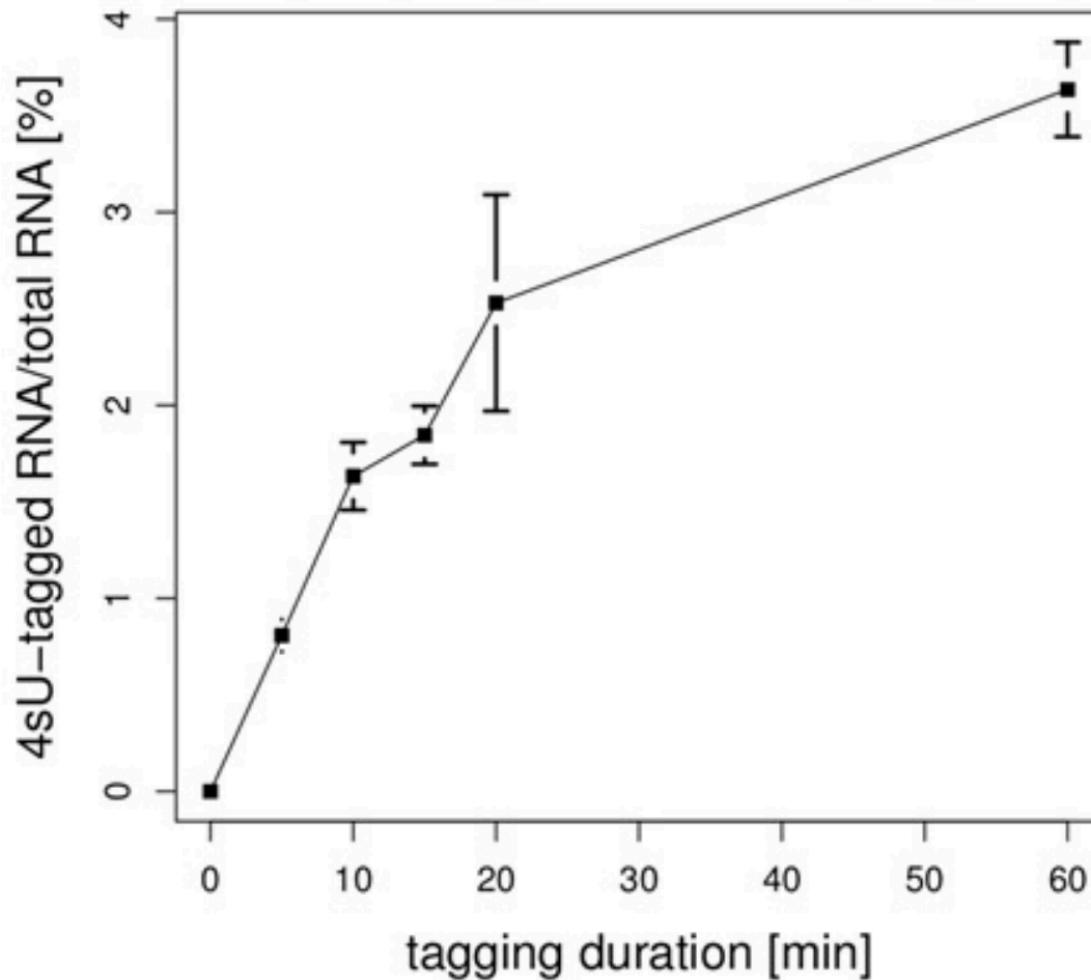
- Conversion of adenosine to inosine (A-to-I)
- It can be identified in RNA-seq data
- REDItools is a suite of tools for its detection
- Discrimination from abundant ALU events is critical

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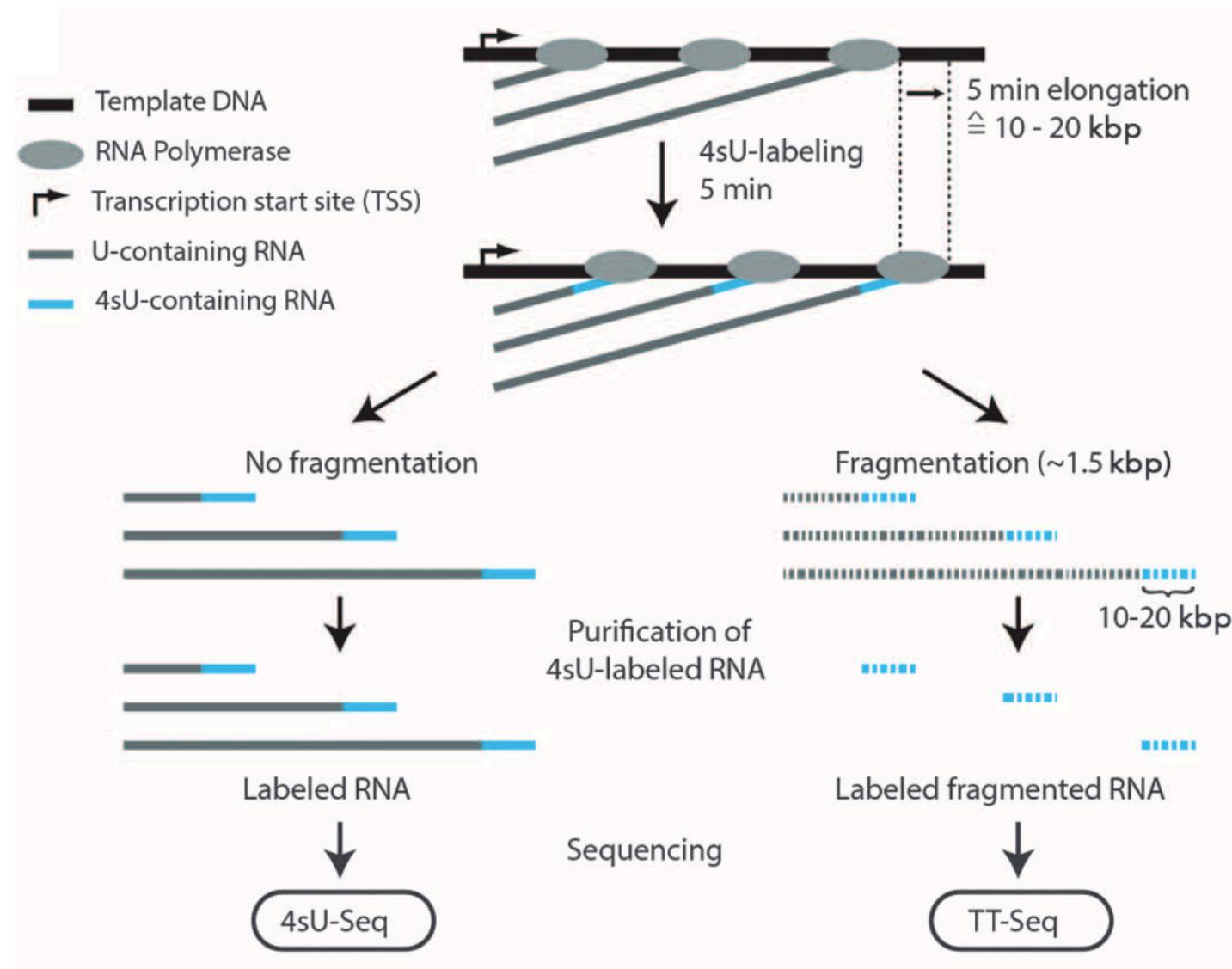
4sU-seq



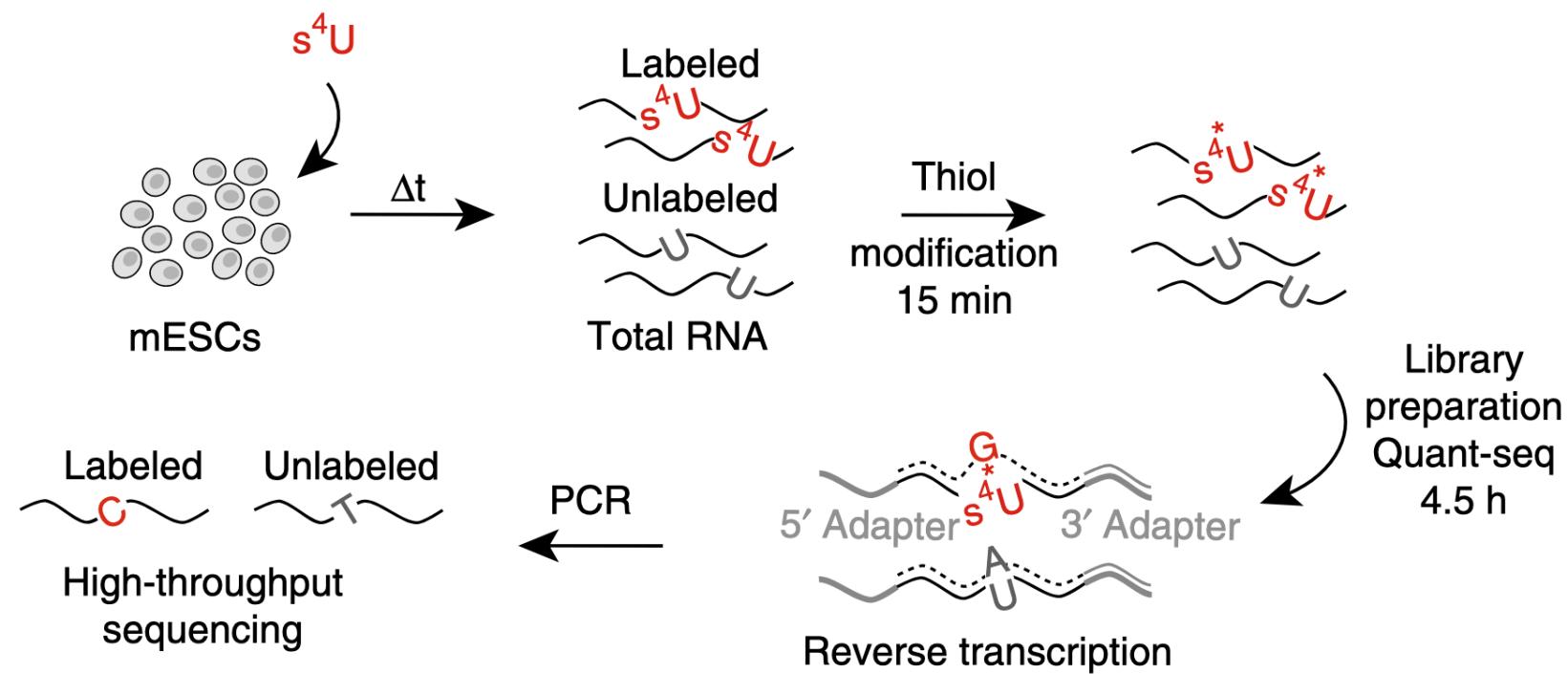
- 4sU gets incorporated in nascent RNA
- 4sU does not interfere with cell metabolism
- Thiol-specific biotinylation leads into tagged (newly transcribed) and untagged (preexisting) RNA
- Biotynilated RNA can be purified using streptavidin-coated magnetic beads



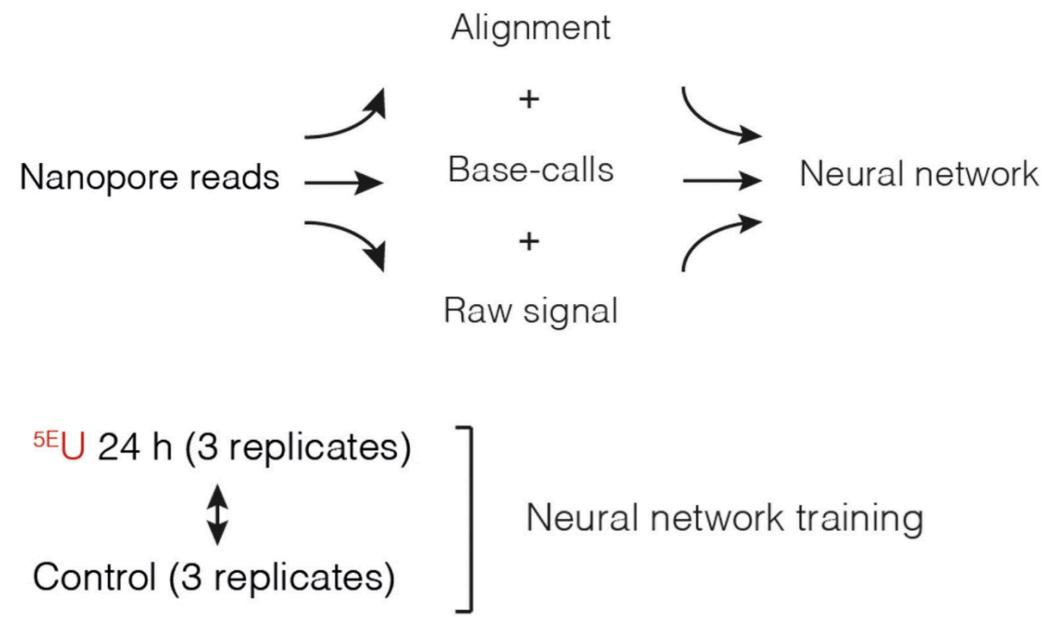
TT-seq



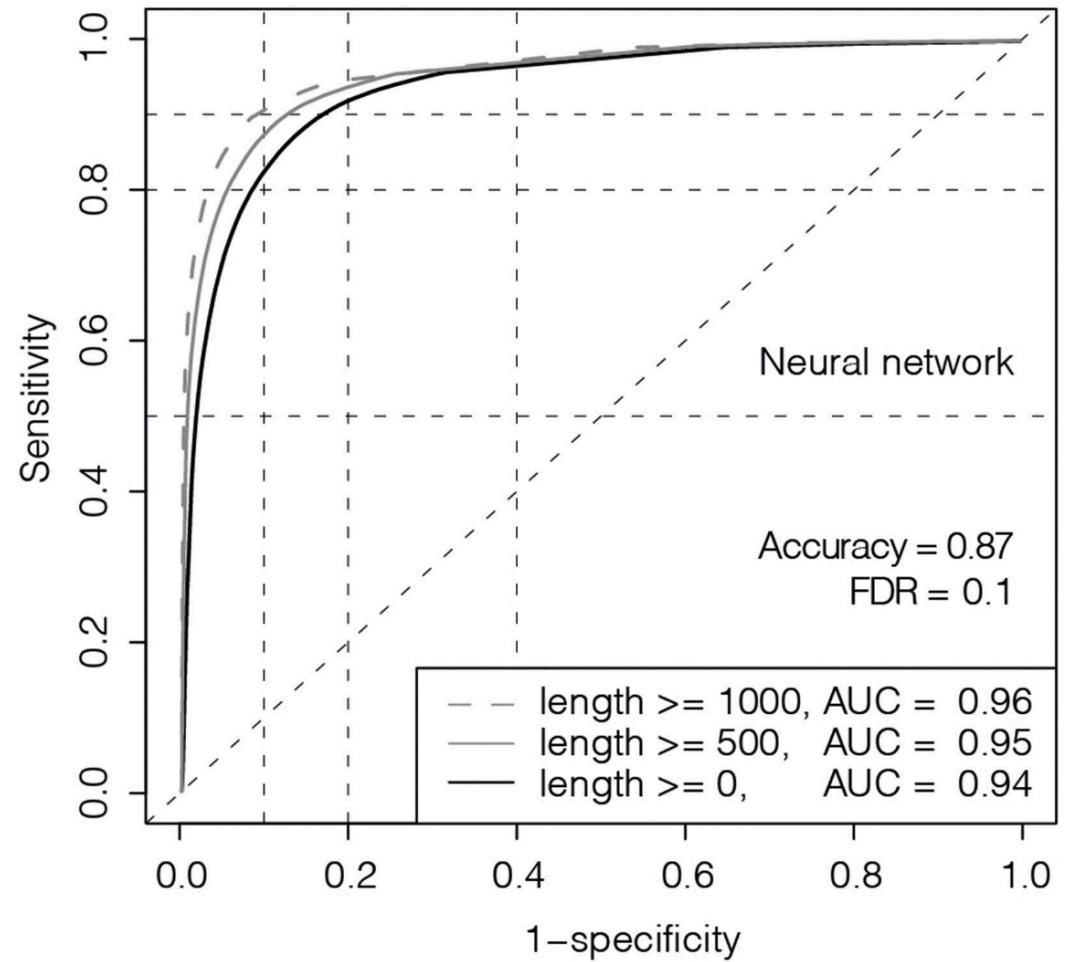
SLAM-seq



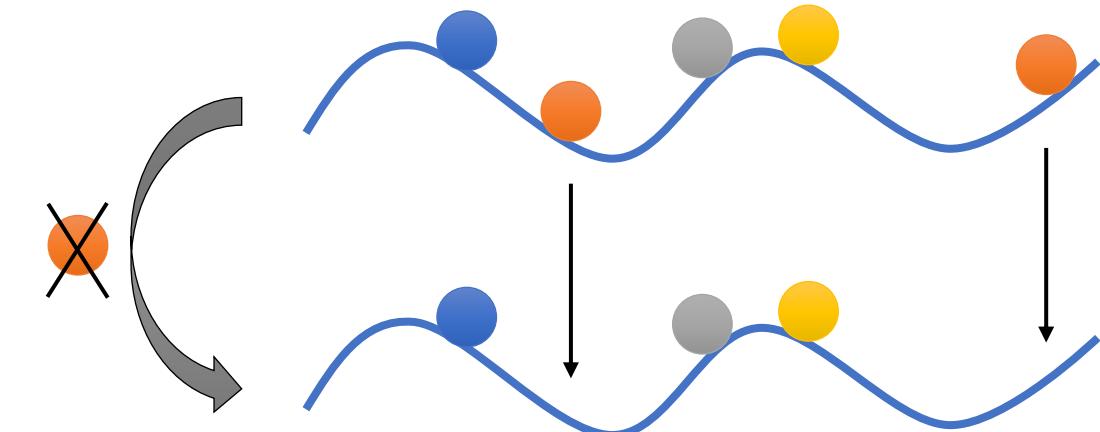
- Thiol-assisted conversion of 4sU incorporated in nascent RNA
- Allows detecting nascent RNA *in silico*
- Does not require physical separation of 4sU+ RNA
- Not fully compatible with short pulses
- Medium / low sensitivity



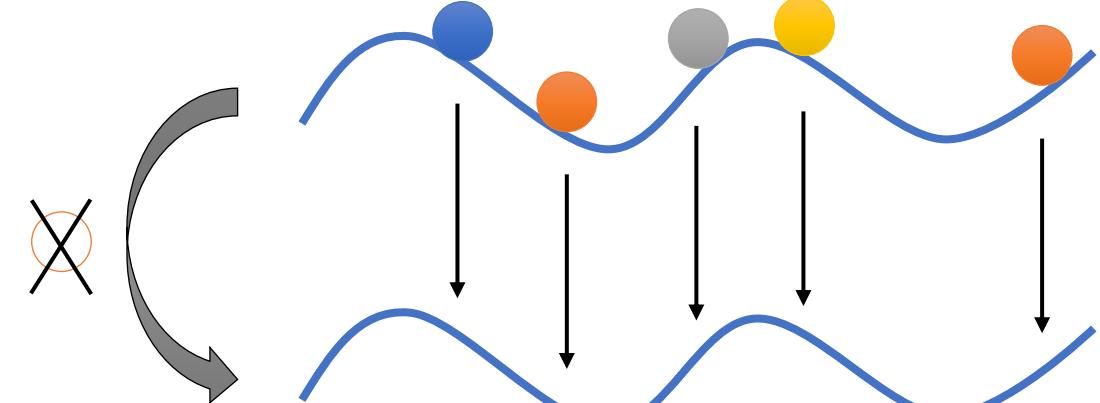
- Detection of 5EU+ reads with Nanopore dRNA-seq
- Does not require chemical treatments
- Does not require physical separation of labelled RNA
- Allows quantifying synthesis and degradation rates



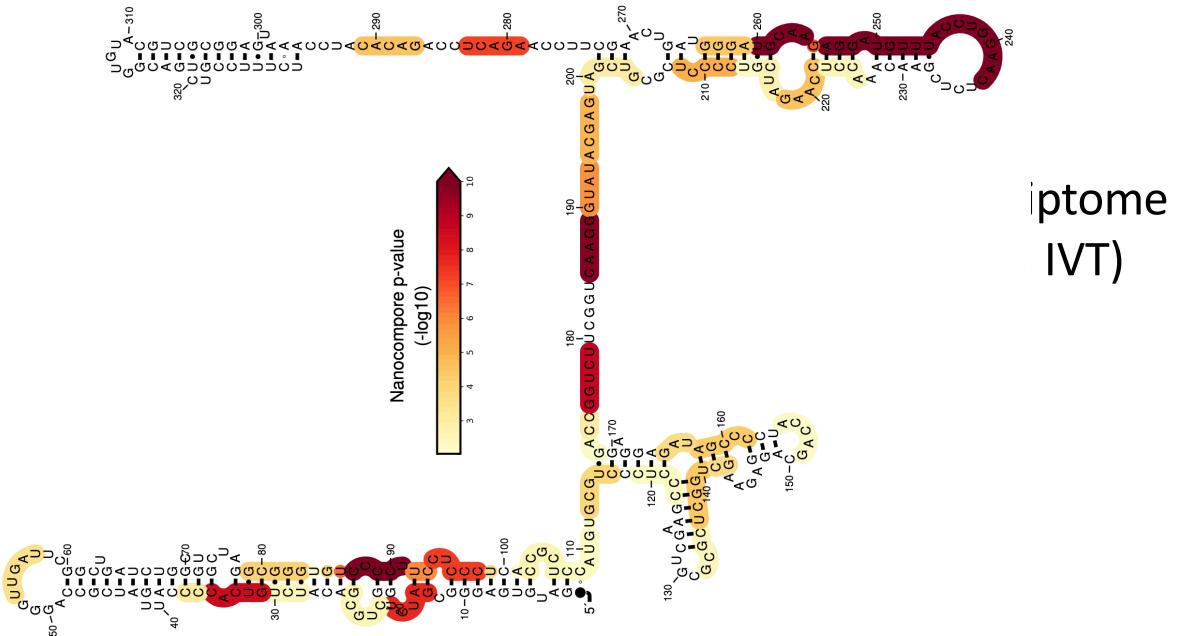
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 - Single cell and molecule – scDART-seq, Nanopore native RNA-seq
- Other mods – m5C, Ψ , m1A, Nm, RNA editing
- Nascent RNA – 4sU-seq, TT-seq, SLAM-seq, nano-ID
- **Combinations of mods** - IVT
- Computational methods – m6A absolute, Differential, Nanopore
- Benchmarking of methods – Short and long reads



Writer knockout baseline



RNA->cDNA -> IVT baseline



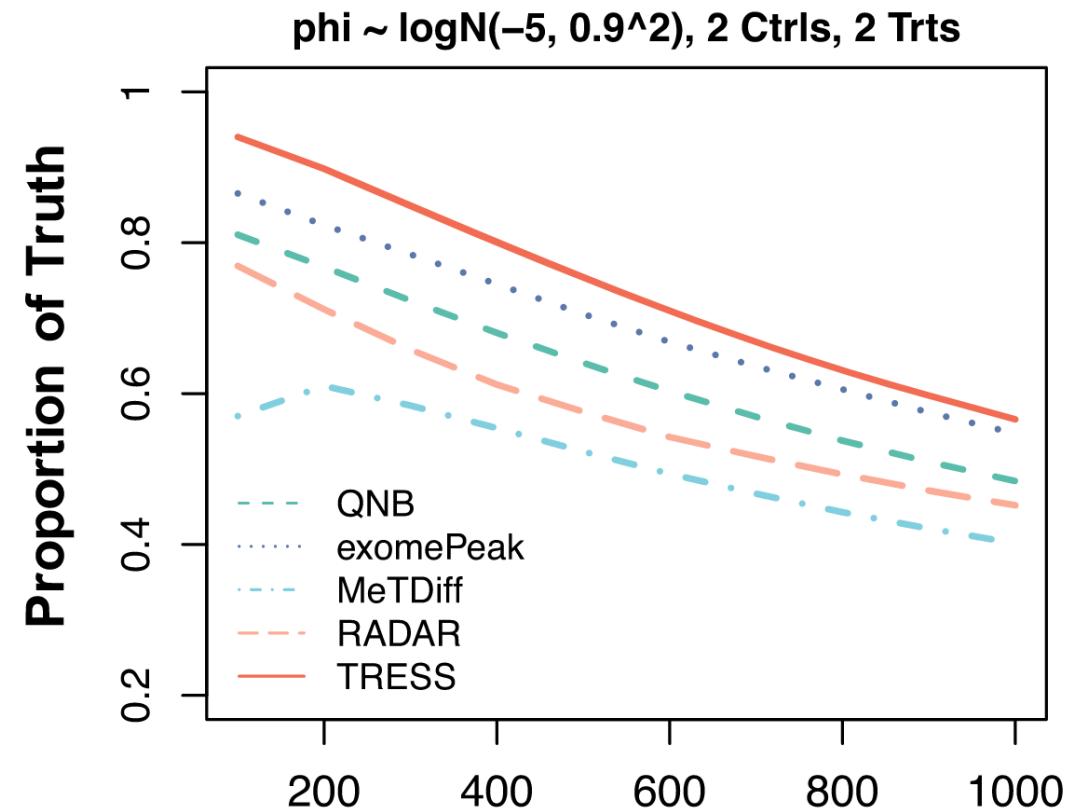
- IVT represents the ideal baseline for the comprehensive analysis of all the marks under ONT radar
- Unbiased mapping of epitranscriptional changes across conditions
- Allows testing the “epitranscriptional code” hypothesis
- Can direct further studies for specific marks (e.g. based on sequence motifs)

- Background on RNA modifications
- Methods to profile m6A
 - Bulk levels - Dot-blot, ELISA/colorimetric, MassSpec
 - Genome-wide, Ab-based - MeRIP-seq, miCLIP, m6A-LAIC-seq, m6A-seq2
 - Genome-wide, not Ab-based - MAZTER-seq, m6A-sac-seq, GLORI, DART-seq
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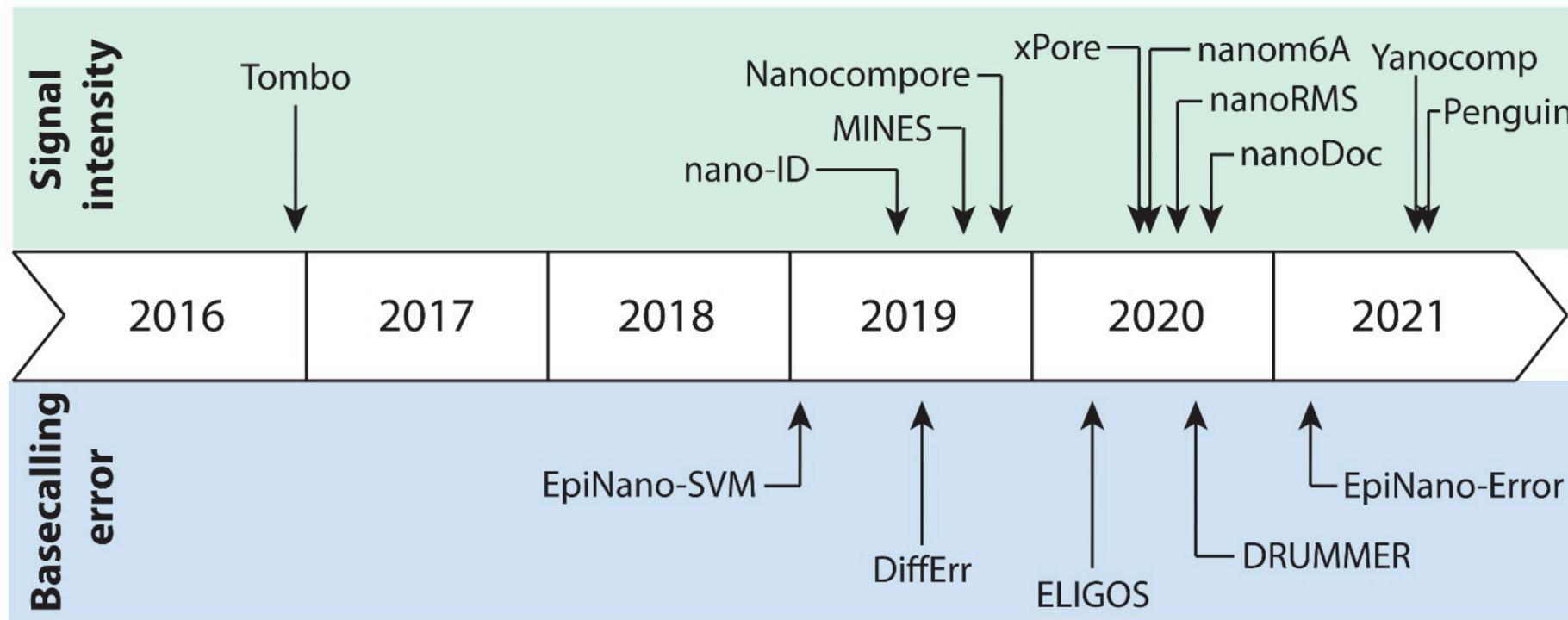
TRESS: Absolute and differential m6A calling (with short reads sequencing)

- Detection of differentially methylated regions from MeRIP-seq data
- count data modelled by a hierarchical negative binomial model
- exploits replicates
- Flexible statistical inference covering various experimental designs
- Incorporate TRES for absolute m6A calling
- available as R/Bioconductor package

Guo Z, *Bioinf* 2022



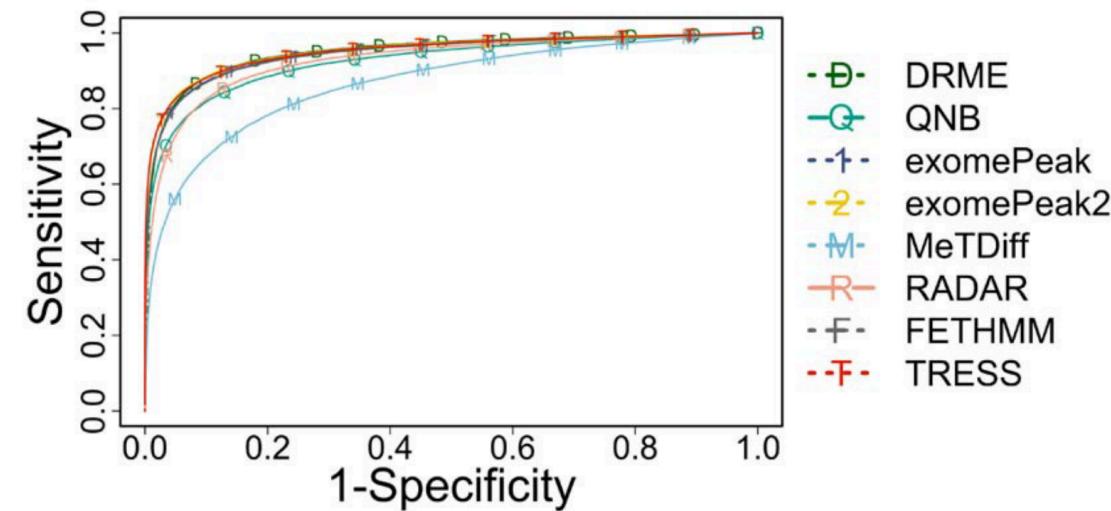
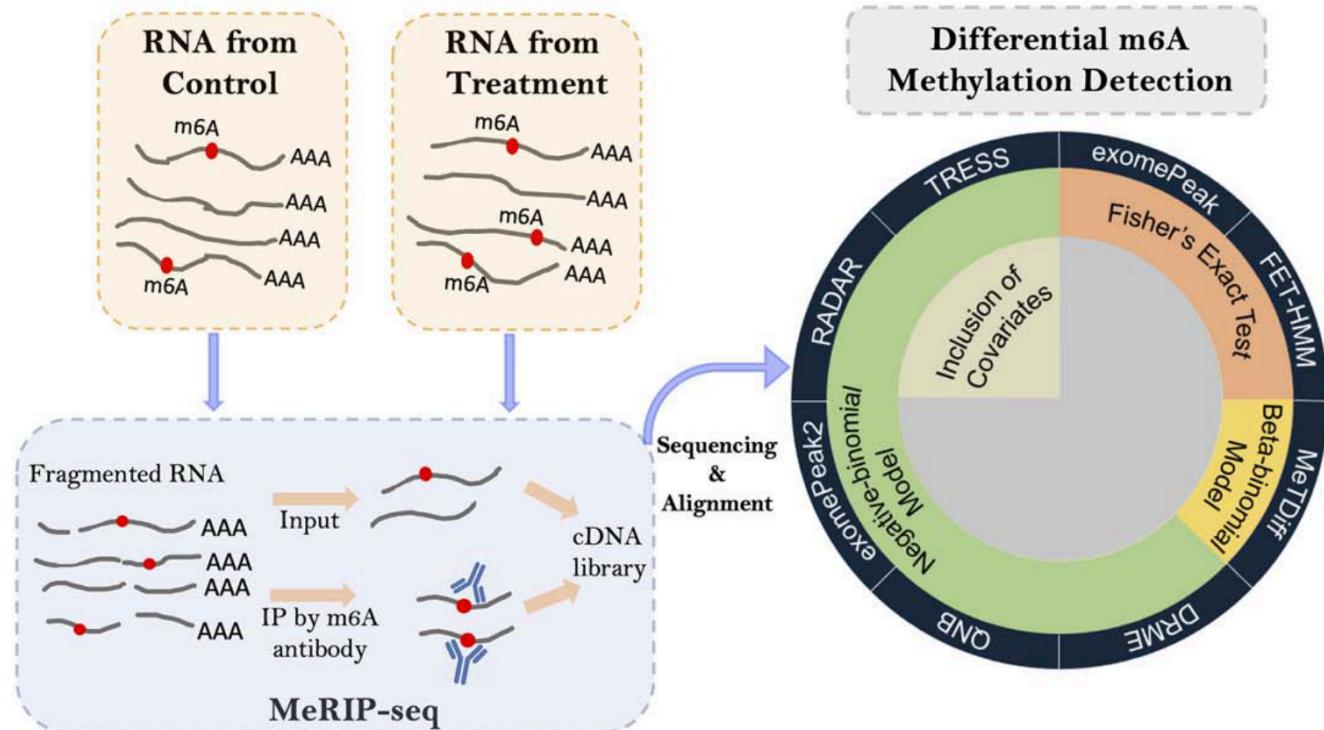
m6A calling with Nanopore dRNA-seq data



15 methods for RNA mods detection on Nanopore dRNA-seq reviewed

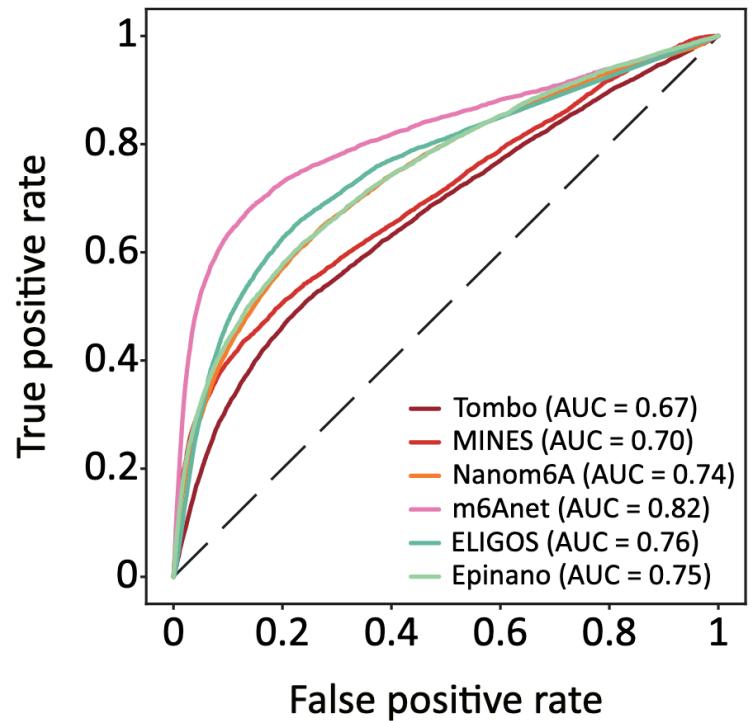
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Benchmarking – short reads

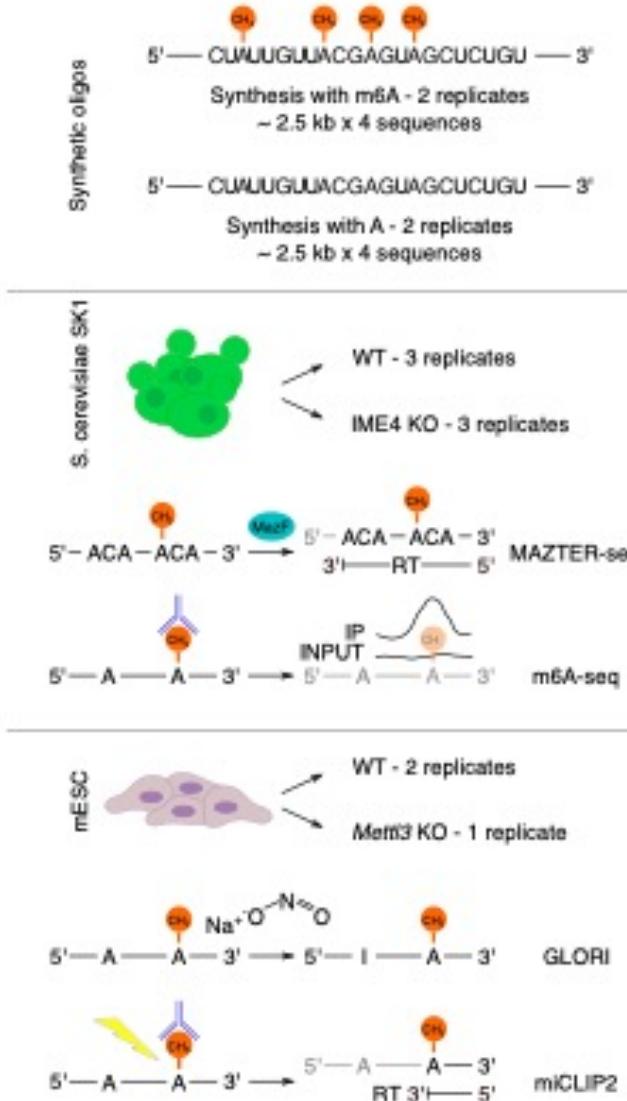


Duan D, *Brief Bioinf* 2023

Benchmarking – long reads / single molecule



Zhong ZD, *Nat Commun* 2023



Pelizzola and Nicassio Labs, in preparation