Mapping of RNA modifications by direct Nanopore sequencing and JACUSA2

Amina Lemsara[1,2], Christoph Dieterich*[1,2,3], and Isabel Naarmann-de Vries[1,2,3]

[1]Klaus Tschira Institute for Integrative Computational Cardiology, University Heidelberg, 69120 Heidelberg, Germany
[2]Department of Internal Medicine III (Cardiology, Angiology, and Pneumology), University Hospital Heidelberg, 69120 Heidelberg, Germany
[3]German Centre for Cardiovascular Research (DZHK)-Partner Site Heidelberg/Mannheim, 69120 Heidelberg, Germany

Abstract

RNA modifications exist in all kingdom of life. Several different types of base or ribose modifications are now summarized under the term the "epitranscriptome". With the advent of high-throughput sequencing technologies much progress has been made in understanding RNA modification biology and how these modifications can influence many aspects of RNA life. The most widespread internal modification on mRNA is m6A, which has been implicated in physiological processes as well as disease pathogenesis. Here, we provide a workflow for the mapping of m6A sites using Nanopore direct RNA sequencing data. Our strategy employs pairwise comparison of base calling error profiles with JACUSA2. We outline a general strategy for RNA modification detection on mRNA and describe two specific use cases on m6A detection in detail. **Use case 1:** a sample of interest with modifications (e.g. "wild type" sample) is compared to a sample lacking a specific modification type (e.g. "knock out" sample, here *METTL3*-KO) or **Use case 2:** a sample of interest with modifications is compared to a sample lacking all modifications (e.g. *in vitro* transcribed cDNA). We provide a detailed protocol on experimental and computational aspects. Extensive online material provides a snakemake pipeline to identify m6A positions in mRNA and to validate the results against a miCLIP-derived m6A reference set. The general strategy is flexible and can be easily adapted by users in different application scenarios.

*Correspondence to: christoph.dieterich@uni-heidelberg.de

INTRODUCTION

Chemical modifications on DNA and histones, also known as epigenetics marks, strongly impact gene expression during cell differentiation and in several other biological programs. In the 1970s, it was recognized that RNA is also subjected to extensive covalent modification, and studies in the late 1980s revealed the widespread deamination of bases (termed RNA editing), which can lead to recoding if it occurs within coding sequences. Impressive development in the RNA modification field occurred during the past eight years, with the discovery of an extensive layer of base modifications in mRNAs. These can influence gene expression and have been already shown to be involved in primary cellular programs such as stem cell differentiation, response to stress, and the circadian clock. The study of RNA modifications and their effects is now referred to as epitranscriptomics, and it reveals striking similarities to what is known for epigenomics. To date thirteen distinct modifications have been identified on mRNA transcripts [Anreiter et al., 2021]. These modifications are catalyzed by a variety of dedicated enzymes and can be divided into two classes: modifications of cap-adjacent nucleotides and internal modifications.

In contrast to the m7G cap, the impact of internal modifications on gene regulation has been less studied apart from RNA editing, which is mediated by RNA deaminases (e.g. the ADAR family). The most widespread internal mRNA modification is N6-methyladenosine (m6A). By modulating the processing of mRNA, m6A can regulate a wide range of physiological processes and its alteration has been linked to several diseases Roignant and Soller [2017], Zaccara et al. [2019], Shi et al. [2019]. The modification is catalyzed co-transcriptionally by a Mega-Dalton methyltransferase complex, which includes the heterodimer METTL3-METTL14 and other associated subunits Garcias Morales and Reyes [2021]. This modification is reversible since two proteins of the AlkB-family of demethylases can remove m6A from mRNA transcripts [Jia et al., 2011, Zheng et al., 2013]. In mammals, m6A preferentially localizes within long internal exons and at the beginning of terminal exons at so-called DRACH motif (D = A/G/U, R = A/G, H = A/C/U) sites [Dominissini et al., 2012, Meyer et al., 2012, Ke et al., 2015]. Once deposited, m6A is recognized by several reader proteins that can affect the fate of mRNA transcripts in nearly every step of the mRNA life cycle, including alternative splicing [Adhikari et al., 2016, Roundtree et al., 2017], mRNA translation [Wang et al., 2015] and decay [Wang et al., 2014, Du et al., 2016, Roundtree et al., 2017]. The best-described readers are the YTH domain family of proteins that decode the signal and mediate m6A functions. By affecting RNA structure, m6A can also indirectly influence the association of additional RNA-binding proteins (RBPs) and the assembly of larger messenger ribonucleoprotein particles (mRNPs) [Patil et al., 2018].

Several approaches have been presented to map RNA modifications on RNA. Herein, we focus on mRNA modification site detection in general and on m6A in particular where antibody-based protocols (miCLIP), methylation-sensitive restriction enzyme assays (MazF) or transgenic approaches (TRIBE, DART) have been presented to map m6A sites. All of the aforementioned approaches rely on high-throughput short read sequencing on the Illumina platform. This typically involves cDNA synthesis by reverse transcription and PCR-based library amplification. One recent addition to the toolbox of RNA modification mapping is direct RNA single molecule long read sequencing on the Oxford Nanopore Technologies platform (dRNA-seq). While our software is able to deal with Illumina and Nanopore-based approaches, the latter is the principal topic of this methods article.

MATERIALS

ONT direct RNA sequencing

This section summarizes all necessary consumables for direct RNA sequencing of poly-adenylated RNA (i.e. mRNA) on the MinION or similar device.

1. 500 ng polyA$^+$ RNA isolated from total RNA e.g. with Oligotex mRNA kit (Qiagen) or Dynabeads oligo dT$_{25}$ beads (Thermo Fisher Scientific) or *in vitro* transcriptome sample. Store RNA at -80 °C and the mRNA purification kit as recommended by the manufacturer.
2. Nuclease-free water. Store at room temperature.
3. Direct RNA-sequencing kit (SQK-RNA002, Oxford Nanopore Technologies). Store at -20 °C.
4. NEBNext Quick Ligation Reaction Buffer (New England Biolabs). Store at -20 °C.
5. T4 DNA Ligase (New England Biolabs). Store at -20 °C.
6. dNTP Mix (10 mM each). Store at -20 °C.
7. SuperScript IV Reverse Transcriptase (Thermo Fisher Scientific). Store at -20 °C.
8. Agencourt RNAClean XP beads (Beckman Coulter). Store at 4 °C.
9. 70 % ethanol, freshly prepared.
10. Qubit dsDNA HS assay kit and Qubit Fluorometer (Thermo Fisher Scientific).

11. Flow cell priming kit (EXP-FLP002, Oxford Nanopore Technologies). Store at -20 °C.

12. Thermocycler.

13. Gentle rotator mixer.

14. Magnetic stand for 1.5 ml tubes.

15. 1.5 ml DNA LoBind tubes (Eppendorf), 0.2 ml PCR tubes.

16. MinION or GridION sequencing device and MinION R9.4.1 Flow cells (FLO-MIN106D, Oxford Nanopore Technologies). Store Flow cells at 4 °C.

Preparation of an *in vitro* transcriptome sample

1. 100 ng polyA$^+$ RNA isolated from total RNA e.g. with Oligotex mRNA kit (Qiagen) or Dynabeads oligo dT_{25} beads (Thermo Fisher Scientific). Store RNA at -80 °C and the mRNA purification kit as recommended by the manufacturer

2. 10 μM oligo(dT)-VN RT primer.
   TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN. Store at -20 °C.

3. 20 μM template switching oligo (TSO). ACTCTAATACGACTCAC-TATAGGGAGAGGGCrGrG+G. Store at -20 °C.

4. 10 μM T7 extension primer. GCTCTAATACGACTCACTATAGG. Store at -20 °C.

5. Nuclease-free water. Store at room temperature.

6. dNTP Mix (10 mM each). Store at -20 °C.

7. Template Switching RT Enzyme Mix (New England Biolabs). Store at -20 °C.

8. Q5 Hot Start High-Fidelity 2X Master Mix (New England Biolabs). Store at -20 °C.

9. RNase H (5,000 U/ml) (New England Biolabs). Store at -20 °C.

10. NucleoSpin Gel and PCR Clean-up, Mini kit for gel extraction and PCR clean up (Macherey-Nagel) or equivalent. Store at room temperature.

11. MEGAscript T7 transcription kit (Thermo Fisher Scientific). Store at -20 °C.

12. RNA Clean & Concentrator-25 kit (Zymo Research). Store at room temperature.

13. Thermocycler.

14. Table top centrifuge for 1.5 ml tubes.

15. Nanodrop spectrophotometer or equivalent.

16. 0.2 ml PCR tubes, 1.5 ml DNA LoBind tubes (Eppendorf).

Hardware requirements

All analyses have been performed/tested on two alternative hardware systems: a standard Linux desktop computer or an Apple iMac (Retina 5K, ultimo 2014). The workflow requires a multi-core processor system with minimal main memory of 16GB RAM and several GBs of free disk space (depending on data set size).

Software dependencies and installation

Our analysis workflow has few requirements, which are detailed in Table 1. Specifically, to execute our workflow, the following prerequisites are necessary: a BASH shell, a JAVA runtime environment, a working PERL and R installation. Additional i.e. non-standard software to process and map Nanopore reads (bedtools, samtools and Minimap2) are obligatory. Table 2 lists some additional R packages, which are required to run the R code. Detailed instructions on how to setup are found under `https://github.com/dieterich-lab/MiMB_JACUSA2_chapter`.

METHODS

Our workflow is based on the pairwise comparison of samples with different modification status (Figure 1). The sample of interest (yellow) may be compared to different samples lacking certain modifications. If available, the wild type (WT) sample can be compared to a knock out (KO) sample lacking specific enzymatic activities (green), as outlined in Use Case 1. Alternatively, a sample lacking all modifications may be used for comparison (blue). This may be either a simulated sample (i.e. with NanoSim) or an *in vitro* transcribed sample derived from cDNA. Such an analysis is detailed in Use Case 2. In any setting, JACUSA2 calculates scores for the Mismatch, Insertion and Deletion rates of the pairwise comparisons as outlined above (Figure 1, right).

One feature of Nanopore sequencing is to read sequences as 5-mers, as always five nucleotides are occupied by the pore protein (Figure 2). Because

of this, a m6A modification may affect basecalling not only if the modified nucleotide is in the central position, but also at neighboring positions (-2 to +2). To account for this, JACUSA2 scores for Deletion, Mismatch and Insertion are calculated for the entire 5-mer context. Depending on the modification-specific signature, a Feature set can be selected to calculate the final JACUSA2 score (Figure 2).

Our workflow can be divided into a wet-lab part (Figure 3A) and a computational part (Figure 3B). Starting from total cellular RNA, polyA$^+$ RNA is isolated and subjected to Nanopore direct RNA-sequencing. Guppy basecalling can be done as well as live basecalling during sequencing on the respective FAST5 files, which results in FASTQ output files (Figure 3A). FASTQ files are aligned to a reference sequence with Minimap2. SAMtools is used to generate BAM files as input for JACUSA2 analysis, which yields candidate m6A sites with the presented workflow in this chapter (Figure 3B).

Nanopore direct RNA sequencing

1. Adjust 500 ng polyA$^+$ RNA to a total volume of 9 μl with nuclease-free water. Complete RT adapter ligation reaction (in 0.2 ml PCR tube) with 3 μl NEBNext Quick Ligation Reaction Buffer, 0.5 μl RNA CS (RCS, from SQK-RNA002), 1 μl RT-Adapter (RTA, from SQK-RNA002) and 1.5 μl T4 DNA Ligase. Incubate 10 min at room temperature.

2. Prepare reverse transcription master mix on ice during ligation: 9 μl nuclease-free water, 2 μl 10 mM dNTPs, 8 μl 5x SuperScript IV first strand buffer, 4 μl 0.1 mM DTT.

3. Add the reverse transcription master mix to the ligation reaction and mix by pipetting. Add 2 μl SuperScript IV reverse transcriptase and mix by pipetting. Incubate in a thermocycler with the following protocol: 50 min at 50 °C, 10 min at 70 °C, cool down to 4 °C.

4. Let the Agencourt RNAClean XP beads come to room temperature during reverse transcription. Carefully resuspend beads before use. Transfer reaction to a 1.5 ml DNA LoBind tube and mix with 72 μl Agencourt RNAClean XP beads. Incubate 5 min at room temperature on a gentle rotator mixer.

5. Collect beads on a magnetic stand and remove supernatant. Wash pelleted beads two times (30 sec) with 200 μl freshly prepared 70 % ethanol. Remove supernatant. Spin sample down and place on magnet again. Remove any residual ethanol.

6. Resuspend beads in 20 μl nuclease-free water by gentle flicking and incubate 5 min at room temperature on a gentle rotator mixer. Collect beads on a magnetic stand and transfer 20 μl eluate in a fresh 1.5 ml DNA LoBind tube.

7. For ligation of the RMX adapter, add the following to 20 μl eluate: 8 μl NEBNext Quick Ligation Reaction Buffer, 6 μl RMX (from SQK-RNA002), 3 μl nuclease-free water, 3 μl T4 DNA Ligase. Mix by pipetting and incubate 10 min at room temperature.

8. Add 40 μl carefully resuspended Agencourt RNAClean XP beads to the reaction and mix by pipetting. Incubate 5 min at room temperature on a gentle rotator mixer.

9. Collect beads on a magnetic stand and remove supernatant. Wash pelleted beads two times with 150 μl wash buffer (WSB, from SQK-RNA002). Resuspend beads by flicking, spin down and return to magnetic stand. Remove supernatant from pelleted beads.

10. Resuspend beads in 21 μl elution buffer (EB, from SQK-RNA002) by gentle flicking and incubate 5 min at room temperature on a gentle rotator mixer. Pellet beads on a magnetic stand and transfer 21 μl eluate in a fresh 1.5 ml DNA LoBind tube.

11. Quantify 1 μl of the library on a Qubit fluorometer with the Qubit dsDNA HS kit according to the manufacturerers protocol. Concentration should be usually in the range of 5 - 10 ng/μl.

12. Insert MinION R9.4.1 Flow cell in the MinION or GridION sequencing device and perform Flow cell check in the MinKNOW software. For successful sequencing of mammalian polyA$^+$ RNA at least 1,000 available pores are recommended.

13. Prepare Priming Mix by adding 30 μl flush tether (FLT, from EXP-FLP002) to a vial of flush buffer (FB, from EXP-FLP002) and mix by pipetting. Open priming port. Remove air bubble from priming port by inserting the tip of a P1000 pipette into the priming port and slowly dialing up, until a small volume of storage buffer enters the pipette tip. Load 800 μl Priming Mix via the priming port and carefully avoid introduction of air bubbles. Close the priming port and wait for 5 min.

14. Mix 20 μl library with 17.5 μl nuclease-free water and 37.5 μl RNA running buffer (RRB, from SQK-RNA002) and mix by pipetting. Open the priming port and the sample port. Load 200 μl Priming Mix via the priming port. Mix library by pipetting just before loading and load dropwise via the sample port. Carefully avoid introduction of air bubbles. Close the sample port and the priming port.

15. Start sequencing for 48 to 72 h in the MinKNOW software. Choose direct RNA-sequencing kit and high-accuracy basecalling as parameters.

Preparation of an *in vitro* transcriptome sample

The *in vitro* transcriptome sample is prepared based on a protocol published by Zhang *et al.* Zhang et al. [2021] with some modifications.

1. Adjust 100 ng polyA$^+$ RNA to a total volume of 6 μl with nuclease-free water. Add 1 μl each of 10 μM oligo(dT)-VN RT primer and 10 mM dNTPs. Mix by pipetting and incubate in a thermocycler: 5 min at 75 °C, 2 min at 42 °C, cool to 4 °C.

2. Assemble 2.5 μl 4x template switching RT buffer, 0.5 μl 20 μM TSO, 1 μl 10x template switching RT enzyme mix and mix by pipetting. Combine with 6 μl RNA and incubate in a thermocycler: 90 min at 42 °C, 10 min at 68 °C, cool to 4 °C.

3. For Second strand synthesis add to First strand synthesis reaction: 50 μl Q5 Hot Start High-Fidelity 2X Master Mix, 5 μl RNase H, 2 μl 10 μM T7 extension primer, 33 μl nuclease-free water. Mix by pipetting and incubate in a thermocycler: 15 min at 37 °C, 1 min at 95 °C, 10 min at 65 °C, cool to 4 °C.

4. Purify double stranded cDNA with NucleoSpin Gel and PCR Clean-up kit according to the manufacturerers protocol and elute in 20 μl elution buffer. Determine concentration on a Nanodrop spectrophotometer. cDNA may be stored at -20 °C.

5. Combine 8 μl cDNA for *in vitro* transcription with 2 μl each of ATP, GTP, CTP, UTP, 10x reaction buffer and enzyme mix from the MEGAscript T7 transcription kit. Incubate 3 h at 37 °C.

6. Digest template DNA by addition of 1 μl Turbo DNase. Mix by pipetting and incubate 15 min at 37 °C.

7. Adjust reaction volume to 100 μl with nuclease-free water and clean up with RNA Clean & Concentrator-25 kit according to the manufacturers protocol, using two volumes of adjusted RNA binding buffer (1:1 RNA binding buffer : ethanol). Elute RNA in 25 μl nuclease-free water. Determine RNA concentration on a Nanodrop spectrophotometer. Store at -80 °C.

Nanopore read processing

1. Base call the ionic current signal stored in FAST5 files using Guppy. For the IVT sample, we applied real-time base calling with the MinKNOW-embedded Guppy basecaller. Otherwise, Guppy basecaller software can be used. In this case, the basecaller requires the path to FAST5 files, the output folder, and the config file or the flowcell/kit combination. The output are FASTQ files that can be compressed using the option "–compress_fastq".

   ```
   $ guppy_basecaller --compress_fastq -i path_to_fast5 -s path_to_output
   -c config_file.cfg --cpu_threads_per_caller 14 --num_callers
   1
   ```

   Set the number of threads "cpu_threads_per_caller" and the number of parallel basecallers "num_caller" according to your resources. Additional details can be found in Gup [2019].

2. Align reads to the transcriptome using Minimap2 software. The output is a SAM file that has to be converted to a compressed form as BAM file using SAMtools command. The alignment requires a reference sequence. Here, we used GRCh38 Ensembl annotation and FASTA file release version 96. To reduce the indexing time of the human genome, save the index with the option "-d" before the mapping and use the index instead of the reference file in the minimap2 command line.

   ```
   $ minimap2 -d reference.mmi reference.fa
   ```

   To enable spliced alignments, use the setting "-ax splice –junc-bed annotation.bed –junc-bonus" where "–junc-bonus" allows to tune the bonus score and the BED file "–junc-bed annotation.bed" provides the splice junctions. The BED file can be generated using the following command:

   ```
   $paftools.js gff2bed annotation.gtf > annotation.bed
   ```

   Use "-ub" to allow alignment to both strands or '-uf' to force the alignment to only forward strand. For Direct RNA Sequencing, it is recommended to set a small k-mer size "-k [=14]" to enhance sensitivity. We recommend outputting primary alignments "–secondary=no". Use the parameter '–MD' to add the reference sequence information to the alignment; this is recommended for the downstream analysis. Customize the number of threads "-t" according to your resources. Check Minimap2 manual for more details [Min].

```
$ minimap2 -t 5 --MD -ax splice --junc-bonus 1 -k14 --secondary=no
--junc-bed final_annotation_96.bed -ub reference.mmi Reads.fastq.gz
|samtools view -bS > mapping.bam
```

3. Map RNA modifications using JACUSA2 pipeline. JACUSA2 [Piechotta et al., 2021] rapidly detects RNA modifications based on a comparative strategy where the mapping features (mismatch, insertion and deletion) of a sample of interest are compared to a reference sequence (call-1) or against a sample without RNA modifications, e.g. a knock-out of an RNA modifying enzyme or an IVT (call-2). Moreover, it allows the integration of information from replicate experiments. The output of JACUSA2 variant calling is a set of scores reflecting the read signatures involving mismatch, insertion and deletion. The analysis of read signature can be used for RNA modification detection. We integrate JACUSA2, in particular call-2 method, with the downstream analysis in one pipeline using the Python-based workflow management system Snakemake [Köster and Rahmann, 2012]. The Snakemake pipeline involves rules for the variant calling using JACUSA2 call-2, detection of RNA modification patterns, prediction of new modified sites and other intermediate rules as shown in Figure 4. The input of the pipeline are BAM files from paired conditions with different replicates. BAM files need to be sorted and may be subjected to many filters before being used by JACUSA2 call2 rule. Here, we suggest to filter out secondary and poor alignments. The output of JACUSA2 call2 is preprocessed (get_features) and subjected to a learning process to extract and visualize modification patterns (resp. get_pattern, visualize_pattern) and make predictions (predict_modification). "split_trani_test" rule allows splitting input data into a training set and a test set. To use our snakemake-based JACUSA2 pipeline a set of parameters should be defined in the "config.yaml" file; mainly: the label of the analysis 'label', the input bam files under 'data', the reference sequence 'reference', a file containing size of chromosomes 'chr_size', JACUSA2 jar file 'jar', plus the path to inputs and outputs under 'path_inp' and 'path_out' fields respectively. Further details on how to use JACUSA2 pipeline is presented within the use cases in the next section. The pipeline could be executed on a high-performance-computing cluster (HPC) using the following command by specifying the number of cores to be used "–cores [=all]" and the rule name:

```
$ srun snakemake --cores all rule_name
```

Check Snakemake documentation for more details [sna].

Use Case 1: Comparison of wild-type and knock-out samples

The JACUSA2 workflow detects RNA modifications using direct RNA sequencing by comparing a modified sample to an unmodified control sample. Here, we used a published dataset of HEK293 cell lines to map m6A modification [Pratanwanich et al., 2021]. The benchmark is composed of samples sets two conditions: wild-type cells (WT, modified RNAs) and Mettl3 knock-out cells (KO, unmodified RNAs) in two replicates (2 and 3). The FASTQ files are mapped using Minimap2 as described in the previous section. The following analysis is validated against m6A sites consistently reported in three miCLIP-based studies Boulias et al. [2019], Koh et al. [2019], Körtel et al. [2021] (Figure 5).

Starting with the preprocessed mapped reads as inputs (BAM files), 'HEK293T-WT-rep2.bam' and 'HEK293T-WT-rep3.bam' represent the wild-type replicates and 'HEK293T-KO-rep2.bam' and 'HEK293T-KO-rep3.bam' the control replicates,

1. Identify read error profile: use "jacusa2_call2" rule to run JACUSA2 in pairwise condition mode (call-2). The method requires BAM files of the paired conditions and the corresponding library information "-P1" and "-P2". In addition to the mismatch score, add "-D" and "-I" to output the deletion and insertion scores. JACUSA2 allows filtering reads according to many parameters. Here, we consider all sites with base calling quality "-q [> 1]", mapping quality "-m [> 1] and read coverage "-c [> 4]". Furthermore, it provides a filter feature to improve sensitivity. Here, we consider filtering sites within homopolymer regions "-a [=Y]". The output (named here, "Cond1vsCond2Call2.out") consists of a read error profile where the format is a combination of BED6 with JACUSA2 call-2 specific columns and common info columns: info, filter, and ref. Check JACUSA2 manual for more details on JACUSA2 filter and output options [JAC, 2021]. The number of threads can be customized via the parameter "-p". All parameters related to the JACUSA2 method can be added under the field "jacusa_params" in the config file by setting the name of the parameter followed by the corresponding value [key: value]. Be aware to set all parameters before running the pipeline.

```
$ srun snakemake --cores all jacusa2_call2  $
```

2. Preprocess JACUSA2 output: from JACUSA2 call-2 output, we select all sites within 5-mer of a central nucleotide 'A' flanked by 2 random nucleotides (NNANN) and we filter out sites of the homo-polymer regions (JACUSA filter: Y). Then, we rebuild the tabular features such that the observations are only sites with a reference base 'A'. Each site is characterized by 15 features corresponding to the mismatch,

insertion and deletion scores for the observed site and its two flanking positions from both sides. The rule "get_features" performs the preprocessing step. Use the parameter 'region' with a file containing target 5-mers to limit the analysis to specific sites. For comparison reasons, we consider common sites between use cases 1 and 2 . The output is an R object "features/features.rds", representing the matrix of Sites×15 features.

Is this the reason why you chose to work on the three outputs together WT_IV,WT_KO, KO_IVT

```
$ srun snakemake --cores all get_features
```

3. Extract m6A modification pattern: given the matrix of Sites×Features, the next step is to learn a model representing the m6A modification pattern. To this end, the conventional non-negative matrix factorization (NMF) analysis is suggested [Lee and Seung, 1999]. Briefly, NMF factorizes a non-negative data matrix X (here: n sites and m features) into two non-negative matrices as $X \approx WH$, such that W is an $n \times k$ matrix containing basis vectors and H is an $k \times m$ matrix containing coefficient vectors. The coefficient vectors and their combination can be viewed as a pattern for m6A modification. The rank of factorization k is a critical parameter that affects the performance substantially. We suggest to select the rank k according to the method of Frigyesi and Höglund [2008] by looking at silhouette [Rousseeuw, 1987] and cophenetic correlation [Brunet et al., 2004] indices. Accordingly, the performance indices are computed for different choices of rank ($k < n, m$) and compared to the performance of a random permutation of the original data. Subsequently, the chosen rank corresponds to the value with the largest difference between slopes of the original and the randomized data. Here, the unsupervised pattern training is based on the consensus set of $1,905$ m6A sites reported in the three miCLIP-based studies mentioned earlier. Based on the silhouette and cophenetic correlation indices, we identified an optimal factorization rank of 6 (Figure 6A). We then analyzed the identified patterns. According to the membership indicator of each site in matrix W, more than 80% of m6A modification sites can be represented by five patterns (Patterns 1,2,3,4,6) (Figure 6B). Interestingly, the linear combination of these five patterns in Figure 6C highlights the importance of position 3 and eventually the implication of all scores.

in Figure 6C this is labeled sum

Using the JACUSA2 pipeline, run rule "get_pattern" to generate patterns and provide the set of modified sites as a ground truth under the field "modified_sites" in the config file. Here, the "miCLIP_union.bed" file contains the m6A sites from the three miCLIP-based studies. A miCLIP annotation, reflecting the consensus sites, is added to each site. A subset of modified sites can be used to generate patterns. Accordingly, the "internal_pattern" field should refer to the annotation

of selected sites from the "modified_sites" file. Plus, multiple combinations of patterns can be defined and appended to the field "combined_pattern" as new patterns. The corresponding outputs are under "patterns" folder.

```
$ srun snakemake --cores all get_pattern
```

The produced patterns and their combinations can be visualized using "visualize_pattern" rule. The corresponding outputs are under "pattern/viz" folder.

```
$ srun snakemake --cores all visualize_pattern
```

4. Predict m6A modifications: the additive linear combination of the coefficient vectors (patterns) with the 15 features can be used to predict m6A modification. We examine the ability of prediction on a subset of data of more than $1,52$ million sites with $17,021$ miCLIP m6A sites. We opt for the linear combination of the five most relevant patterns described in step 3. The empirical Cumulative Distribution Function (eCDF) of the inferred scores shows a significant difference between the different miCLIP m6A categories (miCLIP annotation) and the unmodified sites (Figure 6D). As the number of negative samples is much larger than the number of positive samples, we particularly recommend investigating the Positive Predictive Value (PPV) of the predictions. Here, Figure 6E shows a moderate PPV that increases with the cut-off.

   To perform the prediction based on the selected patterns using the JACUSA2 pipeline, run rule "predict_modification". The patterns can be generated from a subset of the input data according to the field "internal_pattern" or predefined patterns indicated in the "external_pattern" field. The output is a BED file containing the estimated scores as well as the corresponding eCDF and PPV plots. The corresponding outputs are located under a new folder called "prediction".

```
$ srun snakemake --cores all predict_modification
```

Use Case 2: Comparison of wild-type and IVT samples

An alternative way to detect RNA modifications is to compare a modified sample to an *in-vitro* transcribed (IVT) control sample. Therefore, we benchmark JACUSA2 on a sample set of two replicates (2 and 3) from wild-type HEK293 cell lines (modified sample) Pratanwanich et al. [2021] and a modification-free IVT sample from HEK293 cDNA (control sample) (see "Preparation of an *in vitro* transcriptome sample"). The analysis steps

are similar to case 1. We evaluate the analysis against miCLIP m6A sites (Figure 5).

1. Identify read error profile: we use JACUSA2 call-2 with the same parameters as the previously described case. The input BAM files (HEK293T-WT-rep2.bam, HEK293T-WT-rep3.bam) and (HEK293T-IVT-rep1.bam, HEK293T-IVT-rep2.bam)are associated to the wild-type and IVT replicate samples respectively.

   ```
   $ srun snakemake --cores all jacusa2_call2
   ```

2. Preprocess JACUSA2 output: we select all sites within the specific 5-mer (NNANN) and we consider the Y filter that excludes sites within homo-polymer regions. Then, we extract 5-mer features such that the selected sites are represented by the Mismatch, Deletion and Insertion scores for the observed site and its two flanking positions from both sides.

   ```
   $ srun snakemake --cores all get_features
   ```

3. Extract m6A modification pattern: using NMF factorization, we extract patterns from the $1,905$ sites reported as modified in the three miCLIP-based studies. Based on the silhouette and cophenetic correlation indices, we identified an optimal factorization rank of 6 (Figure 7A). We determined the predominant factors from matrix W. Accordingly, more than 80% of m6A modification sites can be represented by four patterns (Patterns: 1,2,3,6) (Figure 7B). In agreement with Use Case 1, the linear combination of the four patterns confirms the importance of position 3 and the implication of all scores as shown in Figure 7C.

   ```
   $ srun snakemake --cores all get_pattern
   ```

4. Predict m6A modifications: we evaluate the prediction ability of the detected patterns on a test set of almost $1,52$ million sites where $17,021$ are miCLIP-m6A modified. We consider the linear combination of the four most relevant patterns (1,2,3,6). Figure 7D shows the eCDF of the inferred scores. The difference between the cumulative distribution of non miCLIP sites and miCLIP sites can be nicely observed, while the PPV plot shows a lower performance as compared to Use Case 1 (Figure 7E). The decrease in performance is likely explained by the absence of all modifications and not exclusively m6A in the control condition, which may induce noise to the score estimation by JACUSA2 call-2 .

   ```
   $ srun snakemake --cores all predict_modification
   ```

The first IVT run hat rel. low coverage –¿ might this impact performance of UC2?

CD:to be confirmed

NOTES

Tips and Tricks

1. The reverse transcription step during library preparation is optional. However, we recommend to include this step to ensure proper sequencing also of RNAs with secondary structures. Superscript IV reverse transcriptase may be replaced by Superscript III reverse transcriptase, which is used in the protocol provided by Oxford Nanopore Technologies.

2. The library preparation protocol contains two bead clean up steps. It is important to remove ethanol and wash buffer completely. However, beads should not be dried for several minutes. Directly add water or elution buffer after washing to prevent sticking of the RNA to the beads.

3. The default filter in current MinKNOW versions is a Q score of 9. For direct RNA sequencing we recommend to adjust the output filter to a minimum Q score of 7, as in previous MinKNOW versions.

4. During preparation of the *in vitro* transcriptome sample, *in vitro* transcription and clean up kits may be replaced by equivalent products. The protocol however has been tested only with the mentioned kits.

5. Configuration of the pipeline should be handled via the config file. All parameters should be set before executing rules.

6. Once the pipeline has run successfully you should expect the following folders with the corresponding outputs in the output directory: bam, jacusa, features, patterns, and prediction.

7. JACUSA2 call2 could be run separately using the command line as described in JACUSA2 manual [JAC, 2021], then put the output under a new folder with the name 'jacusa' under the output directory.

8. In the snakemake pipeline, rules are linked so that the workflows are determined from top (e.g. predict_modification) to bottom (e.g. sort_bam) and executed accordingly from bottom to top (Figure 4). Therefore, running for example "predict_modification" rule leads to executing all rules on its pipeline.

9. Patterns could be generated from a subset of the input data that correspond to known modified sites. Alternatively, predefined patterns as a NMF R object could be used as a prediction model.

ACKNOWLEDGMENTS

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CD: funding?

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FIGURE CAPTIONS

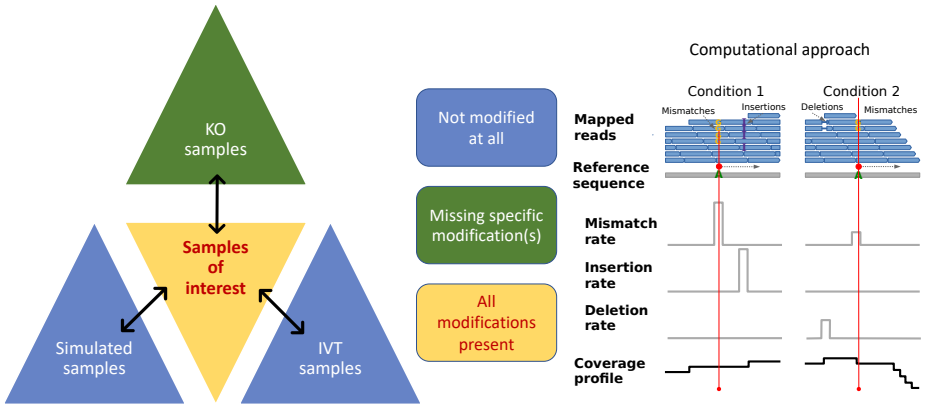

Figure 1: **General outline of RNA modification detection by JACUSA2.** A key feature of our approach is that multiple replicates can be compared as shown on the left. Samples of interests where all modifications are present could be compared with either KO samples where the modification of interest is missing or IVT/simulated samples where all modifications are absent. Read stacks (in blue) are compared head-to-head as shown on the right.

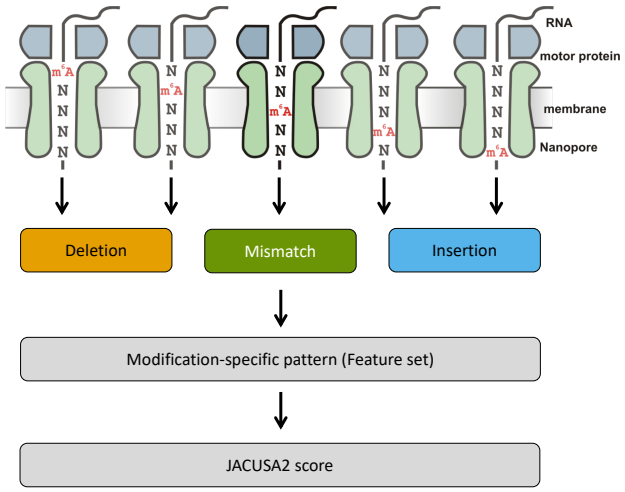

Figure 2: **Motivation of 5-mer context for RNA modification mapping**. The nanopore covers 5 consecutive RNA residues. That is why we consider a 5-mer context and derive 3 principal features for every position within a given 5-mer (15 features in total, with a central A residue in this example). We evaluate each feature set by previously learned patterns and compute a final score for modification site detection.

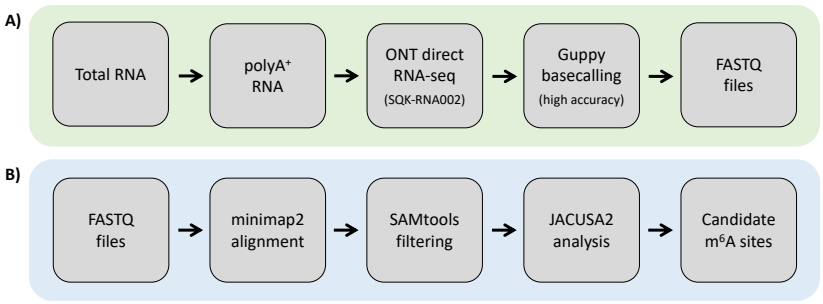

Figure 3: **Experimental and computational workflow**. A) Starting from total cellular RNA, polyA$^+$ RNA is isolated and subjected to Nanopore direct RNA-sequencing. Guppy basecalling can be done as live basecalling during sequencing or after the sequencing run from generated FAST5 files, resulting in FASTQ output files. B) FASTQ files are aligned to a reference sequence with Minimap2. SAMtools is used to generate BAM files as inpug for JACUSA2 analysis, which yields candidate m4A sites.

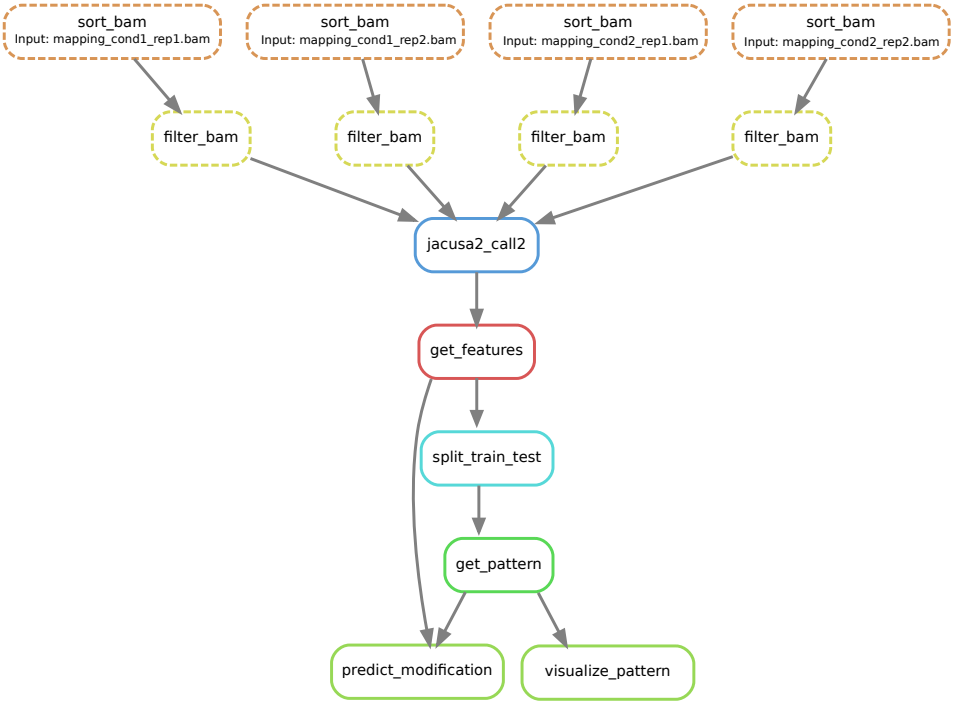

Figure 4: **Computational workflow**. Snakemake workflow for RNA modification detection based on JACUSA2 variant calling.

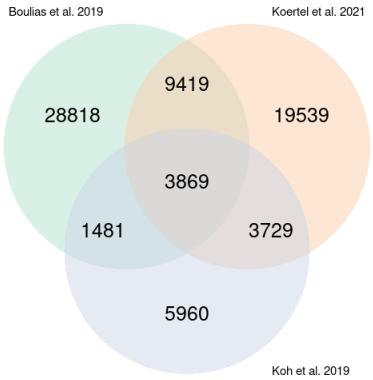

Figure 5: **m6A sites reported in the three miCLIP-based studies** Boulias et al. [2019], Koh et al. [2019] and Körtel et al. [2021].

Software	Version	Description
Minimap2	https://github.com/lh3/minimap2 v2.22 or later	https://lh3.github.io/minimap2/
samtools	https://github.com/samtools/samtools v1.12 or later	http://samtools.github.io/
JAVA	https://openjdk.java.net/ 11.0.12 2021-07-20 - JAVA 11 or later	OpenJDK Runtime Environment
R	https://www.r-project.org/ version 3.5.1 or later	The R Project for Statistical Computing
PERL	https://www.perl.org/ version 5.28.1 or later	Perl is a highly capable, feature-rich programming language
bedtools	https://github.com/arq5x/bedtools2 version 2.29.2 or later	Perl is a highly capable, feature-rich programming language
snakemake	https://snakemake.github.io/ version 6.8.1 or later	The Snakemake workflow management system

Table 1: **Software dependencies**

R Packages	Version	Description
ggplot2	https://cran.r-project.org/web/packages/ggplot2/index.html - ggplot2_3.3.0 or later	ggplot2 is a system for declaratively creating graphics, based on The Grammar of Graphics.
NMF	https://cran.r-project.org/web/packages/NMF/index.html - NMF_0.22.0 or later	Provides a framework to perform Non-negative Matrix Factorization (NMF).

Table 2: **R Package dependencies**

TABLE CAPTIONS

TABLES

snakemake 6.8.1

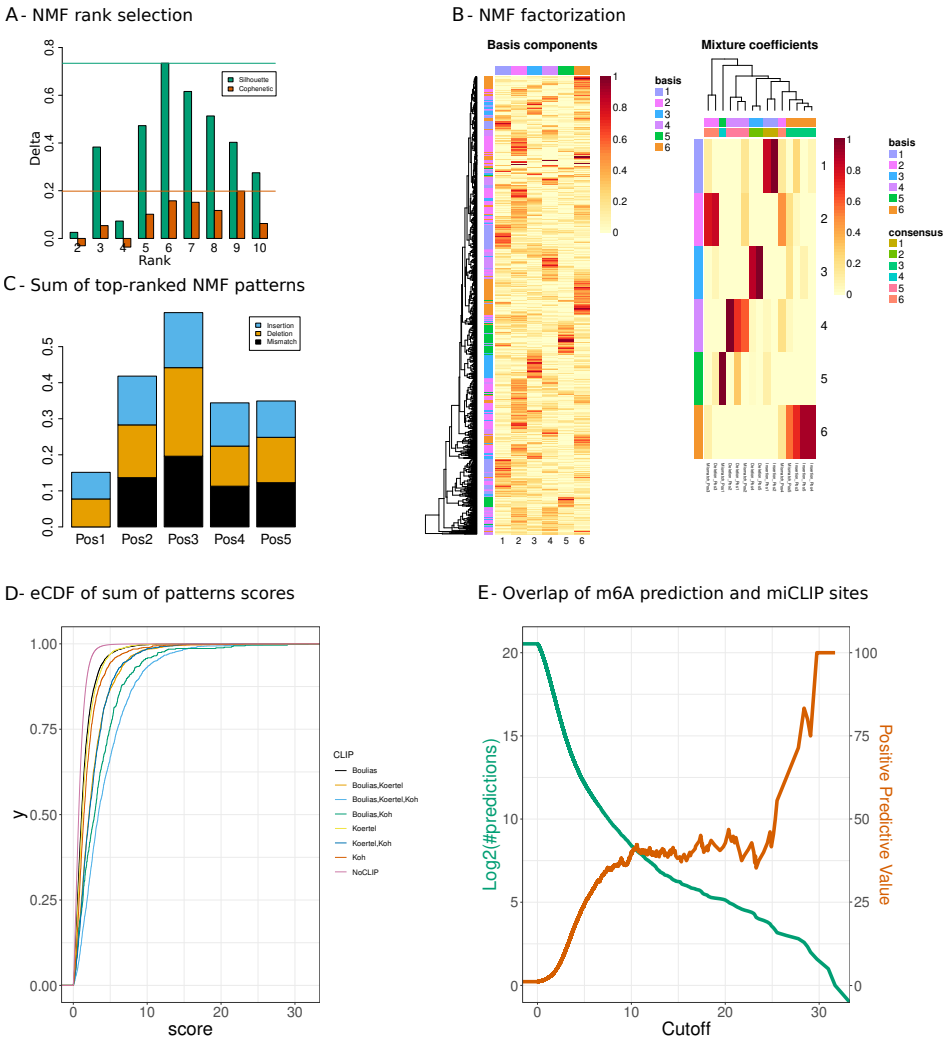

Figure 6: **Case 1. WT versus KO. A**: NMF rank selection. Barplots representing the difference of cophenetic correlation and silhouette indices between the NMF factorization of the original and the randomized data. Ranks with the largest value for both indices are determined, then the smallest rank is selected for the NMF decomposition. **B**: NMF result represented by the basis matrix W and the coefficient matrix H. The matrix H induces the RNA modification pattern. **C**: Barplots representing the linear combination of the top 5 patterns (y-axis) by the number of position in the specific 5-mer context (x-axis) and the score type: mismatch, deletion and insertion (resp. black, orange and blue). The 5 patterns (coefficient vectors: 1,2,3,4,6) are selected according to the predominant columns in matrix W. **D**: Score distribution inferred from the combined patterns, stratified by the different categories of miCLIP validated sites and non miCLIP sites. **E**: Number of predicted m6A sites (green) and Positive Predictive Value (PPV) of predicted m6A sites that overlap with miCLIP sites (orange).

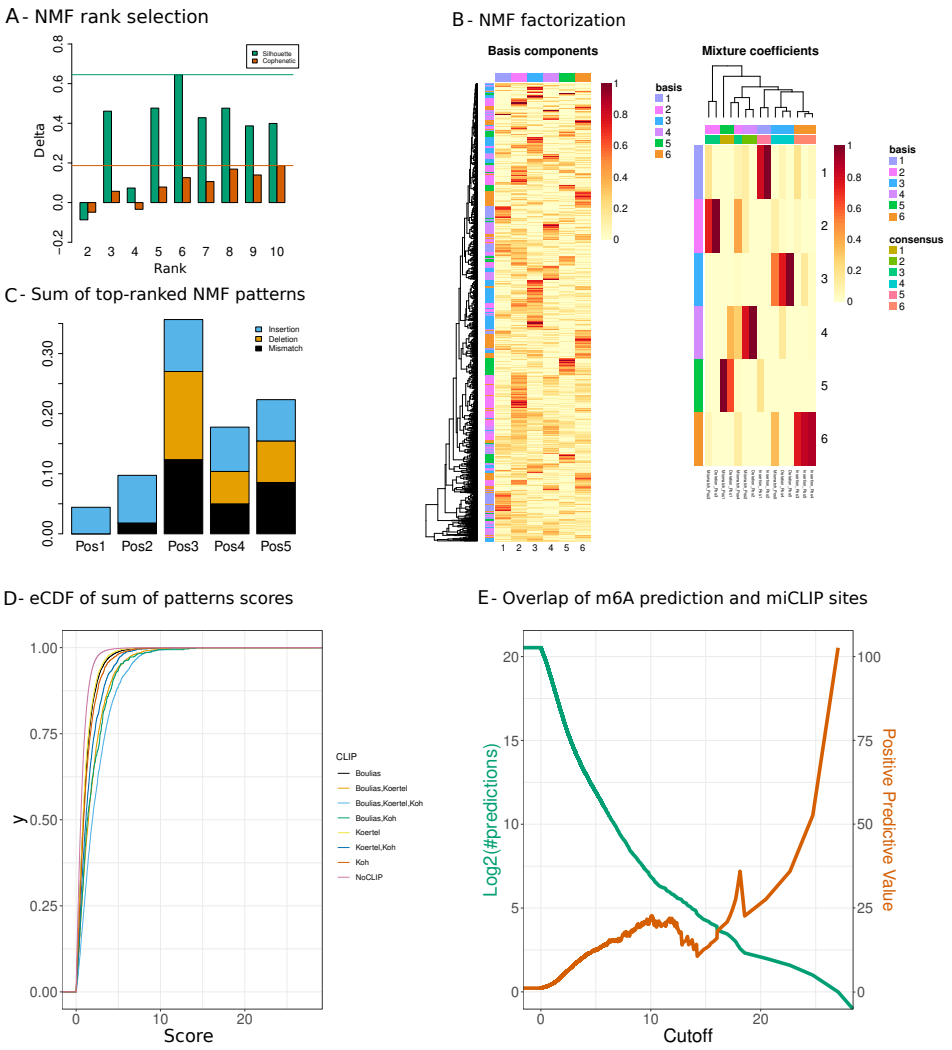

Figure 7: **Case 2. WT versus IVT. A**: NMF rank selection. Barplots representing the difference of cophenetic correlation and silhouette indices between the NMF factorization of the original and the randomized data. Ranks with the largest value for both indices are determined, then the smallest rank is selected for the NMF decomposition. **B**: NMF result represented by the basis matrix W and the coefficient matrix H. The matrix H induces the RNA modification pattern. **C**: Barplots representing the linear combination of the top 4 patterns (y-axis) by by the number of position in the specific 5-mer context (x-axis) and the score type: mismatch, deletion and insertion (resp. black, orange and blue). The 4 patterns (coefficient vectors: 1,2,3,6) are selected according to the predominant columns in matrix W. **D**: Score distribution inferred from the combined patterns, stratified by the different categories of miCLIP validated sites and non miCLIP sites. **E**: Number of predicted m6A sites (green) and Positive Predictive Value (PPV) of predicted m6A sites that overlap with miCLIP sites (orange).