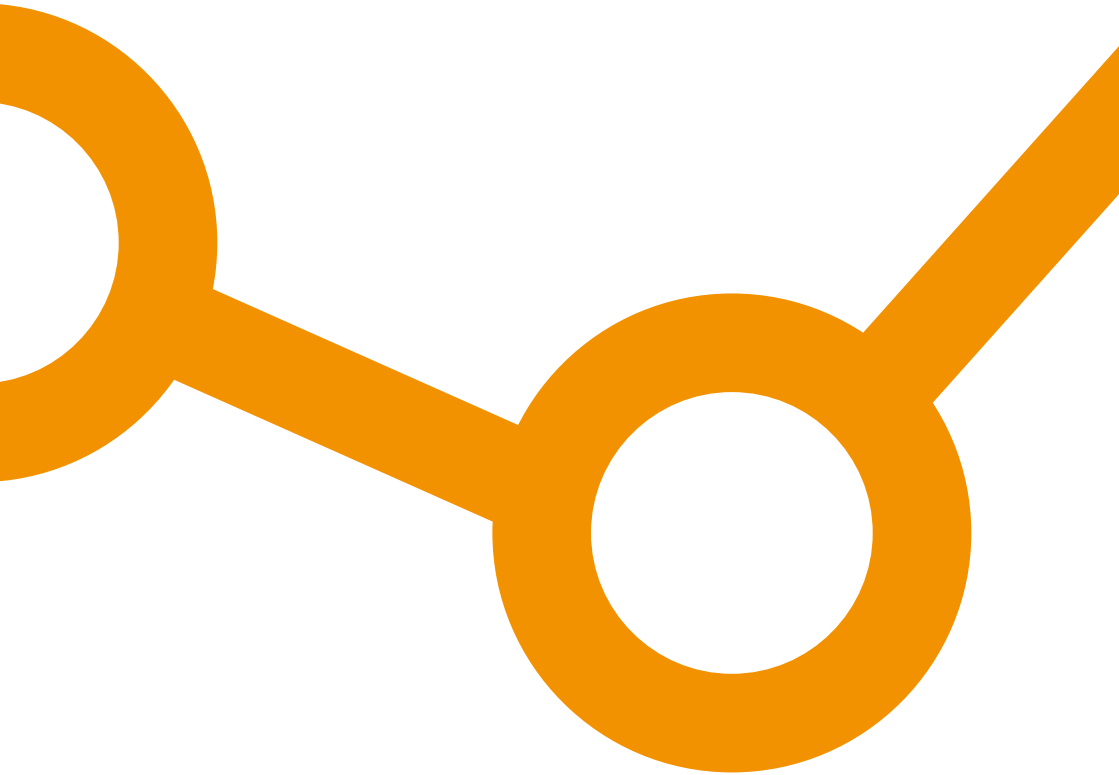

miND

miND® spike-in

Cat no: KT-041-MIND

Instruction Manual v1.5

May 2024

TAmiRNA

stability for life.

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Further information and technical notes can be found at **www.tamirna.com/mind-spike-ins/**

Revision history

Date	Revision
09/2022	1.0
02/2023	1.1
04/2023	1.2
05/2023	1.3
06/2023	1.4
05/2024	1.5

1

Product Summary

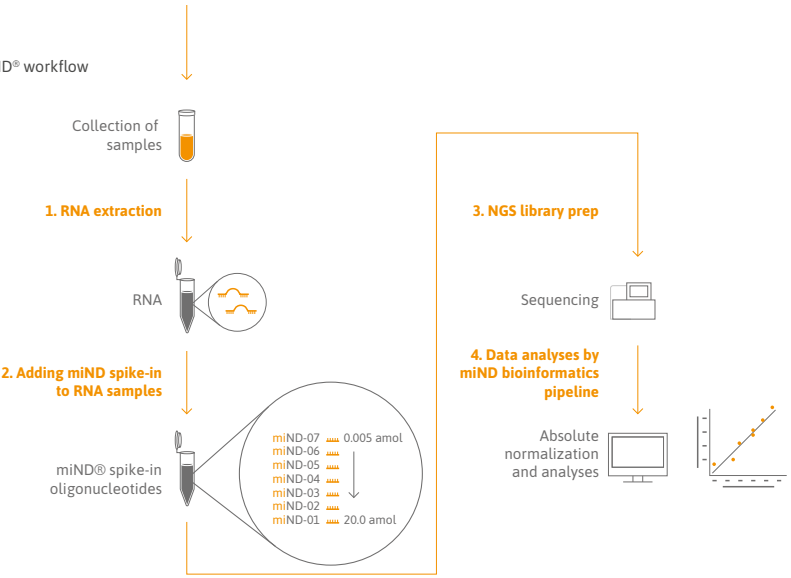
Intended-Use

Intended use

The miND® (**mi**croRNA **N**GS **D**ata Analysis) spike-in has been developed for **quality control** of experiments and **absolute quantitation** of microRNAs in any biological matrix and species (Khamina et al. 2022). The miND® spike-in is added to an RNA sample during the library preparation. The miND® spike-in consists of seven oligonucleotides that are provided in a specific ratio to cover the broad concentration range of endogenous small RNAs. A unique design of the miND® spike-in reduces sequencing bias and ensures precise quantitation of small RNA (Lutzmayer, Enugutti, and Nodine 2017).

The miND® spike-in sequences are detected in the NGS data along with the endogenous small RNAs. Read counts of the miND® spike-in and endogenous miRNAs are used to calculate absolute concentrations (amol/µL or molecules/µL). This conversion can either be achieved by using our miND® NGS data pipeline (Figure 1) (Diendorfer et al. 2022) or by the incorporation of the provided scripts in an already established NGS data analysis workflow.

Figure 1
The miND® workflow



miND® spike-in design features

Each of seven miND® spike-in consists of a unique 13-nucleotide core sequence that is flanked by four randomized nucleotides on the 5' and 3' ends (Table 1) resulting in 65,536 different RNA oligonucleotides per spike-in. The presence of random nucleotides on the 5' and 3' ends of each miND® spike-in sequence is expected to minimize the ligation bias of the core sequence. The miND® spike-in oligonucleotides contain a 5' phosphate group.

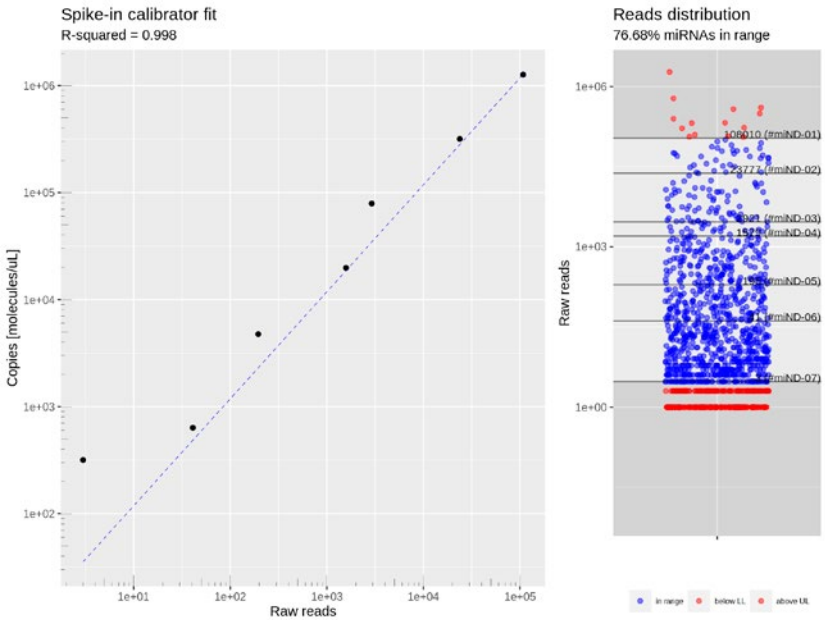
Table 1
The miND® spike-in core sequences

Oligo	Sequence (5' - 3')	Molar amount (amol) in 1 µL of miND spike-in
miND-01	(N)(N)(N)(N)ACGAUCGGCUCUA(N)(N)(N)(N)	20
miND-02	(N)(N)(N)(N)UGAACGUCCGUAC(N)(N)(N)(N)	5
miND-03	(N)(N)(N)(N)UCUCGCGCGCGUU(N)(N)(N)(N)	1.25
miND-04	(N)(N)(N)(N)CGAGUAAUGAACG(N)(N)(N)(N)	0.3125
miND-05	(N)(N)(N)(N)GCUACACACGUCG(N)(N)(N)(N)	0.075
miND-06	(N)(N)(N)(N)UAUUCGCGGUGAC(N)(N)(N)(N)	0.01
miND-07	(N)(N)(N)(N)ACCUCCGUUUACG(N)(N)(N)(N)	0.005

The miND® spike-in simultaneously enables quality control and absolute quantitation of miRNAs across different sample types:

- miND® spike-in serves as a quality control for small RNA-sequencing experiments to confirm the dynamic range and sensitivity of the assay
- miND® spike-in are used to generate a linear regression model to calculate absolute concentrations of endogenous microRNAs (Figure 2)

Figure 2
The miND® spike-in calibrator fit and miRNA spike-in reads distribution



The miND® spike-in was developed based on the principles described in the following publication:

- Lutzmayer et al. Novel small RNA spike-in oligonucleotides enable absolute normalization of small RNA-Seq data. 2017 Sci Rep https://doi.org/10.1038/s41598-017-06174-3

Product Components

The miND® spike-in contains the following components:

KT-041-MIND-250 - One 1.5 mL tube with lyophilized miND® spike-in
KT-041-MIND-48 - One 0.5 mL tube with resuspended miND® spike-in

The entire workflow consists of three main steps:

1. a. KT-041-MIND-250 - Dissolve the dry miND® spike-in in nuclease-free water (NFW) (Note: NFW is not provided with the kit.). Aliquot spike-ins to enhance stability after reconstitution.
   b. KT-041-MIND-48 - The miND® spike-in is ready-to-use
2. Add the miND® spike-in to an RNA sample before the NGS library preparation
3. Immediately proceed with the NGS library preparation according to the manufacturer's protocol

Storage and Stability

The dry miND® spike-in are shipped at ambient temperature and **must be stored at -20°C upon arrival**. The resuspended miND® spike-in are shipped on dry ice and must be stored at -80°C upon arrival. Shelf life of the miND® spike-in is at least 24 months. Avoid repeated freezing and thawing as this may lead to degradation. Consider preparing aliquots for the dry miND® spike-in: each tube is sufficient for 250 NGS library preparations.

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Important Pre-Analytical Considerations

Choice of Sample Type and Protocols for RNA isolation and small RNA-sequencing library preparation

The miND® spike-in has been extensively tested and optimised for plasma samples processed according the following conditions:

- RNA extraction from 200 µL of plasma with either miRNeasy Mini kit (Cat. 217004, Qiagen) or Maxwell RSC miRNA Tissue kit (Promega, Madison, WI, USA, AS1460) following the manufacturer's protocols.
- NGS workflow according to the RealSeq®-Biofluids Plasma/Serum miRNA Library Kit for Illumina® sequencing (Cat. 600-00012, 600-00024, 600-00048) and the RealSeq®-Dual Biofluids Plasma/ Serum miRNA Library Kit for Illumina® sequencing (Cat. 700-00024, 700-00048)
- Illumina sequencing using the following parameters: minimal read length 50 bp, minimal required sequencing depth is 7.5 million reads per sample.

For other sample types as well as RNA isolation and small RNA-sequencing protocols, it is important to take into consideration the following factors:

- The yield and efficiency of small RNA isolation varies between different isolation and purification kits. Therefore, the choice of the RNA isolation or purification kit can impact the results.
- The selection of the small RNA-sequencing library preparation protocols might impact miND® spike-in performance. Therefore, if other than the recommended library kits are used, users need to perform a pilot experiment with a limited number of samples in order to ensure that the miND® spike-in covers the concentration range of the endogenous small RNAs in the target samples.
- The miND® spike-in core sequences were mapped against the following genomes: *Homo sapiens, Mus musculus, Bos taurus, Rattus norvegicus, Sus scrofa, Caenorhabditis elegans, Drosophila melanogaster* and *Danio rerio* demonstrating minimal overlap with the target genomes. If miND® spike-in is planned to be used with samples from other organisms, it is recommended to reach out TAmiRNA team (e-mail: support@tamirna.com) in order to discuss additional bioinformatic analysis.
- We suggest to use one lot of the miND® spike-in within a project in order to reduce any potential impact of the lot-to-lot variability on the generated results.

Storage and Stability of the dissolved miND® spike-in and RNA

- Store the dry miND® spike-in (KT-041-MIND-250) upon arrival at -20°C. Store the re-suspended miND® spike-in (KT-041-MIND-48) upon arrival at -80°C.
- Resuspend, aliquot and store the miND® spike-in at -80°C (KT-041-MIND-250) Always keep the dissolved miND® spike-in on ice when handling and return immediately after use to -80°C. Avoid repeated freeze-thawing cycles.
- All samples and miND® spike-in aliquots should be stored in nuclease-free plastic tubes with minimized absorption rates for nucleic-acids ("low-binding").
- RNA samples must be stored at -80°C for long-term storage and kept on ice when handling and return immediately after use to -80°C.

Working with RNA

- Ensure that you work in RNase-free environment and that you use molecular grade NFW only.
- Always work with fresh, disposable plastic consumables and wear gloves.
- Handle carefully to avoid contamination.
- Spin down all reaction and sample tubes before opening.
- Clean all surfaces with commercially available RNase decontamination solutions.
- Use filter barrier pipette tips to avoid aerosol-mediated contamination.
- Designated solutions, tips, pipets and other materials and equipment should be assigned for RNA work only.

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Lab Protocol

Essential components

miND® spike-in kit

Consumables and Instruments Not Supplied by TAmiRNA

Molecular grade nuclease-free water
Nuclease-free, low nucleic acid binding tubes (1.5 mL)
Nuclease-free, filter pipette tips
Vortexer
Calibrated pipettes
Centrifuge for <2 mL tubes
small RNA NGS library preparation kit

Wet-Lab Protocol

1. Dissolve the dry miND® spike-in in sterile nuclease-free water

		Notes
Step 1	KT-041-MIND-250: Briefly centrifuge the tube containing the dried miND® spike-in to collect the oligo pellet at the bottom of the tube. KT-041-MIND-48: Proceed directly with Step 6. Consider preparing single-use aliquots.	
Step 2	Add 250 µL of the nuclease-free water.	
Step 3	Briefly vortex or mix by pipetting 8-10 times up and down. Leave the tube on ice for 20 minutes.	
Step 4	Prepare aliquots that can be used for a single experiment. The volume of the aliquots can be determined based on the experimental plan (it is recommended to add 1 µL of the dissolved miND® spike-in to an RNA sample before the NGS library preparation).	
Step 5	Store the aliquots of the dissolved miND® spike-in at -80°C.	

2. Add the miND® spike-in to an RNA sample before the small RNA NGS library preparation.

Step 6	Thaw an aliquot of the dissolved miND® spike-in on ice.
Step 7	Mix the miND® spike-in by gently tapping the tube and briefly centrifuge to collect the liquid at the bottom of the tube.
Step 8	Add 1 µL of the miND® spike-in to each RNA sample right before starting the NGS library preparation experiment.
Step 9	Immediately proceed with the NGS library preparation according to the manufacturer's protocol

Data Analysis

The absolute quantification of miRNAs using miND® spike-in requires the following steps:

- mapping and quantification of microRNAs and other small RNAs
- mapping and quantification of miND® spike-in
- calculation of absolute concentrations using a regression model

For easy analysis, a docker based toolkit is available on our public GitHub space: https://github.com/tamirna

Documentation, examples, and updated tools will be published there.

In addition, we have published our in-house miRNA analysis pipeline miND® (Diendorfer et al. 2022) that can be used for the whole process of miRNA mapping from raw NGS data.

Potential Problems

Observation: No miND® spike-in sequences were detected in the generated NGS data
Potential cause:

- A tube with the miND® spike-in was not centrifuged before adding NFW and the oligo pellet was lost
- The miND® spike-in was reconstituted in a wrong volume of nuclease-free water
- Improper storage of the reconstituted miND® spike-in led to degradation
- Contamination of the miND® spike-in with RNases led to degradation
- The miND® data analysis pipeline was not used

Observation: The generated NGS data did not pass the quality control of the miND® spike-in
Potential cause:

- Less than 5 miND® spike-in core sequences detected
- Insufficient sequencing depth
- Degradation of miND® spike-ins due to the use of contaminated reagents
- Low ligation efficiency of spike-ins with the selected small RNA NGS library preparation kit

Related Services

TAmiRNA offers a broad range of high-quality RNA services performed by experts according to GLP standards, including EV/exosome characterization, and RNA isolation from any biological matrix, small RNA and mRNA NGS, qPCR, and customized bioinformatic analyses.

The miND® spike-in is compatible with a broad range of sample types and species:

- **Species compatibility:** our bioinformatic pipeline has been tested with human, mouse, rat, pig, cow, and horse samples. Any species with known microRNAs can be analyzed.
- **Sample types:** besides cells and tissues we have tested conditioned media, plasma (various anti-coagulants), serum, urine, CSF, brain microdialysate, and synovial fluid. This includes enrichment of EV/exosomes from all biofluids.
- **Laser microdissection:** the miND® service can be used to analyze dissected tissue compartments for increased precision. Learn more here: https://www.tamirna.com/space-resolved-rna-profiling-in-complex-tissues/
- **Other RNAs:** PNK-treatment of total exRNA increases coverage of mRNA and long non-coding RNAs in your data.
- **RNA-seq:** we offer mRNA-seq (polyA and total RNA) alongside our small RNA-seq workflow to generate high quality microRNA/mRNA datasets.

Read more about these services at www.tamirna.com/small-rna-sequencing-services/

Related Products

TAmiRNA offers research-use kits for analysis of microRNA biomarkers.
Read more about the products at www.tamirna.com

Further Reading

Below you find a list of publications describing the development and application of the miND® spike-in:

1. Khamina, K. et al. A MicroRNA Next-Generation-Sequencing Discovery Assay (miND) for Genome-Scale Analysis and Absolute Quantitation of Circulating MicroRNA Biomarkers. Int. J. Mol. Sci. 2022,23,1226. https://doi.org/ 10.3390/ijms23031226
2. Diendorfer, A. et al. miND (miRNA NGS Discovery pipeline): a small RNA-seq analysis pipeline and report generator for microRNA biomarker discovery studies. F1000Research 2022,11:233,1226. https://doi.org/10.12688/f1000research.94159.1
3. Gutmann, C. et al. Association of cardiometabolic microRNAs with COVID-19 severity and mortality. Cardiovasc Res. 2022, 118(2):461-474. https://doi.org/10.1093/cvr/cvab338
4. Lutzmayer, Stefan, Balaji Enugutti, and Michael D. Nodine. 2017. 'Novel Small RNA Spike-in Oligonucleotides Enable Absolute Normalization of Small RNA-Seq Data'. Scientific Reports 7 (1): 5913. https://doi.org/10.1038/s41598-017-06174-3.

Notes

Notes

Notes

Notice to purchaser

A patent was filed for the invention related to novel spike-in oligonucleotides for absolute quantitation of nucleotide sequence data under WO2018138334A1.

The purchase of this product conveys to the buyer the non-transferable right to use the purchased product and components of the product only in research conducted by the buyer (whether the buyer is an academic or for-profit entity).

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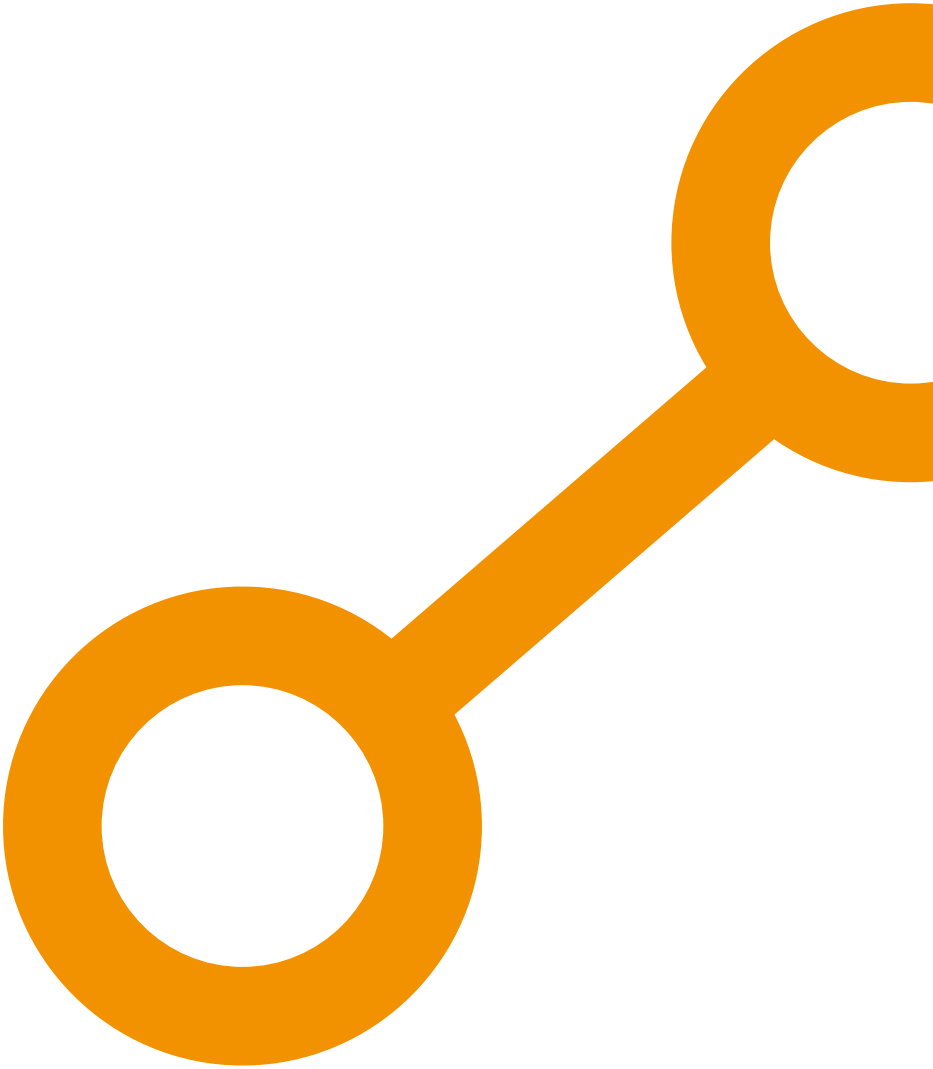

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