



RNAConnect

cDNA Synthesis Protocol for *in vivo* SHAPE-MaP

This protocol is derived from methods described in the literature[1-6], but several adaptations are worth noting:

1. We consistently observe better performance using the alternative acylation reagent 2A3 (2-aminopyridine-3-carboxylic acid imidazolide [5, 6]) rather than NAI. Information for both NAI and 2A3 reagents is provided below. We can provide information on a bulk supplier of 2A3 if needed.
2. Note that MRT and uMRT has the added benefit of providing immediate information on the location of post-transcriptional RNA modifications in natural transcripts, as these also induce mutational profiles upon reverse-transcription. Positions of most natural modifications are apparent from mutational signatures within the DMSO control samples [4, 7]. Preliminary deconvolution of their chemical identities can be performed with MRT-ModSeq [3].

A. ULTRAMARATHONRT

UltraMarathonRT (uMRT) is a highly optimized variant of MarathonRT with improved overall performance and sensitivity under conditions of low RNA input.

B. ENZYME INFORMATION

- Unit definition: One (1) unit is equal to the amount of uMRT that incorporates 1 nmole dTTP at 42°C in 30 minutes, when using poly(rA) as the template and Oligo(dT)18 as the primer.
- Enzyme concentration and storage buffer: The enzyme is supplied at 20U/μL in a buffer that contains 25 mM K-HEPES pH 7.5, 300 mM KCl, 10% glycerol and 1 mM DTT.

C. REACTION BUFFER COMPOSITION (2X)

- 100 mM Tris-HCl (pH 7.5 at 25°C)
- 400 mM KCl
- 2 mM MnCl₂
- 10 mM DTT
- 40% glycerol

Note: (1) The reaction buffer for *in vivo* SHAPE-MaP requires Tris-HCl with pH 7.5, rather than pH 8.3 that is used for standard reverse transcription; (2) MnCl₂ should be separated from the other components for long term storage and then combined with them immediately before performing reverse transcription.

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D. ADDITIONAL REAGENTS REQUIRED

Reagents	Recommendation
dNTP mix	Use a stock that contains 10 mM each of dATP, dCTP, dGTP, and dTTP of high purity (>99%) in a pH 7.0 solution (e.g. New England Biolab, Cat# N0447)
In vivo SHAPE reagent for live cell RNA structure analysis	2A3 (2-aminopyridine-3-carboxylic acid imidazolide; Tocris Cat# 7376) or NAI (2-methylnicotinic acid imidazolide) (e.g. Sigma-Aldrich, Cat# 03-310)
Nuclease-free water	From a reliable source
Primers	Gene-specific primers for reverse transcription and PCR amplification (custom synthesis)
RNA extraction	TRIzol reagent, Thermo Fisher, Cat# 15596026
RNA cleanup	RNeasy Mini column, Qiagen, Cat# 74104

E. STEP-BY-STEP PROCEDURE

a. NAI or 2A3 Modification

- Harvest 1×10^7 – 2×10^7 cells grown in monolayer in a culture dish using a cell scraper
- Gently centrifuge the cultured cells for 5 min at $500 \times g$ to collect the cells
- Discard the supernatant and resuspend the cells in 450 μ L 1x PBS
- Split the cell suspension into two equal halves (225 μ L each)
- *For NAI probing:*
 - Add 25 μ L of 2M NAI stock solution (dissolved in DMSO) and 25 μ L DMSO to each of the two halves of cell suspension respectively. Mix gently using a pipette.
 - The final concentration of NAI for in-cell RNA modification is 200 mM.
 - Incubate the samples at room temperature (20 - 25°C) for 10 minutes.
- *For 2A3 probing:*
 - Prepare 2A3 1M stock by adding 5mg of 2A3 into 26.5 μ L DMSO.
 - Add 25 μ L of 1M 2A3 stock solution (dissolved in DMSO) and 25 μ L DMSO to each of the two halves of cell suspension respectively. Mix gently using a pipette.
 - The final concentration of 2A3 for in-cell RNA modification is 100 mM.
 - Incubate the samples at 37°C for 15 minutes.

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b. RNA Extraction

- Following the treatment of NAI/2A3 or DMSO, immediately add 750 μ L TRIzol into each of the cell suspensions. Pipet the lysate up and down 5-10 times to mix.
- Incubate at room temperature for 5 min.
- Add 150 μ L chloroform to each of the cell lysates, and pipet the lysate up and down 5-10 times to mix.
- Incubate at room temperature for 5 min.
- Centrifuge the samples for 15 minutes at $12,000 \times g$ at 4°C . The mixture separates into a lower phenol-chloroform, an interphase, and a colorless upper aqueous phase.
- Transfer the aqueous phase that contains the RNA to a new tube using a pipette.
- Add 100% ethanol to a final concentration of 80%, securely cap the tube, shake to mix the content, and incubate at -20°C overnight or -80°C for 2 hours to precipitate the RNA.
- Centrifuge for 30 minutes at $12,000 \times g$ at 4°C to pellet the RNA.
- Discard the ethanol with a pipette.
- Briefly spin down the residual ethanol and remove the ethanol with a pipette.
- Dissolve the RNA in 100 μ L ME buffer (10 mM MOPS pH 6.5 and 1 mM EDTA).
- Use an RNeasy Mini column for each sample to clean up the ethanol precipitated RNA by following the manufacturer suggested protocol for RNA cleanup.
 - Elute the RNA using 30–50 μ L RNase-free water.
- Measure RNA concentration with Nanodrop and dilute to $1\mu\text{g}/\mu\text{L}$.

c. Reverse Transcription

i. Anneal RT primers to RNA templates

- » Combine the components as indicated in the table below to a nuclease-free microcentrifuge tube
- » Mix gently by tapping the tube. Collect the contents by brief centrifugation. Incubate at 95°C for 30 sec and snap cool on ice to anneal the primer to the template.

Components	Final amount	Volume
Gene-specific primer (2 μM)	2 pmol	1 μL
NAI/2A3 or DMSO treated total RNA from Step b	1 μg^*	1 μL
dNTP mix stock, 10 mM each	0.5 mM final concentration	1 μL
Nuclease-free water	added to total 6 μL	3 μL

* Total RNA amount can be adjusted based on targeted RNA abundance. Usually 0.5 μg – 1 μg total cellular RNA per 20 μL reaction is sufficient for amplifying even low abundance RNA from cells

ii. Prepare the RT reaction mix

- » Combine the components as indicated in the table below in a nuclease-free microcentrifuge tube
- » Mix the contents gently by tapping the tube and collect the contents by brief centrifugation

Components	Volume
Nuclease-free water	2 μ L
Reaction Buffer (2x)	10 μ L
UltraMarathonRT (20 units/ μ L)	1 μ L
RNaseOUT™ (40 units/ μ L) (optional) (e.g. Thermo Fisher, Cat# 10777019)	1 μ L
Total volume	14 μ L

iii. Carry out the reverse transcription reaction

- » Add the RT reaction mix (14 μ L, prepared in **Section ii**) to the annealed primer and RNA template (6 μ L, prepared in Section i) to make a 20 μ L reaction.
- » Mix gently by tapping the tube
- » Incubate the mixture at 42°C for 3 hours to carry out the reverse transcription.
- » Note that for optimization, one can vary both time (from 1 hour to 4 hours) and temperature (from 25°C to 42°C) of this reaction.
- » Heat up the mixture to 95°C for 1 min to inactivate the enzyme and then snap cool on ice

The cDNA should be stored at -20°C until further use.

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