Genome-wide DMS-MaPseq for in vivo RNA structure determination

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Abstract

Genome-wide dimethyl sulfate mutational profiling with sequencing \(DMS-MaPseq) is a method suitable for the _in_ _vivo_ investigation of RNA folding on a genome-wide scale. Based on the RNA structure-specific _in_ _vivo_ chemical modification by DMS and the encoding of resultant modifications as mismatches during reverse transcription, DMS-MaPseq produces ratiometric RNA structure data that does not require base-by-base correction to control samples. Due to its high signal-to-noise ratio, DMS-MaPseq is the preeminent choice amongst mutational profiling techniques. Here we describe the genome-wide DMS-MaPseq protocol, which produces libraries suitable for Illumina sequencing and can be applied across many model systems.

Introduction

This protocol is used for the _in_ _vivo_ DMS-based chemical probing of RNA structure genome-wide from _S. cerevisiae_ or human cells in culture, providing quantitative RNA structure data encoded as mismatches. In the first step, cells/tissues are treated with DMS, followed by total RNA extraction, and fragmentation. After ligation to a 3’ adaptor and rRNA depletion, the RNA is reverse transcribed using the thermostable group II intron reverse transcriptase \(TGIRT), which encodes structure-specific DMS modifications as mismatches in the cDNA. The cDNA is then circularized and subject to a limited number of PCR cycles before sequencing on an Illumina platform. The data produced has a high signal-to-noise ratio which compares favorably to the existing sequencing-based RNA structure probing techniques currently available[1,2].

Reagents

With the exception of standard laboratory reagents \(such as RNase-free or molecular-biology grade water, ethanol, buffers, salts, etc.), the following items are used this protocol: Dimethyl sulfate \(D186309-5ML, Sigma Aldrich) Beta-mercaptoethanol \(M3148, Sigma Aldrich) Isoamyl alcohol \(W205710, Sigma Aldrich) Acid phenol or Trizol \(AM9720 or 15596026, Ambion) Chloroform \(BP1145-1 Fisher Scientific) RNA Fragmentation Reagent \(AM8740, Ambion) Costar Spin-X columns \(8162, Corning) GlycoBlue Coprecipitant \(AM9516, Ambion) rSAP / Shrimp Alkaline Phosphatase \(M0371S, New England Biolabs) SUPERase Inhibitor \(AM2696, Ambion) CutSmart Buffer \(B7204S, New England Biolabs) T4 RNA ligase 2, truncated \(M0242S, New England Biolabs) Poly\(ethylene glycol) m.w. 8000 \(i.e. PEG; P5413, Sigma Aldrich) Rec J Exonuclease \(RJ411250, Epicentre) 5’ Deadenylase \(DA11101K, Epicentre) Ribo-Zero Gold rRNA Removal Kit \(species-specific product numbers, Epicentre) or Hybridase Thermostable RNaseH \(H39100, Epicentre) TGIRT-III \(Ingex) 0.1 M Dithiothreitol i.e. DTT \(D9163, Sigma Aldrich), prepared the day of reverse transcription CircLigase ssDNA Ligase \(CL4115K, Epicentre) Novex TBE-Urea Sample Buffer, 2X \(LC6876, Invitrogen) Novex TBE-Urea Gels, 6%, 12 well \(EC68652BOX, Invitrogen) Novex TBE Gels, 8%, 12 well \(EC62152BOX, Invitrogen) SYBR Gold Nucleic Acid Gel Stain \(S11494, Invitrogen) Phusion High Fidelity DNA polymerase \(M0530S, New England Biolabs) HiSeq 2500 v2 kit or HiSeq 3000/4000 kit \(varying options, Illumina)

Equipment

With the exception of standard laboratory equipment \(such as centrifuges, thermocyclers, incubators, water baths, etc.), the following items are used this protocol: Eppendorf Thermomixer, for use with 1.5 ml tubes Blue light \(ex. Invitrogen) Magnetic rack for 1.5 ml tubes \(ex. DynaMag-2, 12321D, Thermo Fisher Scientific) Bioanalyzer \(Agilent) or Fragment Analyzer \(Advanced Analytical Technologies) HiSeq 2500, HiSeq 4000, or MiSeq sequencer \(Illumina)

Procedure

1. In vivo dimethyl sulfate \(DMS) modification **Cautions before working with DMS:** DMS is highly toxic and should only be used in a well-ventilated fume hood. We recommend using multiple layers of nitrile gloves such that the exterior layer can be removed and replaced after handling DMS directly. DMS should have a faint yellow color, that will grow darker due to oxidation over time. Open a fresh bottle of DMS every six months to ensure freshness and optimal reactivity. DMS will be quenched during the following procedure by BME and resulting liquid waste should be disposed of per your local hazardous waste regulations. With the exception of the centrifugation steps and final steps of total RNA extraction, all steps should be done in a fume hood. a. _S. cerevisiae_ • Dilute saturated overnight yeast cultures to OD600 of ~0.09, and grow at 30°C to a final log phase OD600 of 0.5-0.7 at the time of DMS treatment. • Pre-chill a 30 ml stop solution on ice for > 1 hour before DMS treatment. Stop solution contains 15 ml 100% isoamyl alcohol, 9 ml BME, and 6 ml ddH2O. Also prepare and pre-chill a 10 ml wash solution containing 3 ml BME and 7 ml ddH2O. \(Solutions will phase separate, invert to mix directly before using.) • Move 15 ml of log-phase yeast culture into a 50 ml conical and place in a rack submerged in a 30°C water bath, such that the entire 15 ml culture is just submerged. • Pipet 750 µl of DMS \(5% v/v) into the yeast culture, stirring immediately stirring with a serological pipet. Treat the yeast for 4 min, with consistent stirring or swirling of the culture. Culture should remain submerged in the water bath during this time. • Pour ice cold 30 ml stop solution into yeast culture with DMS, cap, invert to mix, and place on ice. The DMS will be fully hydrolyzed by the BME at this point. • Centrifuge 50 ml conical for 4 min at 3,500 x g at 4°C. Optional: parafilm the lids to minimize the smell of DMS if centrifuge is located outside of a fume hood. • Decant supernatant into DMS/BME waste, will have a small pellet of yeast at the bottom. Pour in ice-cold wash solution, and shake/pipet to resuspend yeast pellet. • Parafilm lid \(optional) and spin again for 4 min at 3,500 x g at 4°C. • Decant supernatant into waste. Residual wash solution remaining in tube is fine, and traces of remaining BME in tube will quench any remaining DMS released upon cell lysis. • Add 0.6 ml total RNA lysis buffer \(6 mM EDTA, 45 mM NaOac pH 5.5) to tube and resuspend yeast pellet by pipet. Move sample to 1.5 ml Eppendorf tube that contains 40 µl 20% SDS. • Prewarm 0.65 ml of acid phenol in thermomixer at 65°C. • Move tube with yeast/SDS mixture to 65°C thermomixer and shake at 1,400 rpm for 30 sec to ensure mixing of SDS. • Add yeast sample to tube with hot acid phenol. Incubate in thermomixer at 65°C, shaking for 2 min at 1,400 rpm. Note, that DMS-treated RNA can be brittle and high temperature or vortexing steps should be minimized to prevent fragmentation. • Immediately move samples into a dry ice / EtOH bath \($_{\text{(1:1 composition) until frozen,}}$ 3 min. •

Spin tubes 3 min at 20,000 x g at room temp and immediately remove top aqueous phase into new 1.5 ml tube, avoiding material at the phase interface. • Add 0.65 ml acid phenol \(room temperature) to aqueous phase. Incubate at room temperature in thermomixer for 2-3 min, shaking at 1400 rpm. • Spin tubes 3 min at 20,000 x g at room temp and immediately remove top aqueous phase into new 1.5 ml tube, avoiding material at the phase interface. • Add 0.65 ml RNase-free chloroform and vortex at room temperature in thermomixer for 30 sec. Spin tubes 2 min at 20,000 x g at room temp and immediately remove top aqueous phase into new 1.5 ml tube, avoiding material at the phase interface. • Add >1 volume isopropanol \(ex. 700 µl) to final aqueous phase. Invert to mix well and chill >30min at -30°C. • Spin >45 min at 20,000 x g at 4°C to pellet nucleic acids. • Remove supernatant and wash pellet in 0.75 ml 70-80% EtOH. • Pulse spin tube to collect residual EtOH at the bottom, and remove all liquid with small pipet tip. • Air-dry 5 min and resuspend in 50 µl 10 mM Tris pH 7.0. May need to add additional volume if the pellet is large. • Nanodrop a 1:10 or 1:100 dilution of RNA sample. Procedure should yield >50-100 ug RNA. b. Adherent human cells • Begin with a 15 cm2 plate of fully adherent HEK 293T cells in 15 ml media \(other adherent cell lines will work with the procedure too). • Working a fume hood, remove 10 ml media from the plate into a 50 ml conical and add 200-300 µl DMS. Shake vigorously to ensure mixing. • Slowly pipette the DMS/media mixture back on the plate by tilting the plate and pipetting into a corner. Note that this step has to be done very carefully to avoid lifting cells from the plate. • Parafilm the plate, and move it to a 37°C incubator / hybridization oven for 5 min, keeping the plate in a fume hood if possible and swirling occasionally. Some cells may become detached during treatment. • Slowly pipet out the DMS/media into waste. • Add 10 ml of 30% v/v BME \(diluted in 1 x PBS) and collect the cells into a 15 ml conical using a scraper to scrape the cells from the plate. • Centrifuge cells at 1000 x g at 4°C for 3 min; decant the BME solution. • Wash the cells by adding 10 ml of 1 x PBS and repeating the centrifugation step. Decant the PBS. • Add 1 ml Trizol to plate, lysing cells, and extracting total RNA according to the Trizol protocol \(Optional: if using higher amounts of DMS you may add 10 µl 100% BME to the Trizol). **2. Fragmentation** • Start with 10 µg of RNA in 9 µl 10 mM Tris pH 7.0 in a 0.2 ml PCR tube for a 10 µl final reaction volume. • Move tube to thermocycler for 2 min at 95°C to denature the RNA. • Move tube directly to ice. Add 1 µl Ambion Fragmentation Reagent, immediately pipetting up and down to mix well. • Move tube back to 95°C thermocycler for 1 min 40 sec. Move to ice. • Add 1 µl of Ambion Fragmentation Reagent Stop Solution, which contains EDTA to quench the Zn2+ in the fragmentation reagent. As before, pipet up and down to mix well. • Add 10 µl of 2x TBE-Urea Sample Buffer, mix by flicking. • Load all of sample/dye mix into well of a 6% TBE-Urea gel. • Run gel 45 min at 150 V. • Stain gel in 1x SYBR Gold, image on a blue light, and cut RNA fragments of 100-170 nucleotides in size, which removes small RNA contaminants of <100 nucleotides \(e.g. tRNAs). • To extract RNA from gel slice, nest a 0.5 ml Eppendorf tube in a 1.5 ml Eppendorf tube with two holes poked in to the bottom of the smaller tube using a 20-gauge needle. Place the cut gel piece into the small 0.5 ml tube, and spin the nested tubes at room temperature for 3 min at 20,000 x g. • Move any remaining gel pieces into the bottom of the 1.5 ml tube, and discard the 0.5 ml tube. • Add 300 µl 0.3 mM NaCl and shake in a thermomixer at 70°C for 10 min with vigorous shaking \(1,400 rpm). Gel slurry can also be left rotating overnight at 4°C. • Transfer the gel slurry to a Costar Spin-X column, using a 1 mL pipet tip with the end trimmed using a sterile razorblade. Spin Costar column 3 min at 20,000 x g. • Transfer eluate into fresh

1.5 ml Eppendorf tube. • Add 800 µl 100% IsOH and 2 µl GlycoBlue. • Precipitate nucleic acids by placing tube at -30°C for 30 min or more. • Spin 45 min, 4°C at max speed \(20,000 x g). • Remove supernatant. Wash pellet gently with 500 µl 70-80% EtOH and allow pellet to air dry 5 min. • Resuspend pellet in 7 µl 10 mM Tris pH 7.0. **3. 3’ end resolution and Ligation** a. rSAP Treatment • To fragmented RNA solution, add 1 µl 10x CutSmart Buffer and 1 µl SUPERase Inhibitor, mix. Add 1.5 µl rSAP enzyme. Mix. • Incubate 1 hour at 37°C. • Heat inactivate at 65°C for 5 min. Move to ice. b. Ligation to linker-2 • Pre-mix 6.5 µl 50% PEG 8000, 0.5 µl 50 M linker-2, 1 µl 0.1M DTT, 1 µl 10x T4 RNA ligase buffer, and 2 µl T4 RNA ligase, and add the solution to heat-inactivated rSAP reaction. Mix well. • Incubate 2 hours at 25°C. • Add 154 µl 10 mM Tris pH 7.0, 700 µl 100% EtOH, 18 µl 3M NaOAc pH 5.5, and 1.5 µl GlycoBlue. Mix well. • Chill for >30 min at -30°C to precipitate nucleic acids. Spin >45 min at 4°C at max speed \(>20,000 x g). Wash pellet with 70-80% EtOH and air-dry. • Resuspend pellet in 15 µl 10 mM Tris 7.0. c. Linker degradation with Rec J • To 15 µl of RNA/Tris from above, add 2 µl 10x RecJ buffer and 1 µl SUPERase Inhibitor. Mix. • Add 1 µl 5’ deadenylase and 1 µl Rec J. Mix. • Incubate at 30°C for 30 min – 1 hour. • Add 154 µl 10 mM Tris pH 7.0, 700 µl 100% EtOH, 18 µl 3M NaOAc pH 5.5, and 1 µl GlycoBlue. Mix well. • Chill for >30 min at -30°C to precipitate nucleic acids. Spin >45 min at 4°C at max speed \(>20,000 x g). Wash pellet with 70-80% EtOH and air-dry. • Resuspend pellet in 26 µl RNase-free water and proceed to Ribo-Zero. **4. Ribo-Zero, rRNA depletion** Note: this protocol deviates slightly from the manufacturer protocol with a final incubation step at 40°C instead of 50°C. Because the RNA is fragmented, the temperature is lowered to ensure there is no excess denaturation of rRNA bound to the bait oligos. • Prepare and wash Ribo-Zero magnetic beads according to manufacturer instructions. • To 26 µl RNA/water solution, add 4 µl 10x Ribo-Zero Reaction Buffer. Mix. • Add 10 µl Ribo-Zero removal solution. Mix gently. • Incubate solution 68°C for 10 min. • Incubate at room temp for 5 min. • Add the 40 µl RNA/removal mix to 65 µl prewashed beads. Immediately pipet up and down 10x and then vortex at medium speed for 10 sec. • Incubate 5 min at RT. • Vortex sample again, 10 sec at medium speed. • Incubate 40°C for 5min. • May need to do VERY light pop spin to get any liquid out of cap before placing on magnet. Resuspend beads by lightly flicking if necessary, after spin. • Put tube on magnet, ~1 min or until supernatant is clear. • Move supernatant to fresh RNase-free tube. • Add 800 µl 100% EtOH, 11 µl 3 M NaOAc pH 5.5, 2 µl GlycoBlue. • Precipitate. • After precipitation, wash pellet well to remove all salts before reverse transcription. Ex. wash pellet 3-4x with 70-80% EtOH, vortexing the pellet and spinning 5 min, 20, 000 x g, 4°C to recover pellet in between washes. • Resuspend pellet in 5 µl 10 mM Tris 7.0 and move to 0.2 ml PCR tube. **5. Reverse Transcription** • To 5 µl of RNA from above, add 2 µl 5x First Strand Synthesis buffer \(final reaction concentration of 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl2) and 0.5 µl 2 M oCJ200-link2 primer \(PAGE purified). • Denature the RNA at 80°C for 2 min, then move tube to benchtop, allowing templates to pre-anneal for 5 min at room temperature. • Add 0.5 µl SUPERase Inhibitor, 0.5 µl 0.1M DTT, 0.5 µl TGIRT-III enzyme. Mix. *The 0.1M DTT stock is prepared freshly from powder the day of reverse transcription. Additionally, fresh aliquots of all RT components, including the primer and buffer from above, should be used when possible. • Incubate reaction at 57°C for 1.5 hours in a thermocycler with a heated lid. • Add 1 µl 5M NaOH, pipetting up-and-down to mix, and incubate at 95°C for 3 min. • Add 154 µl 10 mM Tris pH 7.0, 800 µl 100% EtOH, 16 µl 3M NaOAc pH 5.5, and 1 µl GlycoBlue. Mix well. • Chill for >30 min at -30°C to precipitate nucleic acids. Spin >45 min at 4°C at max speed \(>20,000 x g). Wash pellet with 70-80%

EtOH and air-dry. • Resuspend pellet in 8 µl 10 mM Tris 7.0. • Add 8 µl 2x TBE-Urea Sample Buffer to sample. • Denature cDNA/dye solution 80°C for 2 min. Keep sample on ice before loading gel. • Load entire sample volume onto 6% TBE-Urea gel, run 45 min at 150 V. • Stain gel in 1x SYBR Gold, and cut RNA fragments of 170-240 nucleotides in size, which removes residual RT oligo which runs at ~80 nucleotides. • Extract cDNA from gel slice as described in fragmentation section, using 300 µl 0.3 M NaCl, Costar columns, and EtOH precipitation. • Resuspend pellet in 15 µl 10 mM Tris 8.0. **6. Circ Ligation** • Make Circ mastermix, with 2 µl of 10 x CircLigase buffer, 1 µl 1 mM ATP, and 1 µl 50 mM MnCl2 for each reaction, which will have a 20 µl final reaction volume. • Add 4 µl Circ mastermix to 15 µl cDNA/Tris 8 sample from above. Mix well. • Add 1 µl CircLigase ssDNA Ligase. Mix. • Incubate 60°C for 2 hours. • Incubate 80°C for 10 min to heat inactivate enzyme. Keep on ice and proceed to PCR or store at -20°C. **7. PCR** • Mix 3.4 µl 5x Phusion High Fidelity buffer, 0.4 µl 10 mM dNTPs, 0.2 µl Phusion, 11.9 µl RNase-free H2O, 0.2 µl 100 M oNTI231, 1 µl 10 M indexing oligo, and 1 µl of circ ligation product \(1/20th of reaction) for an 18 µl final reaction volume. PCR Program Initial denaturation: 98°C, 30 sec 8-13 cycles with the following settings: Denaturation: 94°C, 15 sec Annealing: 55°C, 5 sec Extension: 65°C, 10 sec • Add 3 µl 6x DNA Loading dye, load entire sample on 8% TBE gel, run 50 min at 180 V to remove empty vectors from fragment-containing inserts. • Stain gel with SYBR Gold and image on blue light as previously described. • Cut gel slice from ~200-300 nucleotides, and extract dsDNA from gel slice as described in fragmentation section, using 300 µl 0.3 M NaCl, Costar columns, and EtOH precipitation. • Resuspend pellet in 10 µl 10 mM Tris pH 8.0 and quantify by bioanalysis before sequencing. *Libraries were constructed for use with the custom Read1 sequencing primer oNTI202.

Timing

6 days.

Troubleshooting

This protocol is optimized for the production of fragments suitable for 50 bp single read sequencing. Generating libraries with larger fragment inserts is possible, but may require a scaling of input material given high loss during protocol steps, such as inefficiencies in gel extraction for larger fragment sizes. Generally, it may be preferable to use T4 PNK for 3’ end healing instead of rSAP to also resolve cyclic phosphates after fragmentation. The preferred T4 PNK conditions would be 30 min at 37°C in reaction conditions: 100 mM Tris/acetate pH 6.0, 3 mM MgCl2, and 2 mM DTT.

Anticipated Results

This protocol will produce libraries ready for sequencing on an Illumina platform, with a final library size of ~225 nucleotides and a final molarity of 2-4nM, depending on the number of PCR cycles used in the final step.

References

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Figures

name	purpose	sequence (5' to 3')
Linker 2	3' Cloning adaptor for RNA footprints	5rApp/CACTCGGGCACCAAGGA/3ddC
oCJ200-link2	Primer for reverse transcription of sequencing libraries	5'/5phos/GATCGTCGGACTGTAGAACTCTGAACCTGTCG/iSp18/CAAGCAGAAGACGGCATACGAGATTCCTTGGTGCCCGAGTG
oNTI231	Amplification of sequencing libraries, paired with indexing primer	caagcagaagacggcatacga
Indexing primer with 6bp TruSeq index		aatgatacggcgaccaccgagatctacacgatcggaagagcacacgtctgaactccagtcacNNNNNNcgacaggttcagagttc
oNTI202	Read1 sequencing primer	CGACAGGTTCAGAGTTCTACAGTCCGACGATC

Figure 1

Table 1 Oligonucleotides used in protocol