A Fast One-Step Digestion of DNA or RNA for Global Detection and Characterization of Nucleotide Modifications

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Introduction

The Nucleoside Digestion Mix (NEB #M0649) is an optimized mixture of enzymes that provides a convenient one-step method to generate single nucleosides for quantitative analysis by liquid chromatography-mass spectrometry (LC-MS). It digests ssDNA, dsDNA, and RNA, and tolerates a wide range of base and ribose modifications (1-11). The Nucleoside Digestion Mix has also shown activity towards unnatural nucleobases, as demonstrated by Floyd Romesberg in collaboration with scientists at NEB; this work describes how bases lacking any hydrogen bonds are propagated with high fidelity *in vivo* in *E. coli.* (12). The digestion protocol is fast and very reliable, and has greatly facilitated the characterization and global quantification of DNA and RNA modifications (1-11). Moreover, the low-glycerol formulation (< 1%) significantly reduces glycerol-induced ion suppression during mass analysis.

In this application note, we present examples of how the Nucleoside Digestion Mix has been used to quantify epigenetic DNA modifications, to measure the activity of nucleic acid-modifying enzymes, and to monitor the metabolic incorporation of azido modified nucleosides into cellular RNA.

Protocol

1. Setup the following reaction:

COMPONENTS	VOLUME
Nucleoside Digestion Mix Reaction Buffer (10X)	2 µl
DNA or RNA Substrate	to 1 µg
Nucleoside Digestion Mix	1 µl
Water	to 20 µl*

* Reactions can be scaled up to 50 µl without any loss in activity.

2. Incubate at 37°C for 1 hour.
3. Sample is now ready for LC-MS analysis. NO additional purification is required.

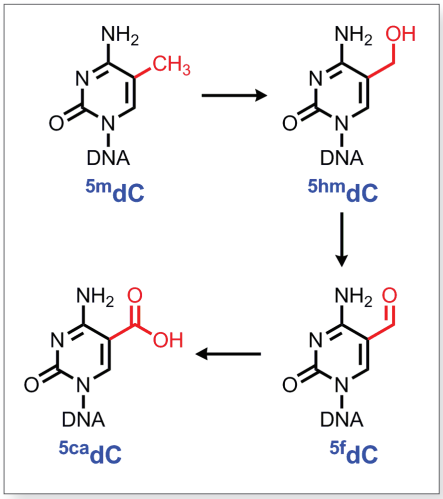
Technical Tips

- For an efficient digestion, **ensure complete removal of ethanol or isopropanol**. Ethanol (or isopropanol) precipitation is often used to purify nucleic acids. Residual ethanol (or isopropanol) in the DNA or RNA sample may affect the performance of the digestion. We recommend air drying the pellet for 5-10 min at room temperature (typically by placing the tubes upside down after decanting most out the ethanol), then speed-vac drying for 5-10 min. If a large amount of DNA/RNA is used, longer times may be required to completely remove all residual ethanol. If vacuum evaporation is used, care must be taken not to over dry the pellet, as this will greatly decrease its solubility. Note that isopropanol is less volatile than ethanol, so it may require more time to dry the nucleic acid pellet.
- The Nucleoside Digestion Mix has reduced activity on DNA and RNA oligonucleotides with highly modified backbones. An example of these are therapeutic oligonucleotides, such as antisense RNAs, which contain clustered ribose modifications like 2´-O-methyl ribose to resist nuclease cleavage. The Nucleoside Digestion Mix will not digest a 5´-3´ bridging phosphorothioester linkage (wherein a sulfur atom replaces a non-bridging oxygen in the phosphate backbone) or an inverted 5´-5´ triphosphate bridge (such as in the mRNA cap structure). If your sample contains complex secondary structures and multiple base and backbone modifications (e.g., as in total RNA extracts), we recommend **increasing the ratio enzyme mix/nucleic acid from 1 µl/µg substrate to 5-10 µl/µg substrate.** Alternatively, the **incubation time may be increased from 1 hour to overnight.**

Quantification of Epigenetic Modifications

Epigenetic modification of DNA nucleobases exists in a wide variety of organisms, and plays important roles in both prokaryotes and eukaryotes. The most studied epigenetic modification is DNA methylation, including *N*6-methyladenosine (^{N6m}A), 5-methylcytosine (^{5m}C), and *N*4-methylcytosine (^{N4m}C). In prokaryotes, these modifications are involved in protecting bacterial genomes from restriction endonucleases, which target invading bacteriophage DNAs. In eukaryotes, cytosine methylation is reported to play important roles in the control of gene expression, parental imprinting, and developmental regulation in both physiological and pathological conditions. Recent studies have shown that ^{5m}C can be successively oxidized to 5-hydroxymethylcytosine (^{5hm}C), 5-formylcytosine (^{5f}C), and 5-carboxylcytosine (^{5ca}C) by the ten-eleven translocation (TET) family of enzymes (Figure 1) (13).

FIGURE 1:
Cytosine modifications in mammalian genomic DNA



Several approaches have been utilized to study DNA modifications, including thin layer chromatography (TLC), liquid chromatography-mass spectroscopy (LC-MS), modification-specific antibodies and restriction endonucleases, and chemical labeling. The main challenge with the quantification of modified nucleobases in genomic samples is the relatively low levels of these modifications. The Nucleoside Digestion Mix has enabled the accurate quantitation of cytosine modifications by liquid chromatography-tandem mass spectrometry (LC-MS/MS) with sensitivity down to < 0.002% relative abundance (~ 2 modifications per 100,000 bases, Table 1) (4).

Table 1: **The Nucleoside Digestion Mix enables accurate quantitation of dC modifications in mouse E14 embryonic stem cell genomic DNA down to < 0.002% natural abundance.**

E14 gDNA was digested with the Nucleoside Digestion Mix, and then analyzed by LC-MS/MS. The relative amounts of ^{5m}dC, ^{5hm}dC and ^{5f}dC were calculated relative to dC, which was set to 10^6. Experimental values were compared to previously reported data (13).

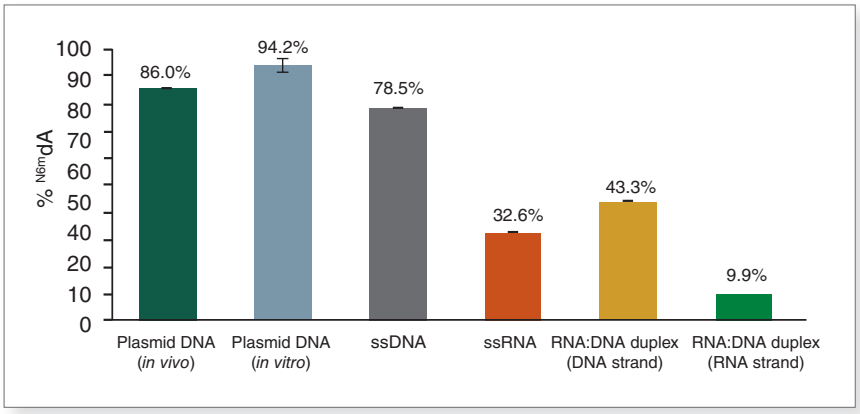
E14 gDNA	dC	^{5m}dC	^{5hm}dC	^{5f}dC
Experimental	10^6 (100%)	33600 (3.4%)	1490 (0.15%)	20.2 (0.0020%)
Reference 13	10^6 (100%)	29600 (3.0%)	1120 (0.11%)	17.9 (0.0018%)

Measuring the Activity of Nucleic Acid Modifying Enzymes

Typical methods for assaying the catalytic activity of enzymes that modify DNA and RNA employ radioactivity, fluorescent substrates, or antibody-based immune assays. Direct detection is difficult since nucleic acids are large, highly structured polymers and their modifications are often silent to commonly-used detection techniques, such as PCR and gel electrophoresis. LC-MS is a direct and accurate method for quantification of DNA and RNA modifications such as methylation, oxidation or glycosylation. As an example, the non-specific nature of the adenine DNA methyltransferase M.EcoGII (NEB #M0603) was determined by LC-MS analysis of M.EcoGII-treated DNA and RNA samples digested with the Nucleoside Digestion Mix. This enzyme can catalyze methylation of up to 86% of dA residues of DNA substrates *in vivo* and 96% *in vitro,* thereby rendering them insensitive to cleavage by multiple restriction endonucleases (11). Additionally, M.EcoGII is able to methylate single-stranded RNA and DNA-RNA hybrid substrates (Figure 2).

FIGURE 2: **The characterization of the non-specific nature of the adenine DNA methyltransferase M.EcoGII (NEB #M0603) using the Nucleoside Digestion Mix.**

EcoGII Methyltransferase-treated DNA and RNA samples were converted to nucleosides using the Nucleoside Digestion Mix, and analyzed in duplicate using LC-MS. In each sample, the relative percentage the dA bases methylated was calculated as ^{N6m}dA/(^{N6m}dA+dA). Samples analyzed were as follows: *In vivo* assay, from a pRRS:M.EcoGII high-copy replicon that constitutively expresses the EcoGII Methyltransferase introduced into methylation-deficient ER2796 *E. coli* cells; *In vitro* assay, from purified pUC19 plasmid DNA; ssDNA, from single-stranded M13mpl8 bacteriophage DNA; ssRNA, from a 1.8 kb *in vitro* transcribed F-luc RNA; RNA:DNA hybrid duplex, from a a synthetic 48 mer DNA:RNA hybrid oligonucleotide substrate (containing 10 dA bases in the DNA strand and 18 rA bases in the RNA strand, respectively).



Monitoring the Metabolic Incorporation of the Azide Functionality into Cellular RNA

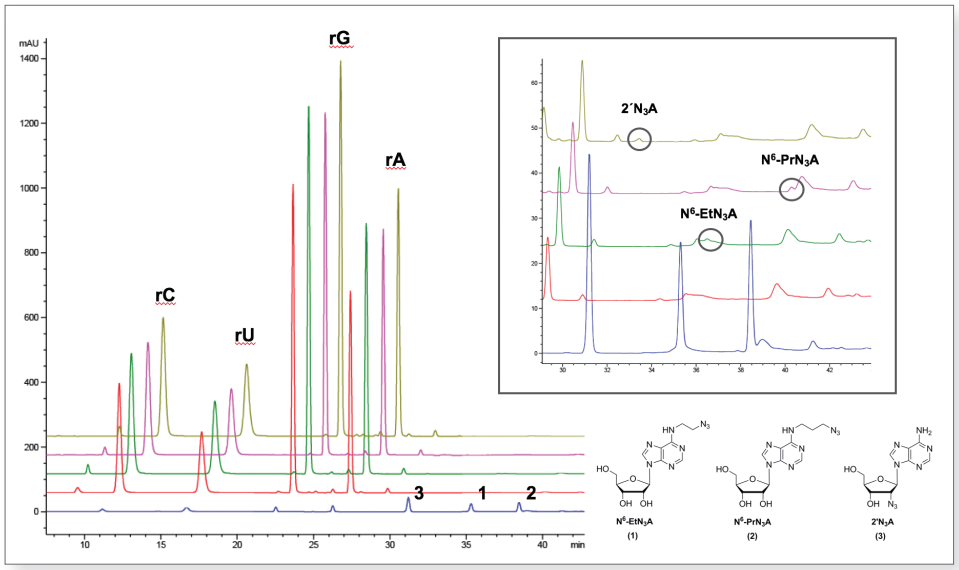
Tracking RNA transcription and post-transcriptional regulation is critical to understanding the cellular mechanisms underlying healthy and diseased states. One of the techniques used to interrogate the function of coding and non-coding RNAs is to incorporate modified nucleosides into cellular transcripts. The combination of the Nucleoside Digestion Mix with LC-MS analysis has been used to detect and quantify the incorporation of azido nucleosides into cellular RNA (9). Cells treated with chemically synthesized N^6-ethylazido adenosine (N^6-EtN_3A), N^6-propylazido adenosine (N^6-PrN_3), and 2´-azidoadenosine (2´N_3A) incorporated 0.2-0.3% of these nucleoside analogues relative to canonical adenosine (Figure 3). Azidonucleosides are utilized for labeling and real-time imaging of nascent RNA (9).

Conclusion

The realization that our knowledge about complete genome and transcriptome sequences is not sufficient to understand how genomic information is decoded or regulated has turned epigenetics and epitranscriptomics into some of the most exciting and fastest-growing areas of biological research. Considerable efforts have been directed towards the development of methods to identify and quantify DNA and RNA modifications in different cell types, tissues, and organisms. The Nucleoside Digestion Mix, which is optimized for rapid and robust hydrolysis of DNA or RNA to single nucleosides, coupled with sensitive LC-MS analysis, provides a valuable tool for genome- and transcriptome-wide profiling of base composition, modification and temporal fluctuation due to environmental changes.

FIGURE 3: **Chemically-modified nucleosides metabolically incorporated into RNA are detected and quantified with the aid of the Nucleoside Digestion Mix.**

RNA from cells treated with N^6-ethylazido adenosine (green), N^6-propylazido adenosine (magenta), and 2´-azido adenosine (light brown) was digested to ribonucleosides using the Nucleoside Digestion Mix and analyzed by LC-MS. Digested RNA from wild-type cells is shown in red. A nucleoside standard mixture containing rC, rU, rG, rA, and the azido nucleoside analogues is shown in blue. The insert shows an expansion of the overlaid chromatograms demonstrating the specific incorporation of the azido nucleosides.



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