Review of: "Purely enzymatic incorporation of an isotope-labeled adenine into RNA for the study of conformational dynamics by NMR"

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Potential competing interests: The author(s) declared that no potential competing interests exist.

Feyrer et al present a method for the enzymatic incorporation of a site-specific (the initiating) adenosine-5'-monophosphate that is labeled with stable isotopes into a 30nt RNA. Using segmental labeling, a larger 46nt RNA was created and with NMR they were able to unambiguously assign the resonances of the adenosine residue and confirm the presence of a second conformation that is in slow exchange with the ground state. The idea of using (labeled) AMP as initiating nucleotide in transcripts produced with T7RNAP is interesting and also holds promise for labeling RNAs for applications in other fields of research besides NMR. However, I have several suggestions and comments for the authors to improve their manuscript.

The style and grammar can be improved throughout the entire manuscript. A few examples:

Line 46: use 'although' instead of 'while

Line 75: 'making it highly specific for the 5' position' is redundant here.

Line 82: replace 'uses' for 'describes'

Line 165: 'illuminated in' replace for 'imaged using'

Line 254: 'stoichiometric amounts of'

Line 255: 'contains all' instead of 'uses the entirety'

Line 335: What do the authors mean with 'diminishing returns'?

- The introduction of the manuscript is a bit unorganized. In lines 63-68, the authors briefly describe segmental labeling as applied by Duss et al, but this paragraph is in between two paragraphs for site-specific labeling methods. It would be better placed around lines 94-95 and, since this technique is of importance for the author's work, explained into more detail and maybe also refer to other groups besides Duss et al alone.
- In the discussion, the authors could give a vision of what use their method is in other research fields.
- The authors claim to thorough report on yields (line 365), but in my opinion that is not yet the case. I would expect a table with total input template, total RNA yield, NTP incorporation yield, purification yield etcetera for both labeled and unlabeled fragments next to each other so that the reader has a direct overview of the differences between the three RNA strands produced.
- In lines 71 and 86 the authors use the words 'often' and 'many' but to my knowledge NMR studies that employ the incorporation of labeled NMPs as initiating nucleotide are not that widespread.
- Rephrase sentences 86-88. It reads like the authors are going to use something that is expected to give them

problems. This point could be sold to the reader in a better way.

- Has the described 46nt RNA any biological origin or function, or is it completely synthetic?
- Where have the authors purchased their reagents other than the DNAs and enzymes?
- Why do the authors emphasize on the ‘Standard Desalting’ for their DNA oligos in lines 119 and 188? I can imagine that for the ligation splint a certain degree of inhomogeneity is acceptable, but I would expect that for transcription the templates should be as homogeneous as possible. T4 DNA ligase is capable of ligating gaps (Nilsson, NAR, 1982) which may occur due to impurities in the produced RNAs.
- The part describing the denaturing PAGE (lines 152-160) needs to be checked. It is written sloppy.
- Page 8, line 173: is this the Prep (22 x 250mm) or the Semi-Prep column (9 x 250mm)?
- Page 9, RNA ligation: how long and at what temperature was the ligation reaction carried out? Which splint has ultimately been used for the ligation reaction? It is a bit redundant to mention 2 splints that work equally well.
- Line 203: Rephrase sentence
- Line 207: samples were refolded at 10-50 uM? How is that?
- Line 230: ligated to the 5’-RNA
- Lines 257 – 260: This should be rephrased or rewritten. The ligation yield is obviously much more than 45%, concluding from the gel image.
- Lines 333+334: The authors claim a low yield due to sample loss during HPLC purification. Have the authors considered PAGE purification? Or at least they could mention this as alternative.
- Lines 345,346: The authors really should change this remark. There is no evidence to date that T7 RNAP is able to start with other residues than purines (Chamberlin). It may even skip the first pyrimidine residue(s) after the promoter and initiate with a downstream templated purine instead (Imburgio, Biochemistry 1999).
- Line 349: labels closer to the 5’-termini would not pose a problem, only the 3’-termini, right? Next sentence: It ‘could potentially’
- Wouldn’t a construct flanked on the 3’-end with a (hammerhead) ribozyme also be a potential workaround for short sequences?
- Line 352: references to these statements are missing.
- Line 357: remove ‘spin’ here. It is confusing and readers may think it concerns a paramagnetic label.
- The authors should also rephrase lines 363-365: the weak….. RNA strand. This doesn’t sound attractive.