Letter to the Editor



# Identifying intronic circRNAs: progress and challenges

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In an insightful editorial (1), Bogard and colleagues discussed a recent publication from our laboratory describing the method 'RNase R treatment followed by Polyadenylation and poly(A)$^+$ RNA Depletion' (RPAD) to isolate highly enriched circular RNAs (circRNAs) from heterogeneous total RNA pools (2). With accumulating evidence that circRNAs influence gene expression by modulating the function of regulatory noncoding RNAs (e.g., microRNAs) and RNA-binding proteins (3-5), interest in circRNAs has escalated in recent years. The function of a circRNA depends on its sequence (6), and this has traditionally been inferred by locating the junction sequence and predicting bioinformatically the intervening exon sequences flanked by the junction. Unfortunately, this assumption may not be accurate in every case, and thus a number of methods to enrich circRNAs experimentally have been developed.

Depletion of ribosomal (r)RNA coupled with treatment with an exonuclease such as RNase R and/or removal of endogenous polyadenylated RNAs have been widely used to augment the concentration of circRNAs. However, these methods are inefficient and do not completely remove highly structured and non-polyadenylated RNAs, leaving large populations of linear RNAs intact. Given that the abundance of most circRNAs is lower than that of their linear RNA counterparts, even a small proportion of linear RNAs left in the circRNA-enriched population may interfere with downstream analyses. Considering these important obstacles, RPAD was designed to eliminate linear RNAs to near completion. In the RPAD procedure, after RNase R digestion, most of the remaining RNAs with a free 3'OH end were removed by introducing an additional step of polyadenylation followed by depletion of poly(A)-containing transcripts using oligo-d(T) beads and rRNA depletion. The actual full-length circRNA sequences could then be generated by aligning the RPAD-generated RNA-sequencing reads between the back-splice junction coordinates, instead of relying on bioinformatic predictions (2). This procedure effectively removed highly structured and non-polyadenylated RNAs and led to the identification of known and novel exonic circRNA (EcircRNA) isoforms as well as many novel intronic circRNAs (IcircRNAs) (2).

RPAD revealed an unexpectedly high amount of circRNAs in the latter class, those circRNAs derived from introns (IcircRNAs). Intronic sequences arising from lariats, splicing intermediates with 2',5' phosphodiester bonds, were previously shown to form circular intronic RNAs (ciRNAs) (7), but since lariats have free 3'OH ends, they were predicted to be depleted by RPAD. However, RPAD did not eliminate 100% of all linear RNAs, and even after polyadenylation and depletion of polyadenylated RNAs, small percentages (typically less than 2%) of linear RNAs remained. Given that some lariats remained in the RNA pool that escaped poly(A) depletion, the IcircRNAs identified by RPAD likely included a proportion of lariat ciRNAs with 2',5' junctions. Intense efforts are underway in the field to characterize the mechanisms that govern the biogenesis of circRNAs of all types, including IcircRNAs. The development of methods such as RPAD to improve the isolation of circRNAs will go a long way towards facilitating the identification of circRNAs, elucidating their function, and enabling their use in therapy.

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