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## Methods for analysis of circular RNAs

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### Abstract

Eukaryotic cells express a myriad of circular RNAs (circRNAs), many of them displaying tissue-specific expression patterns. They arise from linear precursor RNAs in which 5' and 3' ends become covalently ligated. Given these features, biochemical and computational approaches traditionally used to study linear RNA must be adapted for analysis of circular RNAs. Such circRNA-specific methodologies are allowing the systematic identification of circRNAs and the analysis of their biological functions. Here, we review the resources and molecular methods currently utilized to quantify circRNAs, visualize their distribution, identify interacting partners, and elucidate their function. We discuss the challenges of analyzing circRNAs and propose alternative approaches for studying this unique class of transcripts.

This article is characterized under:

RNA Structure and Dynamics > RNA Structure, Dynamics, and Chemistry

RNA Methods > RNA Analyses in vitro and In Silico

RNA Methods > RNA Analyses in Cells

### Keywords

backsplice junction; circRNAs; divergent primers; ribonucleoprotein complex

## 1 | INTRODUCTION

The ENCODE Project Consortium has discovered that while >80% of the human genome is transcribed, only ~2% of the transcribed RNAs are translated into proteins, indicating that vast portions of RNA are noncoding and may play regulatory roles (Atianand & Fitzgerald, 2014). Much of this noncoding (nc)RNA is linear, including ribosomal (r)RNAs, long

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#### CONFLICT OF INTEREST

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noncoding (lnc)RNAs, transfer (t)RNAs, and micro (mi)RNAs. However, a vast class of ncRNAs with a circular structure (circRNAs) has emerged over the past few years through high-throughput RNA sequencing (RNA-seq) coupled with novel bioinformatic analyses. Circular RNAs are ubiquitous and highly abundant (tens of thousands in a given cell type), display tissue specificity, and are more evolutionarily conserved than lncRNAs (Memczak et al., 2013; Rybak-Wolf et al., 2015; Salzman, Chen, Olsen, Wang, & Brown, 2013).

Most circRNAs are believed to be generated from precursor (pre-)RNAs through backsplicing of exonic sequences involving the canonical splicing machinery or from intronic lariat precursors (Cocquerelle, Mascrez, Hetuin, & Bailleul, 1993; Jeck et al., 2013; Kristensen et al., 2019; Liang & Wilusz, 2014; T. Li et al., 2018; Nigro et al., 1991). Due to the lack of free ends, circRNAs are substantially more stable than their linear counterparts (Enuka et al., 2016). Although the molecular functions of only a small fraction of circRNAs have been elucidated to-date (discussed in Section 1.2), they generally influence gene expression patterns by binding proteins and nucleic acids. By affecting gene expression programs, circRNAs modulate key cellular processes, including proliferation, differentiation, senescence, innate immune response, and the maintenance of stem cell pluripotency (Bachmayr-Heyda et al., 2015; Gu, Li, Jin, Liu, & Wei, 2017; C. X. Liu et al., 2019; Panda et al., 2017; Yu et al., 2017). Accordingly, circRNAs have been implicated in several pathologies, including cancer, Alzheimer's disease, Parkinson's disease, cardiovascular disease, and atherosclerosis (Floris, Zhang, Follesa, & Sun, 2017; Holdt et al., 2016; Lukiw, 2013; D. Yang, Yang, & Yang, 2018; Zhao & Shen, 2017). In addition, as efforts intensify to find new biomarkers for a range of conditions, circRNAs are emerging as promising candidates (Y. Li et al., 2015). Here, we review the current analytical tools and molecular biology approaches available to investigate circRNA levels, distribution, sequence, and interaction partners.

## 1.1 | Biogenesis

There has been substantial progress in elucidating the mechanisms of circRNA biogenesis (Jeck et al., 2013; Liang & Wilusz, 2014; T. Li et al., 2018; Kristensen et al., 2019). CircRNAs can be generated from exons (ecircRNAs), introns (ciRNAs) or a combination of both (eicRNAs) (Zhao & Shen, 2017). Spliceosome-dependent backsplicing uses the canonical spliceosome machinery to join the downstream 5' donor site of an exon and the upstream 3' acceptor site (L. L. Chen & Yang, 2015; Vicens & Westhof, 2014). Intron-pairing and lariat-driven circularization rely on reverse complementary motifs of introns to promote the circularization through alternative 5' to 3' splicing (Ivanov et al., 2015; X. O. Zhang et al., 2014). In addition, circularization can also be mediated by RNA-binding proteins (RBPs) forming circRNAs under particular conditions or stimuli. These RBPs bind to specific RNA sequences and bring donor—acceptor ends in close proximity, enabling circularization (Ashwal-Fluss et al., 2014; Errichelli et al., 2017; X. Li et al., 2017).

## 1.2 | Functions

**1.2.1 | microRNA sponge**—circRNAs that possess binding sites for microRNAs can function as competing endogenous RNAs (ceRNAs), as they can act as microRNA sponges and thereby prevent microRNAs from binding to target mRNAs. One of the earliest

circRNAs identified, *ciRS-7*, possesses over 50 binding sites for miR-7, while the mouse circRNA sex-determining region Y, *Sry*, has 16 binding sites for miR-138; by binding to these microRNAs, *ciRS-7* and *Sry* reduced miRNA interactions with their mRNA targets (Hansen et al., 2013). Other circRNAs with similar functions have been described, including *circHIPK3*, which can potentially sponge miR-124, miR-30a-3p, and miR-558 (Hansen et al., 2013; Y. Li et al., 2017; Shan et al., 2017; Zheng et al., 2016). By sponging microRNAs, several circRNAs have been found to modulate cell growth, proliferation, and differentiation (Hansen et al., 2013).

**1.2.2 | Protein sponge**—CircRNAs can also bind proteins and these interactions can modulate RBP activity. *CircMbl* acts as a sponge for muscle blind (MBL) protein, an essential factor for the alternative splicing of *Mbl* pre-mRNA, reducing *Mbl* pre-mRNA alternative splicing. When MBL is abundant, a feedback loop mechanism is triggered whereby *Mbl* pre-mRNA preferentially yields *circMbl*, which in turn binds to MBL and decreases its bioavailability (Ashwal-Fluss et al., 2014). In another example, HuR binding to *circPABPN1* prevents HuR from binding *PABPN1* mRNA, in turn leading to a reduction in PABPN1 production and diminished proliferation (Abdelmohsen et al., 2017). Recently, endogenous circRNAs were found to form 16–26 bp imperfect RNA duplexes that bind the innate immune dsRNA receptor (PKR) and repress its activity (C. X. Liu et al., 2019). These findings further support the notion that circRNAs can regulate protein homeostasis.

**1.2.3 | Transcriptional regulation**—Some nuclear circRNAs, particularly eicRNAs, can interact with RNA Pol II at the promoter region of select host genes. Illustrating this function, *circEIF3J* and *circPAIP2* bind the *U1* snRNP in the nucleus (Z. Li et al., 2015), and the resulting complex then interacts with RNA Pol II in the promoter region of the host genes (*EIF3J* and *PAIP2*), promoting transcription.

**1.2.4 | Translation potential**—There is increasing evidence that some circRNAs can be translated partially. For example, *circZNF609* in mammalian myoblasts and *circMbl* in fruit fly were recently shown to have coding potential (Legnini et al., 2017; M. Zhang et al., 2018). Each circRNA has an IRES (internal ribosome entry site) through which ribosomes translate segments of the circRNA independently of the 5′-cap required for linear RNA translation. In addition, Y. Yang et al. (2017) reported that N<sup>6</sup>-methyladenosine (m<sup>6</sup>A) was capable of mimicking an IRES to trigger the initiation of translation. In a more high-throughput manner, a translational landscape analysis of the human heart has identified 40 circRNAs encoding novel microproteins from 39 genes (van Heesch et al., 2019).

## 2 | APPROACHES TO STUDY circRNAs

With increasing need to investigate circRNAs with depth and precision, it has become essential to utilize tools and methodologies that investigate accurately circRNA sequence, length, subcellular localization, biological function, disease implication, interacting molecules, and therapeutic potential. In this section we summarize the resources and techniques available at present to study circRNAs, and we discuss the strengths and limitations of each method.

## 2.1 | Online circRNA resources

Several databases and tools have been established to provide key bioinformatic knowledge about circRNAs. For instance, circBase informs about circRNA identity and tissue specificity based on RNA-seq data (Memczak et al., 2013) and CircInteractome can be used to design divergent primers and siRNAs (Dudekula et al., 2016). CircInteractome, starBase, CircView, and TRcirc can identify potential interacting factors, including microRNAs, RBPs, and transcription factors (TFs) (Dudekula et al., 2016; Feng et al., 2018; J. H. Li, Liu, Zhou, Qu, & Yang, 2014; Tang et al., 2018). Similarly, circRNABase and CircNet can provide information on potential interactions with microRNAs based on global sequencing approaches like HITS-CLIP (crosslinking immunoprecipitation), PAR-CLIP, iCLIP, and CLASH (Y. C. Liu et al., 2016). Given that circRNAs are highly conserved, CIRCpedia can be exploited to find circRNA conservation in several species, cell types, tissues, and disease conditions (Dong, Ma, Li, & Yang, 2018). In addition, ecircRNAs potentially translated into proteins can be studied using CircRNADb. Given their potential link to diseases, several databases have been developed to explore circRNAs in diseases, including Circ2Traits, CSCD, TSCD, and CircR2Disease (Fan, Lei, Fang, Jiang, & Wu, 2018; Ghosal, Das, Sen, Basak, & Chakrabarti, 2013; Xia et al., 2018). These resources, compiled in Table 1, can be used to obtain valuable details for the experimental analysis of circRNAs, as indicated below.

## 2.2 | circRNA sequencing

Advances in high-throughput circRNA sequencing (Circ-seq) analysis have enabled the identification of thousands of circRNAs. Starting from a pool of mixed RNAs, circRNAs can be highly enriched by digestion with RNase R to eliminate most linear transcripts while circRNAs are left intact (Jeck et al., 2013; Salzman et al., 2013). As many linear RNAs, particularly those with strong secondary structures, are refractory to RNase R digestion, Circ-seq can be improved by increasing the purity of the circRNA preparation through an additional polyadenylation and poly(A)+ RNA depletion (the RPAD technique; Panda, De, et al., 2017; Pandey, Rout, Das, Gorospe, & Panda, 2019) and by improving buffer conditions ( $\text{Li}^+$  instead of  $\text{K}^+$ ) to render RNAs containing G-quadruplexes and structured 3' ends susceptible to degradation by RNase R (Xiao & Wilusz, 2019).

Pretreatments to enrich in circRNAs the RNA preparation are not necessary if sequencing is performed at high depth (i.e., 100–150 million reads), since the analysis mainly relies on junction reads that do not exist in canonically spliced linear transcripts. Although Circ-seq analysis detects circRNA junctions, it does not reveal the full sequence of a given circRNA nor the number of circRNA transcripts with the same junction within a given preparation (Szabo & Salzman, 2016). Other assays (below) have been employed to overcome these limitations.

It is important to note that circRNA annotations and analysis algorithms are still evolving. Current annotation strategies begin with the identification of the circularizing junction, and the body of the circRNA is then inferred from known sequences between the circularizing ends. Other annotation methods, based on de novo assembly of the circRNA, are less accurate, as the remnants of linear RNA molecules (possibly also circRNAs with shared

junctions) can confound the assembly. In addition, the nomenclature of circRNAs is also not standardized. CircRNAs are computationally annotated and can be named by default after the circularizing junction; however, in some cases, they are named according to the parent gene annotation (e.g., *circPVT1*), regardless of the actual circularizing junction. CircBase and the cancer-specific circRNAs database (CSCD) have tried to give known circRNAs a unique circRNA ID, but this strategy is not widely used.

### 2.3 | circRNA microarrays

Microarrays are useful tools for high-throughput analysis and multiple comparisons of the expression levels of specific circRNAs. Microarray probes specifically designed to recognize the junction sequences of circRNAs are immobilized on a solid support and incubated with mixed populations of circular and linear RNA. As with Circ-seq, microarray analysis typically includes prior treatment of the RNA sample with RNase R to reduce the presence of linear RNAs and enhance circRNA detection and quantification. This method was successfully employed for investigating several circRNAs, including *circPABPN1*, *circEPSTII*, and *circMTO1* (Abdelmohsen et al., 2017; B. Chen et al., 2018; Han et al., 2017). Microarray use for circRNA profiling has some drawbacks, including the facts that microarray platforms include only a limited number of known circRNAs, identify circRNAs based only on the junction sequences, do not inform on the internal sequence of a given circRNA, and cannot distinguish among circRNAs with shared junction sequences.

### 2.4 | Northern blotting

To investigate circRNAs using Northern blot analysis, short probes can be employed that span the splice junction, although longer probes complementary to the entire circRNA can also be used, as shown for *circHIPK3* (Zheng et al., 2016). In some cases, Northern blot analysis can be employed to distinguish between a circRNA and its linear counterpart, as electrophoresis through denaturing polyacrylamide gels mobilizes linear RNAs at the expected size, while circRNAs tend to run more slowly. Polyacrylamide pore spaces limit the sizes of circRNAs that can be analyzed on polyacrylamide gels to those in the range of 0.2–1 kb, although longer circRNAs can be studied using agarose gels (Tabak et al., 1988). With appropriate adaptations, Northern blot analysis can be used to assess circRNA size, isoforms, processing, sequence, and abundance (Pamudurti et al., 2017).

### 2.5 | PCR-based analyses

**2.5.1 | RT-qPCR analysis**—Circ-seq analysis provides essential information regarding circRNA junctions and annotation, but it is important to perform RT-qPCR (reverse transcription followed by quantitative polymerase chain reaction) analysis to validate high-throughput results. RT-qPCR analysis of circRNA is performed using divergent primers spanning the circRNA junction, which can be designed using online resources such as CircInteractome (Dudekula et al., 2016). In some cases, circRNAs extracted from cells must be enriched by RNase R treatment followed by RT-qPCR analysis, but often this step can be avoided if the circRNA is abundant, as divergent primers do not amplify linear RNA. Another advantage of RT-qPCR analysis is that the products can be sequenced to further verify the junction sequence and may reveal the full sequence of the circRNA of interest

(Panda, Grammatikakis, Kim, et al., 2017). Furthermore, it offers a quantitative estimation of circRNA abundance under different conditions such as circRNA depletion, stress, and disease conditions (Huang et al., 2017; Y. Li et al., 2015; Panda, Abdelmohsen, & Gorospe, 2017) as well as circRNA abundance in different subcellular compartments. Among its limitations, RT-qPCR analysis is designed to be low-throughput.

**2.5.2 | Digital droplet PCR analysis**—Digital droplet PCR (ddPCR) is a relatively new technology that can be used to quantify circRNA copy number. It partitions nucleic acids into nanoliter-sized droplets containing the target sequence for PCR amplification. The ratio of positive to negative droplets is analyzed to assess RNA concentration (Hindson et al., 2011; Quan, Sauzade, & Brouzes, 2018). X. Li, Yang, and Chen (2018) successfully used ddPCR to measure the absolute levels of circRNAs in the plasma of patients with gastric cancer relative to healthy controls, identifying two circRNAs, *hsa\_circ\_0001017* and *hsa\_circ\_0061276*, as potential biomarkers of gastric cancer. Similarly, Panda, Grammatikakis, Kim, et al. (2017) calculated the number of *circPVT1* molecules per cell by ddPCR analysis in senescent and proliferating fibroblasts. While ddPCR and conventional RT-qPCR analyses follow the same principles, ddPCR is more accurate, provides absolute numbers, and measures low-abundance RNAs, which may be particularly valuable for circRNA quantification. However, it requires special instrumentation, software, and proprietary reagents, and therefore its use is somewhat limited.

## 2.6 | RNA fluorescence in situ hybridization

RNA fluorescence in situ hybridization (RNA-FISH) coupled with high-resolution microscopy is widely used to visualize the abundance and subcellular localization of RNA molecules in cells and tissues (Itzkovitz & van Oudenaarden, 2011). RNA-FISH probes targeting circRNA junctions have been used to detect and quantify several circRNAs, including *cdt1AS* and *circHIPK3* (Jeck & Sharpless, 2014; Piwecka et al., 2017; Zheng et al., 2016). Combined with immunohistochemistry, FISH may also be used to assess the colocalization of circRNAs with proteins. A major limitation of this technique is that FISH probe design is restricted to the backspliced junction in order to avoid recognition of the linear RNA counterpart. Thus, unlike the strong FISH signals that often identify linear RNAs thanks to the use of multiple FISH probes recognizing long RNA segments, circRNA FISH signals tend to be weaker, as only one or a small number of FISH probes on the junction sequence can be used for visualization.

## 2.7 | Depletion and overexpression of circRNAs

A major challenge to study circRNA function is reducing circRNA levels effectively, given that, in principle, only the circRNA junction can be targeted by the siRNA without affecting the linear mRNA counterpart. Accordingly, siRNAs/shRNAs are designed specifically to target the circRNA junction, evenly targeting each side of the junction or slightly on one side or the other (Figure 1). This strategy successfully knocked down circRNAs like *circPVT1* in gastric cancer and *circQKI* in primary myoblasts (J. Chen et al., 2017; Legnini et al., 2017). While siRNAs can be manually designed around the junctions, the CircInteractome tool provides multiple options for circRNA siRNAs (Dudekula et al., 2016). In addition, the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology can be

used to delete intronic complementary regions and potentially generate circRNA knockouts (Abudayyeh et al., 2017; Cox et al., 2017). Although most circRNA knockouts are not feasible because the linear counterparts would be disrupted, a *Cdr1as* knockout mouse was successfully created because the linear counterpart was not expressed (Piwecka et al., 2017).

Since circRNA-directed siRNAs may target the linear mRNA or other circRNAs with the same junction sequence, it is essential to perform gain-of-function approaches through overexpression. Backsplicing is triggered when intronic repeats, which usually complement each other, are base-paired to bring the intervening splice sites in close proximity (L. L. Chen & Yang, 2015; Ivanov et al., 2015). This strategy has been used to design expression vectors for the ectopic expression of circRNAs using the cellular splicing machinery (Kramer et al., 2015). For example, the vector pcDNA3.1(+) ZKSCAN1 has been successfully used to express specific circRNAs (Kramer et al., 2015) and was used to investigate the coding potential of *circ-ZNF609* (Legnini et al., 2017). Additionally, circR (pcDNA3.1 His C vector backbone 6.3 kbp) contains an upstream intron and its reverse complementary sequence downstream (~800 bp) to promote circularization. The circRNA of interest and its endogenous flanking sequence, which contains the splicing sites (~200 bp), can be cloned between the two complementary introns. This strategy has been used to overexpress the brain-specific *cdr1AS* and the testis-specific *circSry* (Hansen et al., 2013). Collectively, these tools have been instrumental for studying circRNA functions.

## 2.8 | circRNA affinity pulldown

Several studies have shown that circRNAs form ribonucleoprotein complexes with RBPs, TFs, and miRNAs (Panda, Grammatikakis, Munk, Gorospe, & Abdelmohsen, 2017). CircRNA pulldown can be performed using biotinylated antisense oligomers (ASOs) that specifically recognize the junction sequence of a target circRNA. Using streptavidin-coated beads, the biotinylated probes are pulled down together with interacting RNA molecules and associated proteins. The protein component of the pulldown can be analyzed by mass spectrometry and further assessed by Western blot analysis. The RNA component can be studied by sequencing and RT-qPCR analysis. When studying low-abundance circRNAs, overexpression followed by pulldown may increase the coverage of the interacting partners. Recently, this method was successfully used to investigate several circRNAs. For example, an ASO against *circPVT1* (Panda, Grammatikakis, Kim, et al., 2017) revealed its interaction with *let-7*. Similarly, ASOs against *circFoxo3* uncovered its association with ID1, E2F1, FAK, and HIF-1 $\alpha$  (Du et al., 2016, 2017), *circMbl* ASOs revealed that the circRNA interacted with MBL1 (Ashwal-Fluss et al., 2014), and ASOs that pulled down *circPABPN1* revealed its interaction with HuR (Abdelmohsen et al., 2017).

This technique can provide essential knowledge at the molecular level of the network of circRNA-interacting molecules. The limitations of this approach include background detection of other molecules associated with the ASOs, and inaccessible junction sequences due to circRNA folding.

## 2.9 | Immunoprecipitation of circRNA-RBP complexes

Ribonucleoprotein (RNP) immunoprecipitation (RIP) analysis is another strategy for assessing circRNA interactions with particular proteins. In this assay, an antibody is used to immunoprecipitate an RBP of interest, and the RBP-associated RNAs, including circRNAs, are then detected by RNA-seq, Northern blot, or RT-qPCR analyses. The major advantage of this method is that it allows the study of endogenous complexes in cells or tissues. Using this approach, insulin-like growth factor 2 binding protein 3 (IMP3) was found to bind 34 circRNAs, and HuR was found to bind several circRNAs including *circPABPN1* (Abdelmohsen et al., 2017; Schneider et al., 2016). While RIP followed by RT-qPCR analysis or sequencing can identify target circRNAs, the sites of interaction are not identified. To overcome this limitation, CLIP methods can be employed to determine the specific binding sequences.

## 2.10 | In vitro circRNA synthesis

In vitro synthesized circRNAs have a wide range of applications in molecular biology and potentially in therapeutic applications. They can be synthesized chemically using cyanogen bromide or enzymatically using T4 RNA ligases as well as by using an RNA cyclase ribozyme (L. L. Chen & Yang, 2015; Petkovic & Muller, 2015). In vitro synthesized circRNAs have been introduced into cells to study the innate immune response, as well as to explore ways to synthesize protein in eukaryotic cells (Y. G. Chen et al., 2017; Wesselhoeft, Kowalski, & Anderson, 2018).

## 3 | CONCLUDING REMARKS

Recent advances in RNA-seq and computational analyses have critically increased our knowledge of circRNA biology through the identification and annotation of a large number of circRNAs. However, virtually all circRNAs remain to be characterized in molecular and functional detail. In this review, we highlight key online resources and tools that facilitate the initial steps of circRNA analysis, including circRNA sequence, design of silencing molecules, and association with other molecules. We also summarize and discuss common techniques to assess circRNA identity, abundance, subcellular localization, biochemical interactions, and functions, as well as the challenges and limitations of each approach (Table 2).

Going forward, it will be important to understand in depth how circRNA biogenesis is regulated, including the pathways and factors governing the tissue-specific expression of circRNAs, the kinetics and developmental cues driving biogenesis, the specific RNA segments included, and other mechanistic details. With rising recognition that some circRNAs may have important diagnostic, prognostic and possibly therapeutic value, it will be critical to develop more specific and sensitive detection methods, advanced tools to produce them in tissues and in vitro, and animal models to investigate their full impact in physiology and disease.



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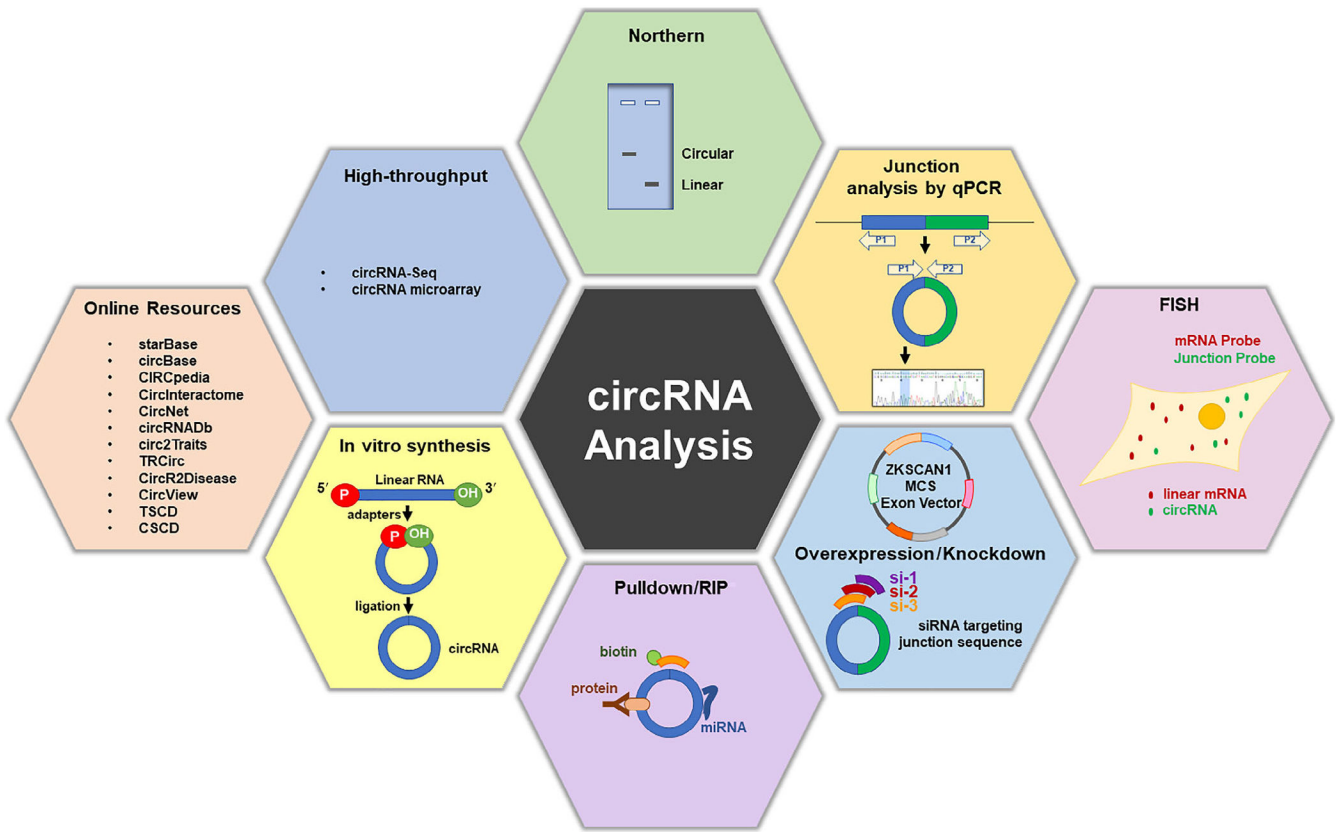
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**FIGURE 1.** Schematic representation of the different approaches and tools to study circular RNA

TABLE 1

## Databases and online resources for circular RNA research

Database	Website	References	Suitable for analysis of
circBase	<a href="http://www.circbase.org">http://www.circbase.org</a>	Glazar, Papavasileiou, and Rajewsky, 2014	circRNA expression levels and sequence
CircInteractome	<a href="http://circinteractome.nia.nih.gov">http://circinteractome.nia.nih.gov</a>	Dudekula et al., 2016	Identification of interacting miRNAs and RBPs, design of divergent primers and siRNAs
CircNet	<a href="http://circnet.mbc.nctu.edu.tw/">http://circnet.mbc.nctu.edu.tw/</a>	Y. C. Liu et al., 2016	circRNA–miRNA interactions
CIRCpedia	<a href="http://www.picb.ac.cn/rnomics/circpedia">http://www.picb.ac.cn/rnomics/circpedia</a>	Dong et al., 2018	circRNAs expressed in different species, cell types, and tissues
circRNADb	<a href="http://reprod.njmu.edu.cn/circrnadb">http://reprod.njmu.edu.cn/circrnadb</a>	X. Chen et al., 2016	Human exonic circRNAs
CircRNABase	<a href="http://www.hzrna.com/circrna-shujuku/circrnabase">http://www.hzrna.com/circrna-shujuku/circrnabase</a>	Y. C. Liu et al., 2016	circRNAs associated with other molecules
CircR2Disease	<a href="http://bioinfo.snnu.edu.cn/CircR2Disease/">http://bioinfo.snnu.edu.cn/CircR2Disease/</a>	Fan et al., 2018	circRNAs associated with disease conditions
CircView	<a href="http://gb.whu.edu.cn/CircView/">http://gb.whu.edu.cn/CircView/</a>	Feng et al., 2018	Identification of interacting miRNAs and RBPs
Circ2Traits	<a href="http://gyanxet-beta.com/circdb/">http://gyanxet-beta.com/circdb/</a>	Ghosal et al., 2013	circRNAs associated with disease conditions
CSCD	<a href="http://gb.whu.edu.cn/CSCD/">http://gb.whu.edu.cn/CSCD/</a>	Xia et al., 2018	circRNAs expressed, interacting miRNAs and RBPs, circRNAs with coding potential
starBase	<a href="http://starbase.sysu.edu.cn/">http://starbase.sysu.edu.cn/</a>	J. H. Li et al., 2014	circRNA–miRNA interactions
TSCD	<a href="http://gb.whu.edu.cn/TSCD">http://gb.whu.edu.cn/TSCD</a>	Xia et al., 2017	Tissue-specific expression (human, mouse), circRNA markers of organogenesis
TRcirc	<a href="http://www.licpathway.net/TRCirc/">http://www.licpathway.net/TRCirc/</a>	Tang et al., 2018	circRNA-transcription factor regulatory network

TABLE 2

## Approaches to study circRNAs

Method	Pros	Cons
Online resources	Allow the identification, annotation and potential associations with <i>trans</i> -acting factors and diseases	Experimental validation required
circRNA sequencing (Circ-seq)	Allows identification and annotation, high-throughput screening, and profiling under different conditions	Does not inform on variants and may include linear RNAs resistant to RNase R
Microarrays	Assess differential expression and circRNA profiling under different conditions	Analysis is limited to the probes on the array, relies on junction sequences, and does not inform on mature sequences
Northern blot analysis	Detects circular versus linear, informs roughly on size and abundance	Not suitable for high-throughput screening
RT-qPCR	Permits Circ-seq validation, may be used to elucidate full sequences	May recognize linear trans-spliced RNAs, not suitable for high-throughput screening
Digital droplet PCR (ddPCR)	Informs on absolute copy number and measures low-abundance circRNAs	Not cost-effective
RNA-FISH	Aids in circRNA visualization, subcellular localization, quantification	Possible detection of linear RNAs
Circ siRNA	Loss of function analysis	May silence linear RNAs
Overexpression of circRNAs	Gain of function analysis	May generate concatemer products
Pulldown with ASOs	Detects <i>trans</i> -acting factors	May recognize linear mRNA counterparts
Immunoprecipitation (RIP)	Detects interaction with RBPs	Does not identify binding site(s)
Synthetic circRNAs	Can be used to study circRNAs and develop therapeutic applications	Are expensive to produce, may contain linear byproducts

Abbreviations: ASO, antisense oligomer; RBP, RNA-binding protein; RIP, ribonucleoprotein immunoprecipitation; RNA-FISH, RNA fluorescence in situ hybridization; RT-qPCR, reverse transcription followed by quantitative polymerase chain reaction.