

YAOHAIBIO

CircRNA

CRO SERVICE PLATFORM

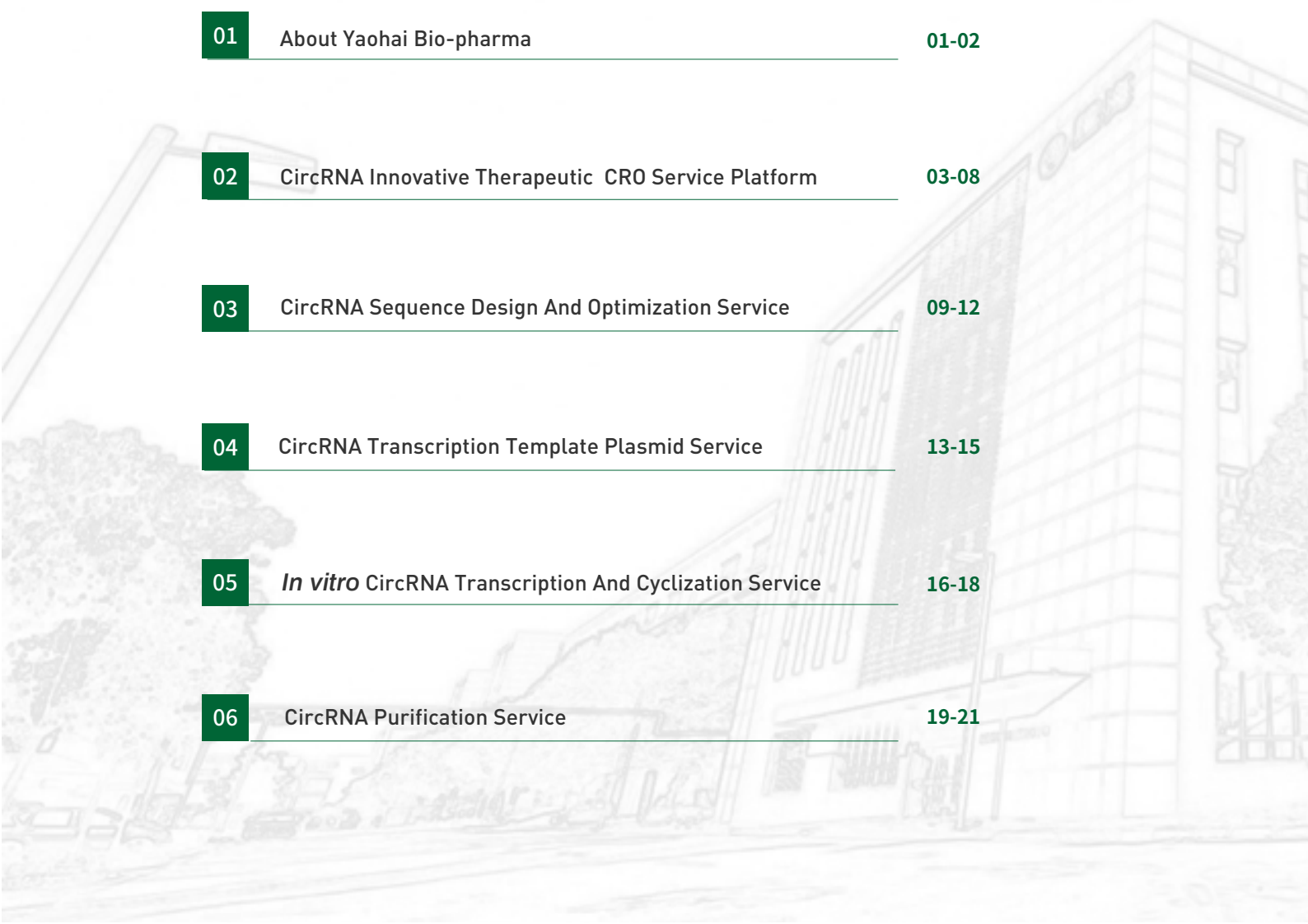


YAIHAI BIO-PHARMA



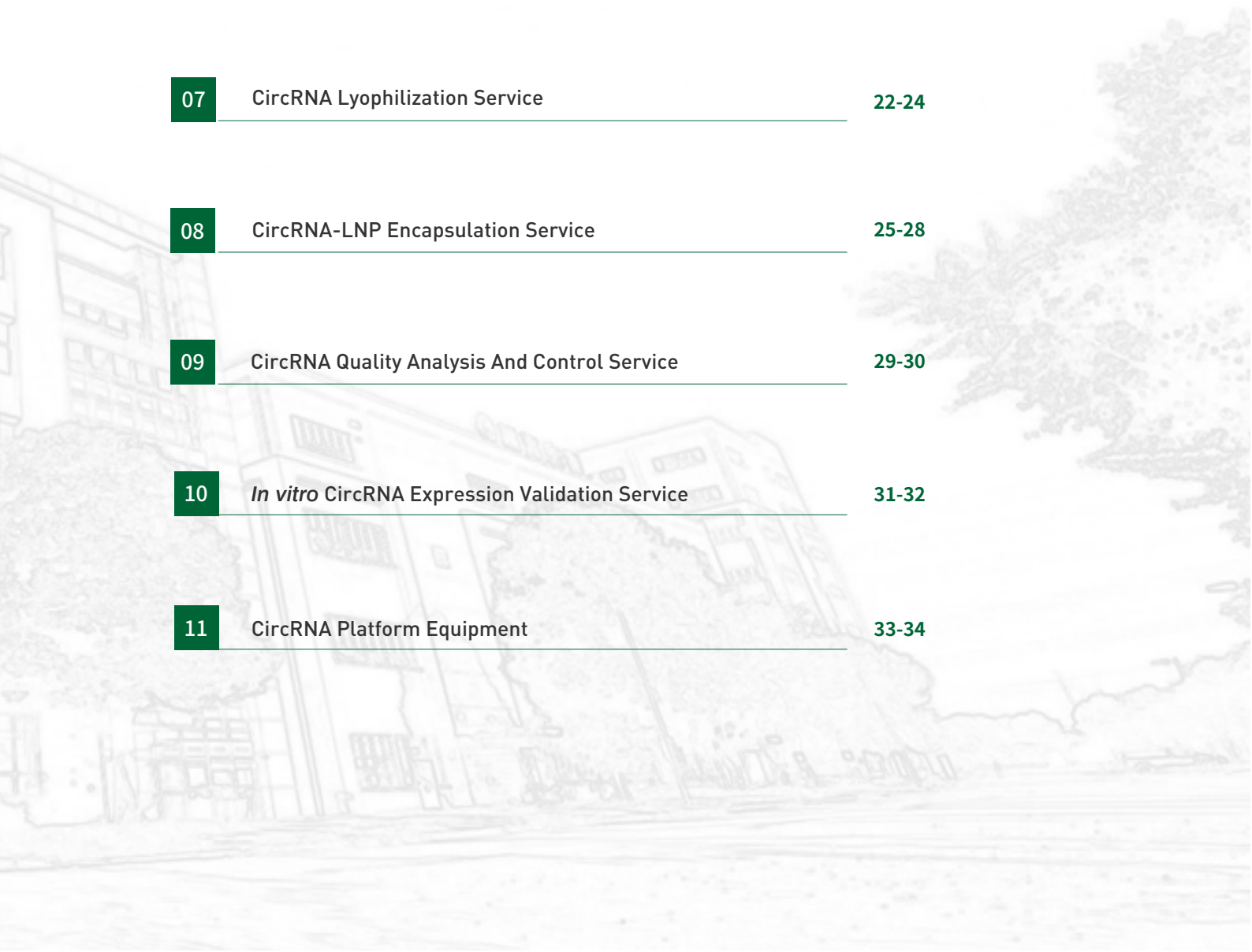
CONTENTS

01	About Yaohai Bio-pharma	01-02
02	CircRNA Innovative Therapeutic CRO Service Platform	03-08
03	CircRNA Sequence Design And Optimization Service	09-12
04	CircRNA Transcription Template Plasmid Service	13-15
05	<i>In vitro</i> CircRNA Transcription And Cyclization Service	16-18
06	CircRNA Purification Service	19-21





07	CircRNA Lyophilization Service	22-24
08	CircRNA-LNP Encapsulation Service	25-28
09	CircRNA Quality Analysis And Control Service	29-30
10	<i>In vitro</i> CircRNA Expression Validation Service	31-32
11	CircRNA Platform Equipment	33-34





ABOUT YAOHAI BIO-PHARMA

Founded in August 2010, Jiangsu Yaohai Biopharmaceutical Co., Ltd. is a national high-tech enterprise based in China Pharmaceutical City Park, Taizhou, Jiangsu Province, China. It is a CDMO service provider specializing in microbial expression systems, focusing on "recombinant proteins/peptides, nucleic acid drugs, Nano-antibodies, cell & gene therapy, novel recombinant vaccines and other fields", and is committed to building an open and integrated CRO/CD-MO/MAH service platform. The company's business covers one-stop CMC services such as engineering bacteria construction, strain library establishment, lab-scale process development and optimization, pilot process scale-up production, clinical sample equipment, specification establishment, analytical method development and validation, GMP compliance, and registration, etc.

The Company adheres to the service concept of "Service with heart and create the future", with the mission of "Create global standards, facilitate the process of new drugs and achieve a healthy life", and continues to empower the creation of new drugs worldwide.



End-to-end Microbial Expression Systems CRDMO / MAH

One-stop service platform

<p>Recombinant Proteins/peptides</p>	<p>Strain development and library building services</p>
<p>Nucleic Acid Drug</p>	<p>Process development and optimization services</p>
<p>Nanobodies</p>	<p>Method development and quality control services</p>
<p>Cell & Gene Therapy</p>	<p>Clinical & Commercial GMP production</p>
<p>Novel Recombinant Vaccines</p>	<p>Registration for application services</p>

CircRNA Applications

The successful development of mRNA COVID-19 vaccine has catapulted RNA, a messenger located in the central dogma, to the forefront of drug research. Compared with linear mRNA, circular RNA (circRNA) is more stable in structure and is a major hot spot in current nucleic acid drug research.

Coding circRNA

- Prophylactic vaccines for infectious diseases
- Therapeutic oncology vaccines
- Therapeutic oncology drugs
- Protein replacement therapy
- Regenerative medicine
- Cellular and Gene Therapy

Non-coding circRNA

- Molecular sponge role
- RNA aptamer

Therapeutic Areas

- Cardiovascular diseases
- Oncology

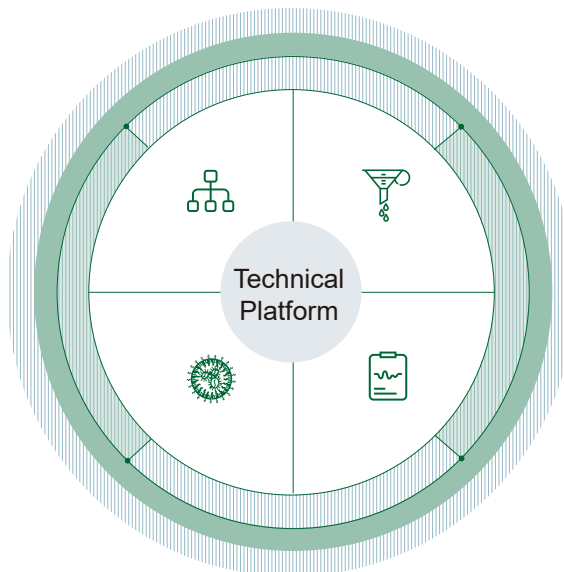
CircRNA Innovative Therapeutic

CRO Service Platform

Technical Platform

RNADes
circRNA structure design
and optimization Platform

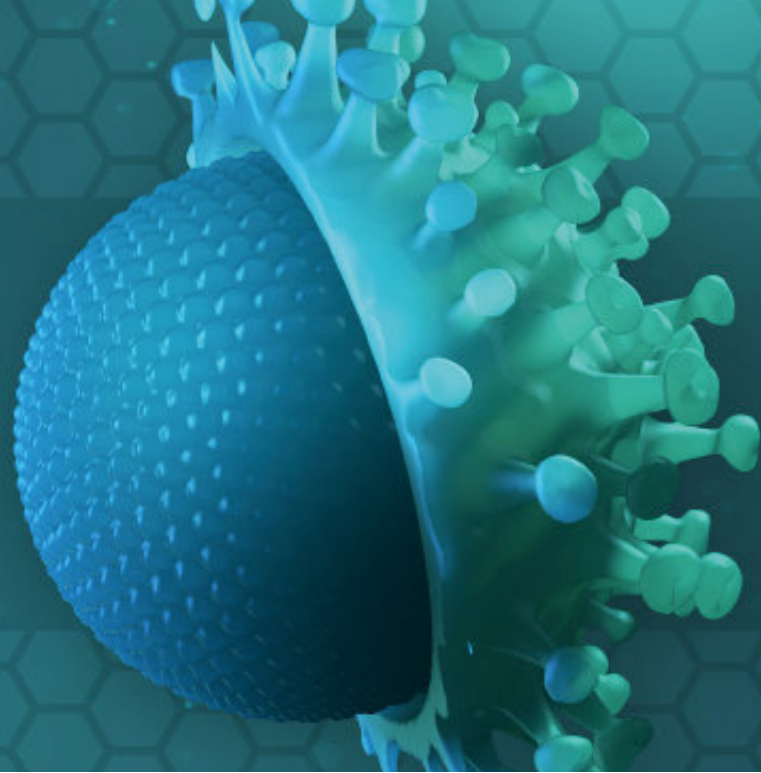
RNASyn
circRNA synthesis and
modification platform



RNAPua
circRNA purification
platform

RNAQua
circRNA quality analysis
and control platform

Yaohai Biopharmaceutical has built a mature CRO service platform for circRNA innovative therapeutics, which can realize efficient preparation and purification of circRNA, and is committed to providing the whole process and *in vitro* preparation technology service of circRNA with high quality for universities and research institutions.



Platform Features

Structure Design And Optimization Platform

Cutting-edge "PIE" loop-forming technology, efficient intron and exon combination
CDS, IRES optimization design

CircRNA Synthesis And Modification Platform

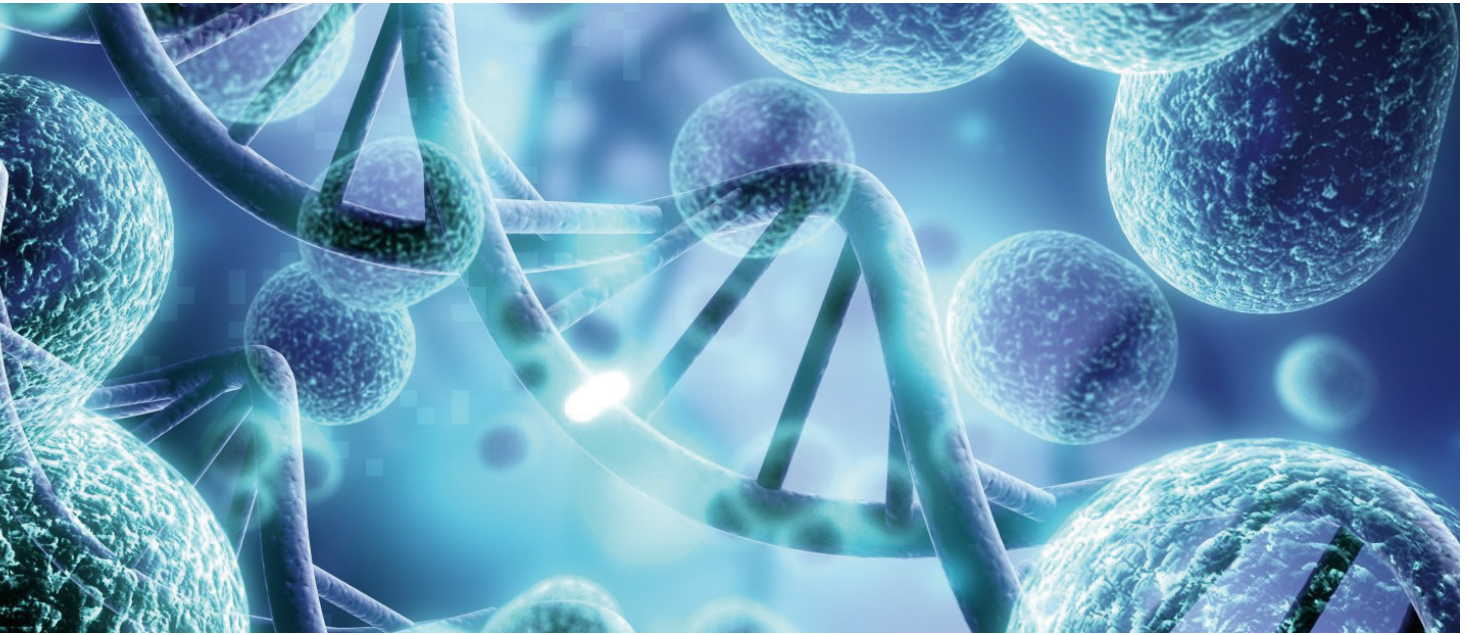
circRNA template plasmid design and construction
circRNA synthesis solution with a loop formation rate of >80%

CircRNA Purification Platform

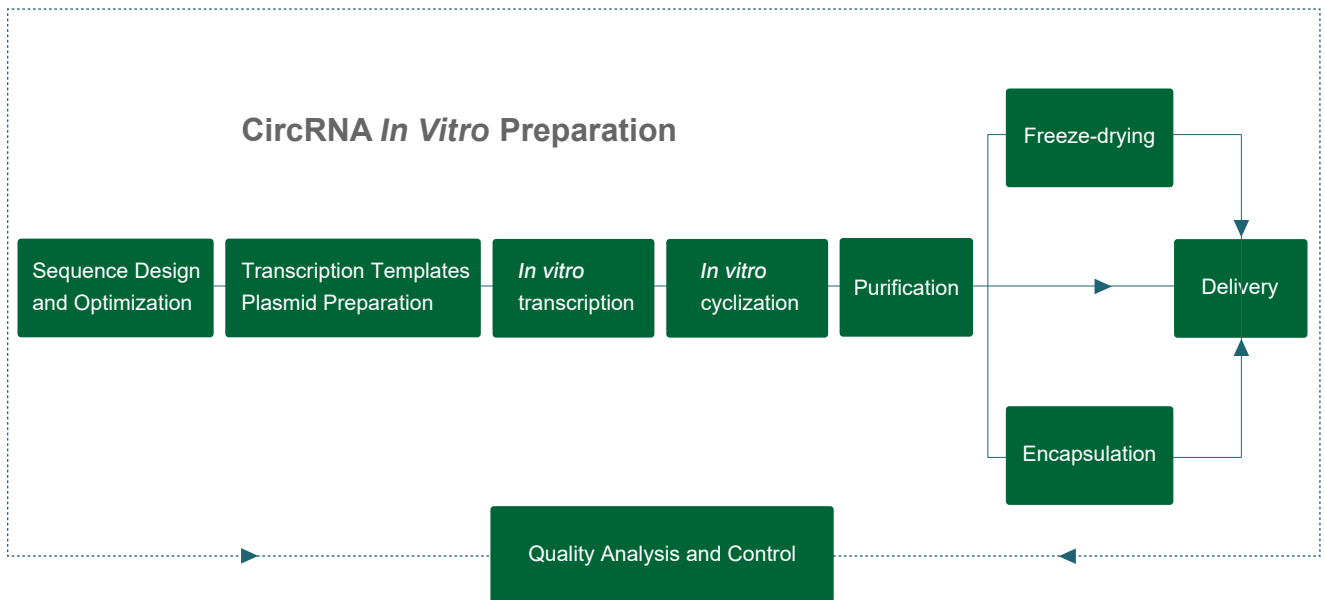
Conventional purification solution of trial grade
Self-developed chromatography column purification process

CircRNA Quality Analysis And Control Platform

Multiple purity assays
High-performance loop formation rate assay



Process Development Flow



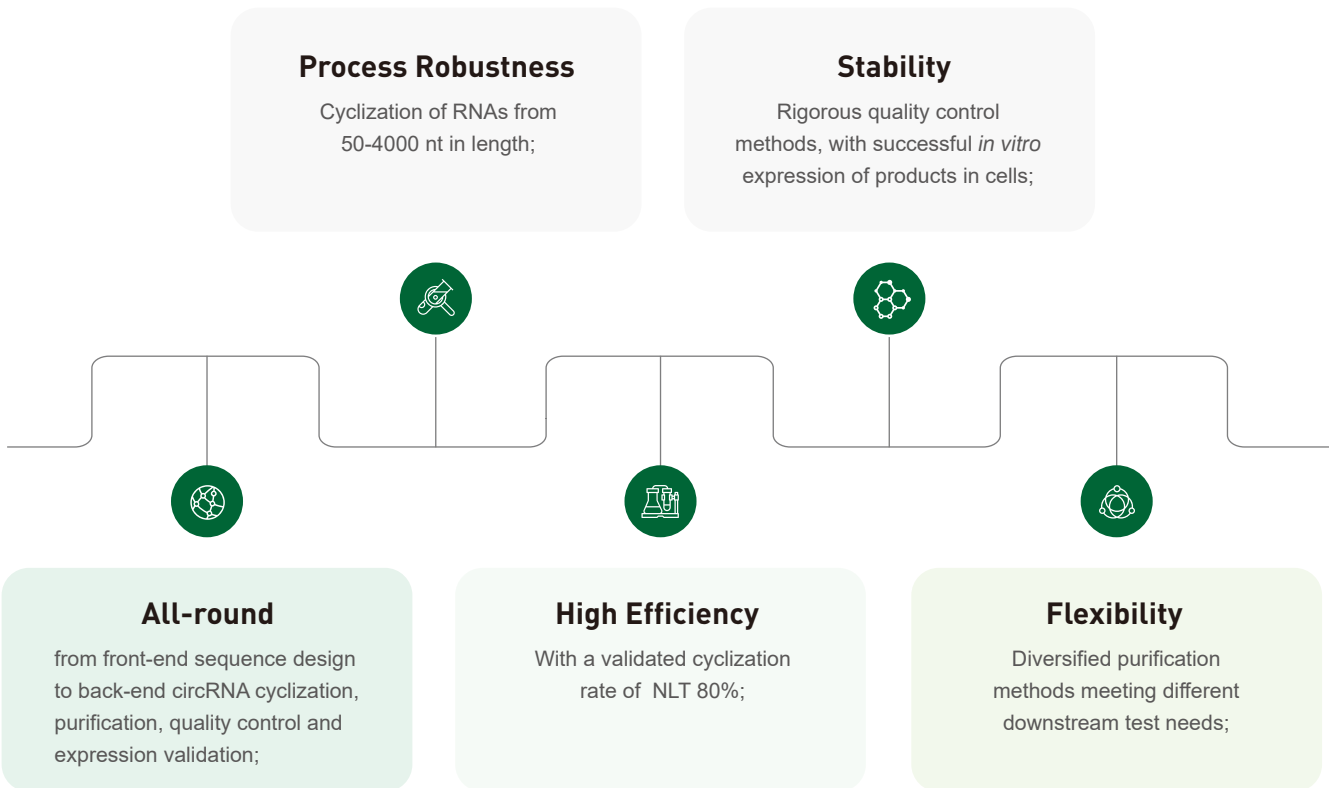
Service Details

Service items	Optional items	Service Details	Delivery Period (days)	Delivery Content
circRNA sequence design and optimization	Design and optimization of coding sequences	CDS codon optimization	1-3	Sequence file
	Design and optimization of non-coding sequences	Design and optimization of IRES, intron		
circRNA transcription template plasmid service	Plasmid DNA preparation	Gene synthesis	7-10	
		Plasmid amplification and extraction	4	
		Plasmid linearization and purification		
circRNA <i>in vitro</i> transcription and cyclization	<i>In vitro</i> transcription	<i>In vitro</i> transcription (IVT)	2-3	N/A
	<i>In vitro</i> cyclization	RNA cyclization based on PIE system		
		circRNA enrichment		
circRNA purification	Conventional purification solutions	Lithium chloride precipitation	1	circRNA drug substance
		Magnetic bead purification		
	Self-developed purification solutions	Self-developed purification solutions	1-2	
	Solution exchange	Ultrafiltration liquid exchange	1	
circRNA lyophilization	Lyophilization	Pre-freezing	2-3	circRNA lyophilized powder
		Primary sublimation		
		Secondary Sublimation		
circRNA encapsulation	LNP encapsulation	LNP encapsulation	2-3	circRNA-LNP Drug product
		Concentration liquid exchange and filtration		
circRNA quality analysis	circRNA drug substance/ lyophilized powder	Concentration, purity	1	CoAs
		Cyclization rate	2-3	
	circRNA-LNP drug product	Encapsulation rate	1	
		Particle size and distribution detection		
		Surface charge detection		
circRNA expression validation	293T cell evaluation	Cell plating	4	CoAs
		Transient transfection of cells		
		Fluorescence signal observation	1-3	
		Western blot/ELISA		

Pre-Products Catalog

Product Name	Test Uses	Delivery Form	Product Specification
circRNA_eGFP circRNA_mCherry circRNA_luciferase circRNA_OVA circRNA_IL-2 circRNA_Cas9	<ul style="list-style-type: none"> Reference standard In vitro or in vivo tests 	<ul style="list-style-type: none"> Lyophilized powder Drug substance (500 ng/μL) 	<ul style="list-style-type: none"> 100 μg 1 mg 10 mg

Service Advantages

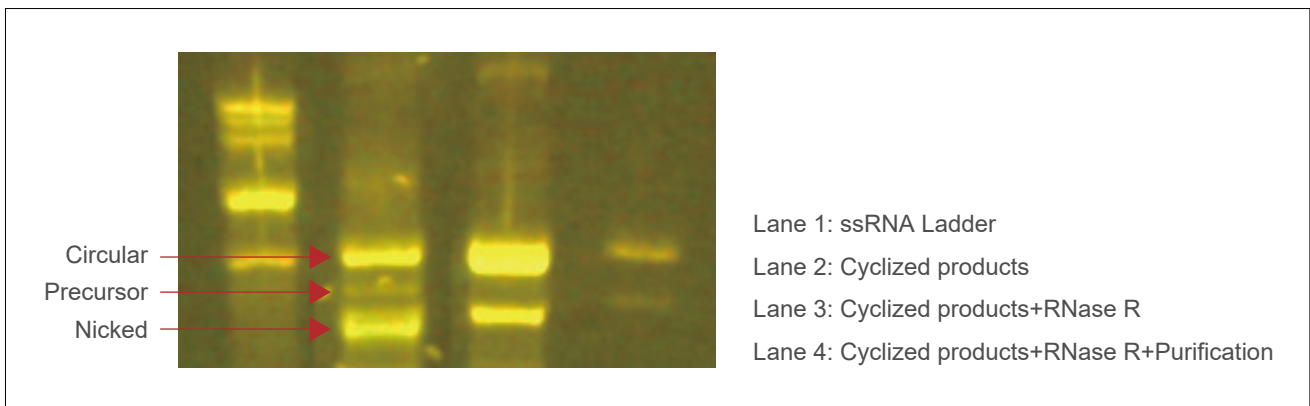


Case Studies

circRNA enrichment and purification

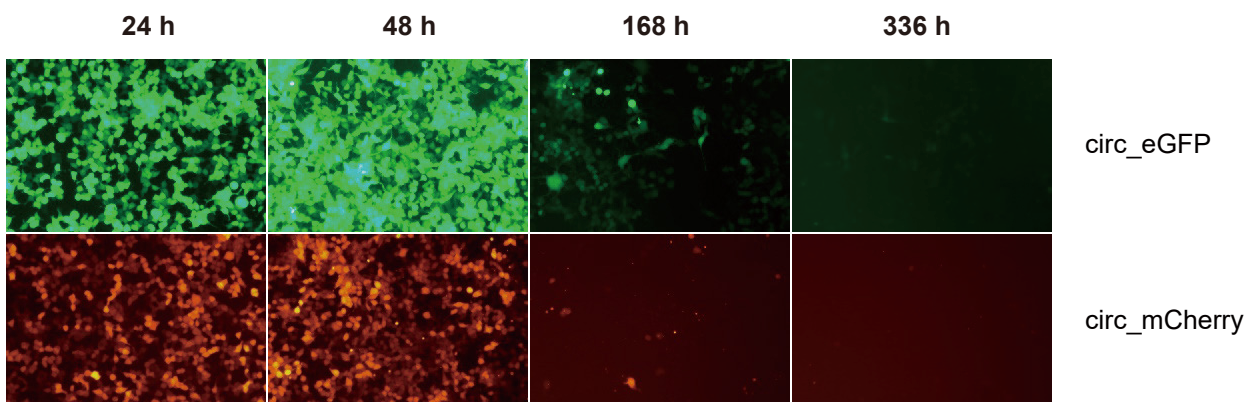
To enrich circRNA, PIE cyclized products are treated with RNase R. The electrophoresis results show that the linear RNA precursors are digested; and after further purification*, most of the nicked circRNA can be removed.*

The purification solution is self-developed by YAOHAI circRNA platform.



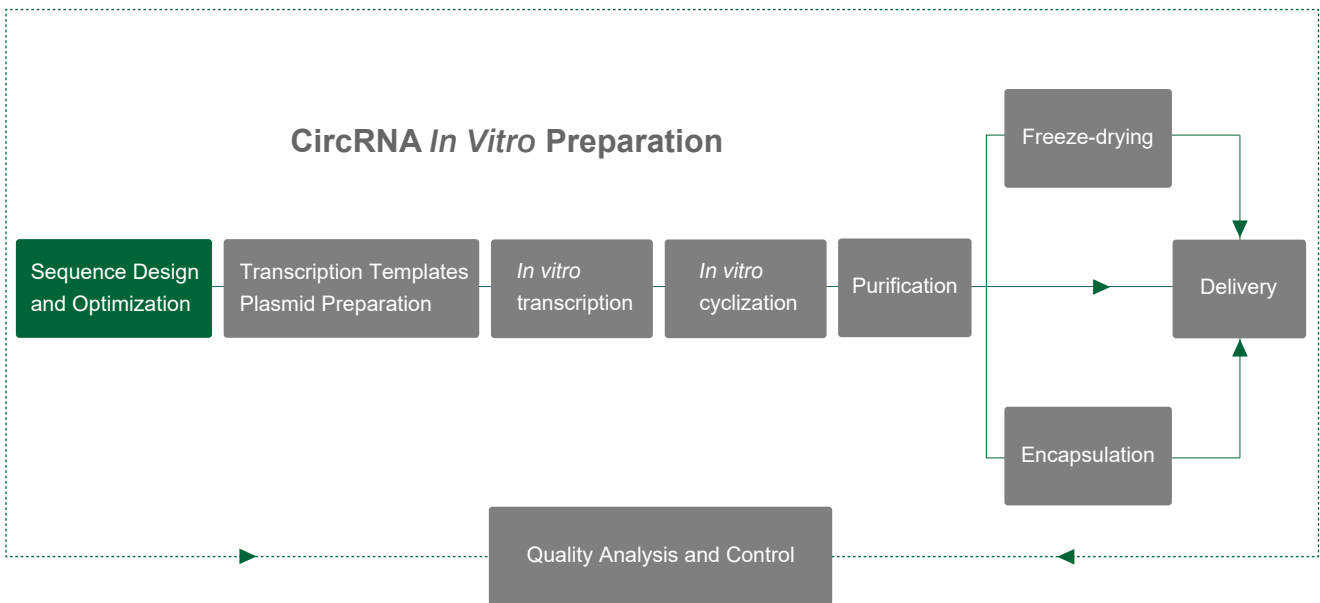
circRNA expression validation

The purified circRNA eGFP are transfected with circRNA Cherry into 293T cells, and fluorescence signal can be observed after 24 h, which will continuously enhanced after 48 h. The fluorescence signal can still be observed after 7 days and 14 days of transfection.



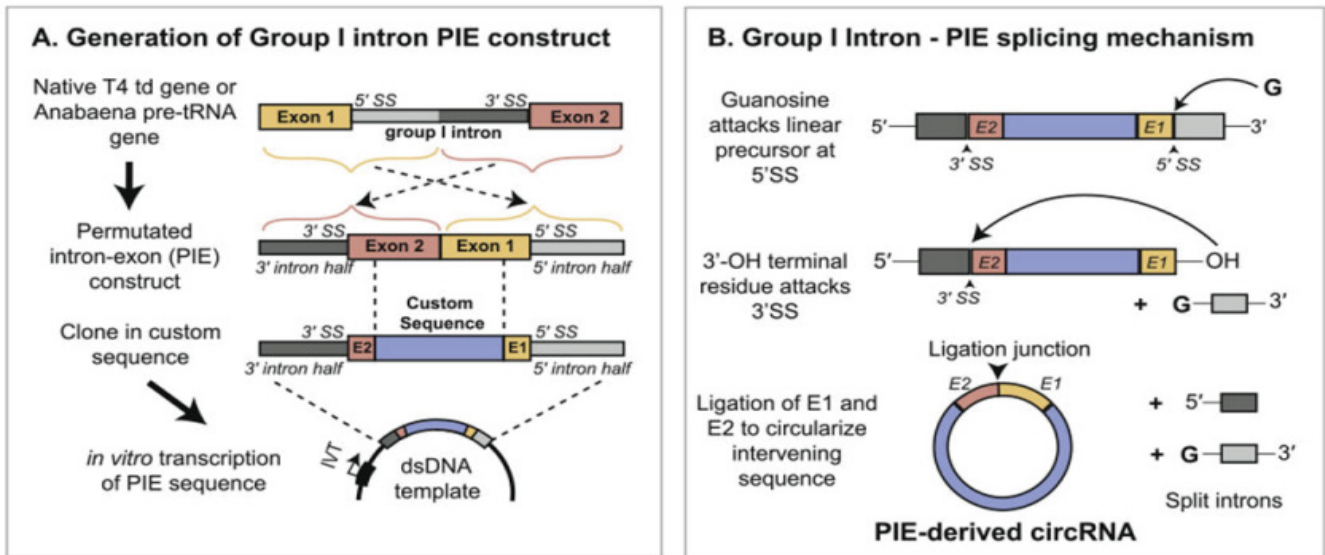
CircRNA

Sequence Design And Optimization Services



Yeohai Biopharmaceutical prepares circRNA based on the PIE system (alignment of exons and introns), which relies on the self-splicing function of type I introns to achieve RNA cyclization. The PIE structure is designed using the T4 td gene or fishy tRNA precursor gene, and the arrangement is as follows: The RNA intron and supporting exon fragment are divided into two parts (5' terminal and 3' terminal), where the 5' terminal sequence is transferred to the tail of the target sequence, the 3' terminal sequence is inserted into the front of the target sequence, and the target gene sequence is inserted in the middle.

Under GTP catalysis, the PIE structure leads to the cyclization of sequences other than introns. Combined with a reasonable enhancement strategy of cyclization rate, YAOHAI can achieve cyclization of sequences up to 4 kb with a cyclization rate of more than 80%.



The functions and design strategies of each component of circRNA are referenced below

CircRNA Components		Biological Functions	Design Strategy
	Two-terminal intron and exon sequences	GTP-catalyzed intron self-splicing for cyclization of sequences outside the intron.	Designed according to the T4 td gene or fishoil tRNA precursor gene.
Coding	IRES	Internal ribosome recognition site that regulates the translation of circRNA.	Screening of IRES sequences from different viral sources, e.g. EMCV, CVB3 sources.
	CDS	Protein-coding regions, sequences coding for antigens, antibodies or other functional proteins.	Codon optimization increases the level of translation; note that certain non-optimal codons may play a role in protein folding.
Non-coding	Non-coding sequences	Target miRNAs or proteins to exert gene or protein regulation.	Targeting specific binding sites for miRNAs or proteins can repeat the sequences of the binding site.

[1] Chen X, Lu Y. Front Bioeng Biotechnol. 2021 Nov 30;9:787881..

Service Details

Service Items	Optional Items	Detailed Steps	Delivery Period (Days)
circRNA sequence design and optimization	Design and optimization of coding sequences	<ul style="list-style-type: none"> CDS sequence matching CDS codon optimization 	1
	Design and optimization of non-coding sequences	<ul style="list-style-type: none"> Intron and exon sequence design and optimization Homologous arm sequence design and optimization Interval sequence design and optimization 	1-2

Service Advantages

Optimized PIE cyclization system

Combined with reasonable optimization strategies to achieve a cyclization rate of NLT 80%;

Cutting-edge CDS optimization team

Cooperation with professional AI algorithm team to complete the optimization of CDS region codons;

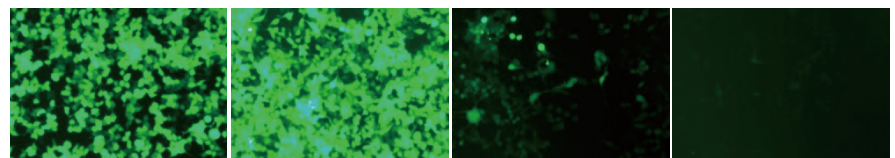
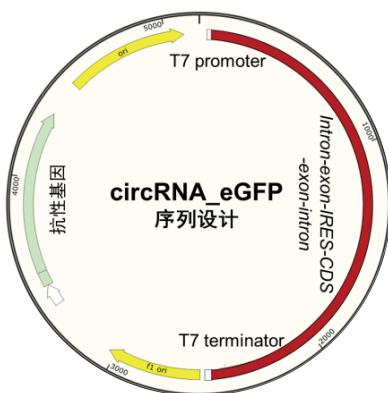
Mature and perfect process

circRNA can be achieved with a high loop formation rate, high stability and high translation efficiency.



Case Studies

Yaohai Biopharmaceutical launched the control product circRNA_eGFP, which is based on the PIE system to achieve the cyclization of RNA. Using a conventional transfection reagent, circRNA_eGFP is transfected with 293T cells, and eGFP (green) fluorescent signal can be detected after 24h, which will be enhanced after 48h, and the fluorescent signal can still be detected after 7 days and 14 days of transfection.



After transfection 24 h

48 h

168 h

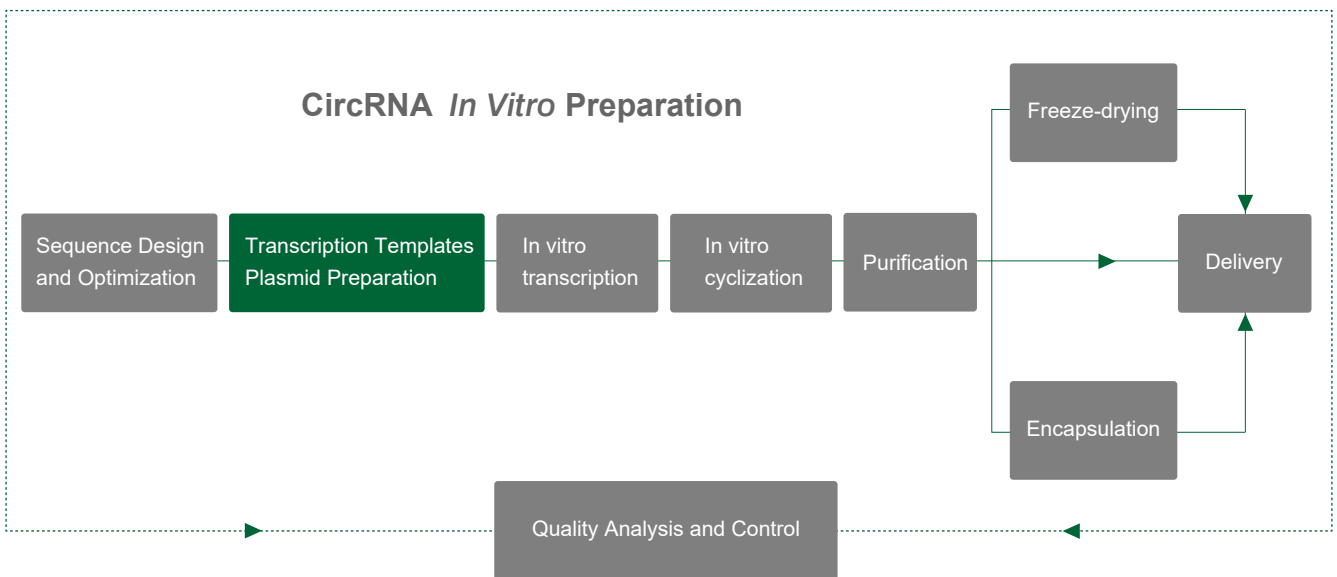
336 h

Sequence design and in vitro expression validation of circRNA_eGFP

CircRNA

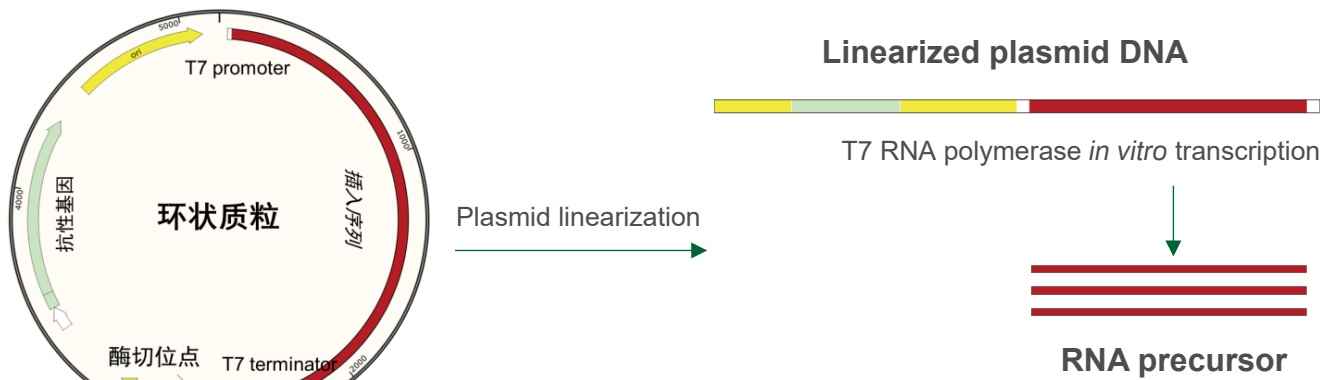
Transcription Template Plasmid Service

Process Development Flow



CircRNA preparation requires linear RNA as precursor material (RNA precursor) for cyclization, where the RNA precursor is usually prepared using linearized plasmid DNA as transcription template and transcribed in vitro with the help of T7 RNA polymerase.

High quality plasmid DNA is essential for downstream in vitro transcription (IVT). Based on the mature plasmid preparation service platform, YAOHAI BIO can provide high purity and standard linearized plasmid DNA preparation service to ensure the integrity of downstream IVT products.



Schematic diagram of in vitro transcription using linearized plasmid DNA as template

Service Details

Service Items	Optional Services	Service Details	Delivery Period (Days)
Cyclic plasmid preparation	Gene synthesis	Gene synthesis (outsourced)	7-10
	Plasmid amplification	Plasmid amplification	2
		Plasmid extraction	
Linearized plasmid preparation	Plasmid linearization and purification	Plasmid linearization	1
		Linearization product purification	
Plasmid DNA quality control	Concentration purity	Ultraviolet spectrophotometry (UV)	1-2
	Plasmid conformation	Agarose gel electrophoresis (AGE)	
		Capillary electrophoresis (CE)-Optional	
	Plasmid integrity	Restriction enzyme identification (AGE)	

Service Advantages

Freecut Template Plasmid

Flexible selection of linearization methods

High Recovery Rate

Continuous optimization of DNA extraction and purification methods to achieve high recovery rates.

Stringent Quality Control

Stringent process control specifications, with a superhelical conformation rate of plasmid samples for research of >80%.

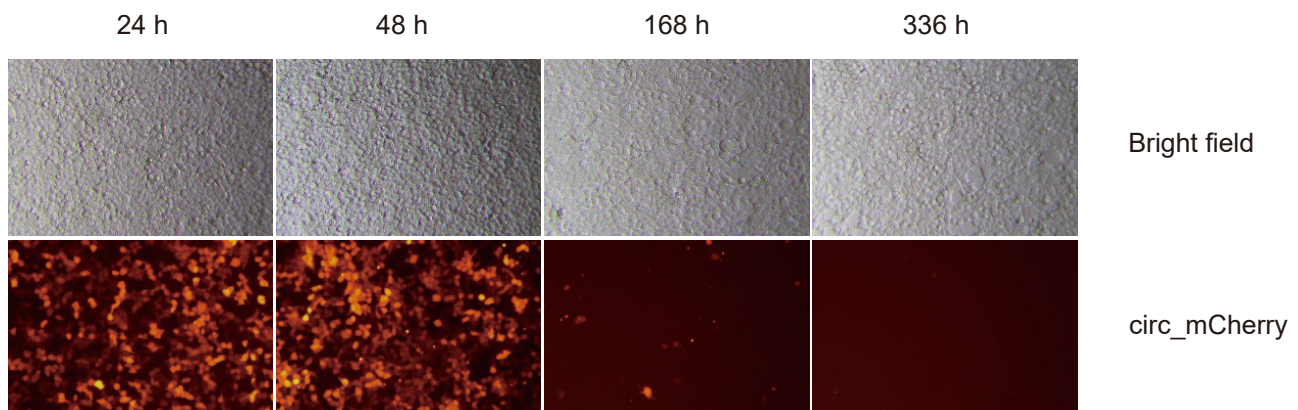
Mature Platform Process

High standard and high efficiency plasmid preparation and quality control services to meet downstream test needs.

Case Studies

Take YAOHAI's pre-product circRNA_mCherry as an example, the superhelix rate of transcription template plasmid samples (research grade) is more than 70%, with a linearization rate of close to 100%.

CircRNA_mCherry is prepared with linearized plasmid as template and transfected into 293T cells. High level of fluorescence expression (red fluorescence) is detected after 24 hours of transfection, which will be continuously enhanced after 48 hours, and can still be detected after 7 and 14 days of transfection. mCherry protein is stably and efficiently expressed, and the transcriptional template purity can meet the requirement of circRNA drug product with high quality.



In vitro expression validation of circRNA-mCherry

CircRNA

in vitro transcription and *in vitro* cyclization services

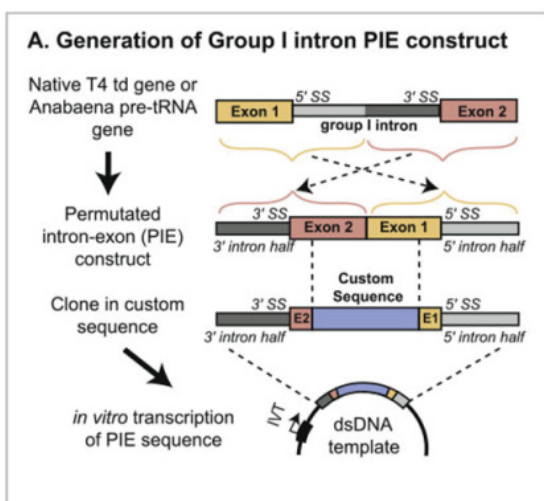
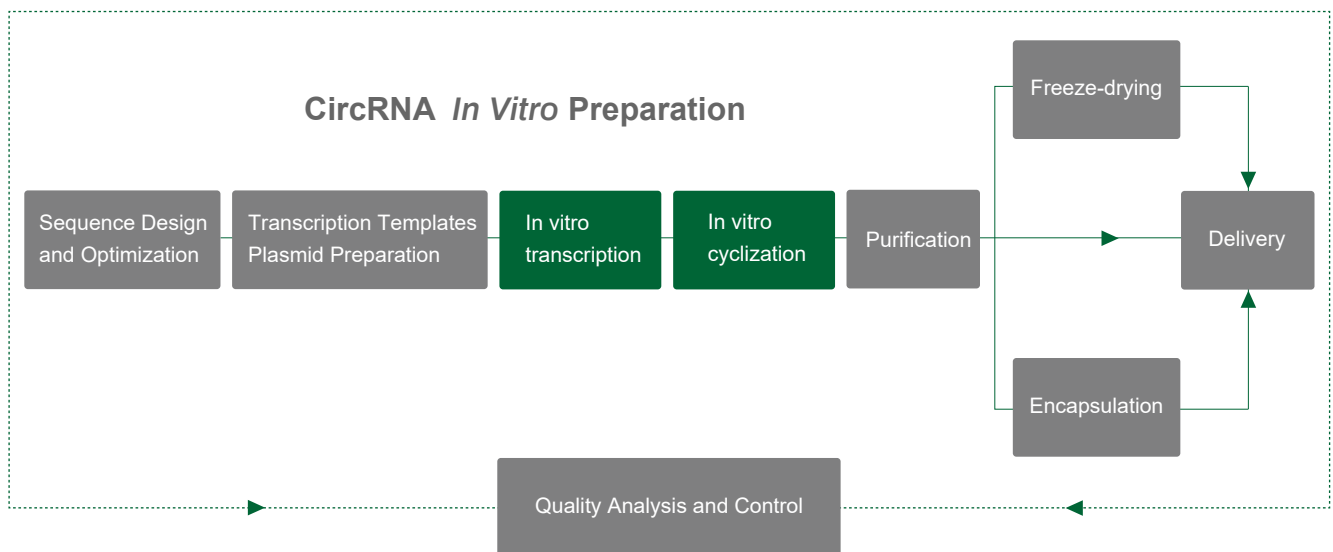


Figure: PIE sequence diagram

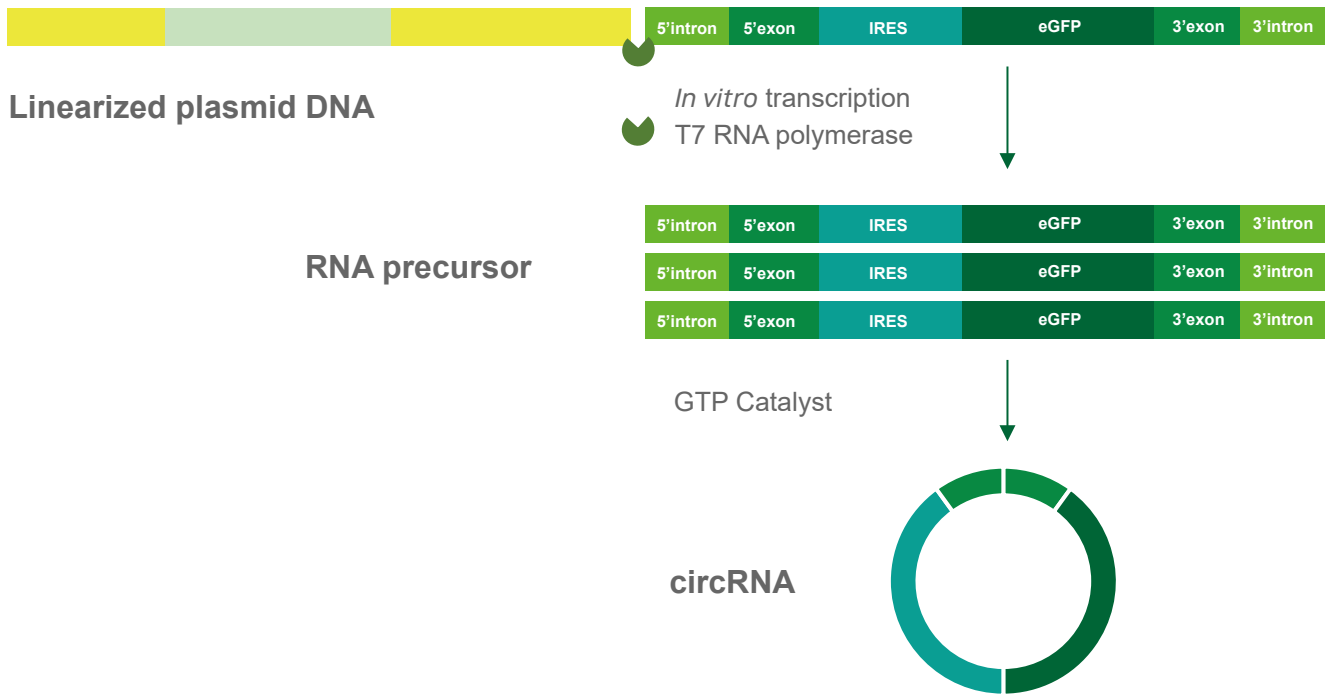
Regarding the batch preparation of RNA precursor, the commonly used method is *in vitro* transcription (IVT, *In Vitro* Transcription). IVT reaction uses linearized plasmid DNA containing T7 promoter as a template and synthesizes RNA precursor with nucleoside triphosphates (NTPs) as a substrate in the presence of T7 RNA polymerase.

In vitro cyclization methods include chemical ligation, enzymatic ligation and PIE nuclease method. Chemical ligation and enzymatic ligation are suitable for the cyclization of shorter RNAs, and the cyclization rate decreases significantly for fragments larger than 100 nt, while the nuclease method based on the PIE system can achieve cyclization of 5 kb sequences.

Under GTP catalysis, the PIE structure undergoes cyclization of extra-intron sequences. Combined with a reasonable enhancement strategy of cyclization rate, YAOHAI can achieve cyclization of up to 3 kb sequences with a cyclization rate of more than 80%.

The *in vitro* cyclization reaction of RNA flows as follows:

RNA precursor is synthesized by *in vitro* transcription, and the PIE component completes self-splicing under GTP catalysis to form circRNA.



Service Details

Service Items	Detail Steps	Delivery Period (Days)
circRNA <i>in vitro</i> transcription and <i>in vitro</i> cyclization	Reaction system confirmation	1-2
	<i>In vitro</i> transcription and cyclization reactions	
	RNase R enrichment	
Conditions optimization	Reaction system design and optimization	2-5

Service Advantages

Rigorous test design and optimization

Up to 4 kb RNA cyclization can be achieved.

High cyclization efficiency

A cyclization rate of more than 80% can be achieved through a rational sequence optimization strategy.

Stringent enzyme control specification

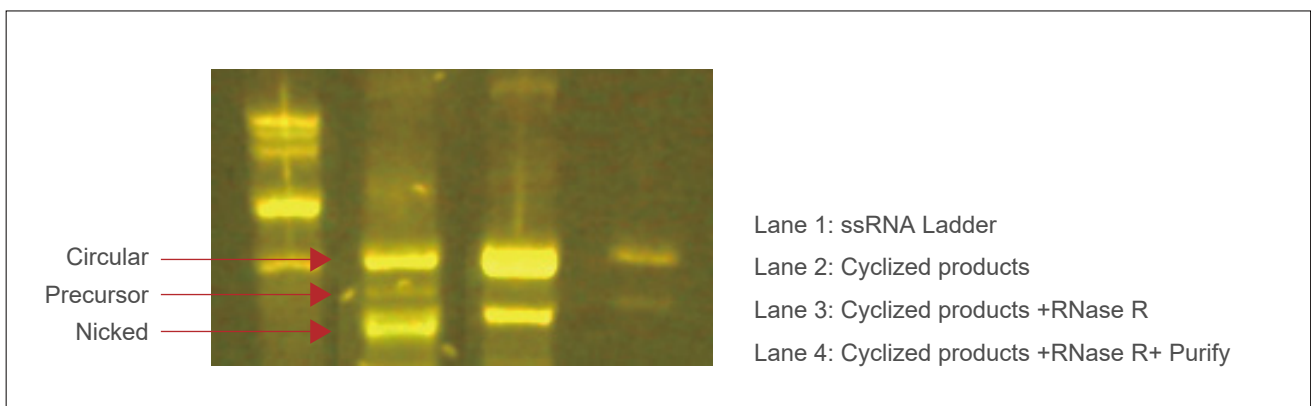
Stringent enzyme control on test environment and consumables to effectively prevent RNA degradation.

Case Studies

In vitro cyclization and enrichment of circRNA

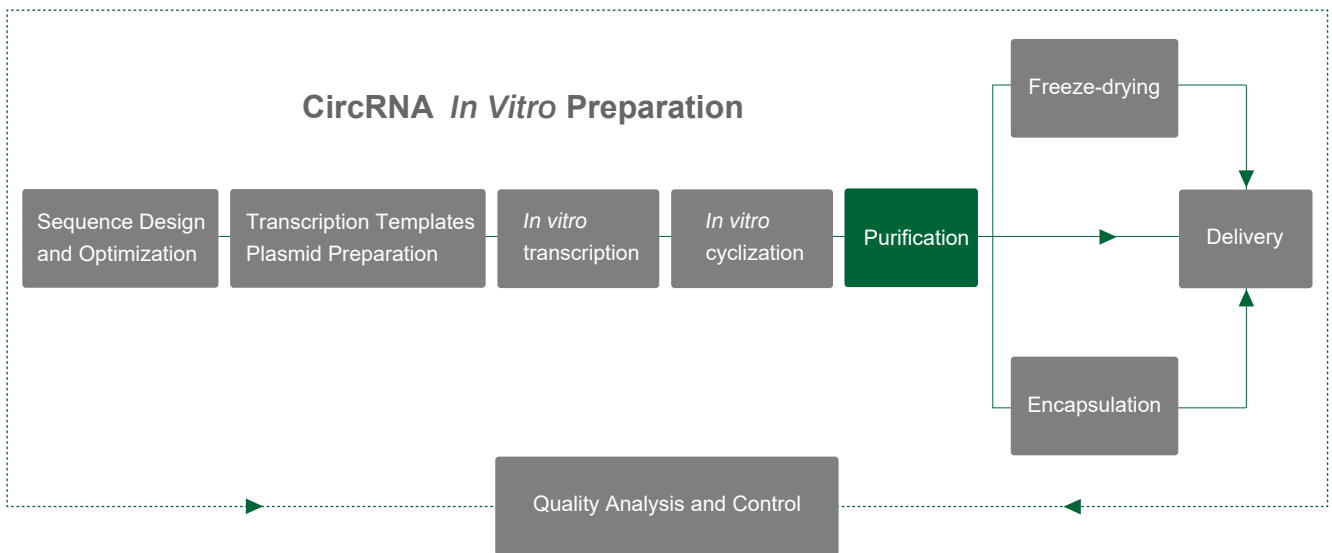
Based on the PIE system, Yaohai Biopharmaceutical has optimized the sequence of circRNA, with a cyclization rate of more than 80% by agarose gel electrophoresis.

Using RNase R to enrich circRNA, E-gel electrophoresis results show that the linear RNA precursor is digested, and after further purification*, most of the nicked circRNA can be removed.* The purification solution is self-developed by the circRNA platform of Yaohai Biopharmaceutical.



CircRNA

Purification Services



The products obtained by *in vitro* cyclization and RNase R enrichment require to be further removed from IVT, unconsumed substrates in cyclization reaction, reaction by-products and nicked RNA to meet the downstream test requirements.

Yaohai Biopharmaceutical has mature LiCl precipitation, magnetic bead purification, self-developed purification and other diversified purification solutions to effectively remove various impurities and prepare high purity circRNA.



LiCl precipitation method

Simplified purification solution for small amounts of circRNA for cell transfection and part of animal experiments.



Magnetic bead purification method

Small amount of circRNA purification solution for cell transfection, and part of animal experiments.



Yaohai purification solution

Meeting downstream application scenarios with higher quality requirements;

Stable and scalable purification process to meet downstream GMP production requirements ☆ ☆ ☆ ☆ ☆

Service Details

Service Items	Optional Items	Detailed steps	Delivery Period (Days)	Delivery
circRNA purification	Conventional purification solution	Lithium chloride precipitation	1	circRNA drug substance
		Magnetic bead purification		
	Purification solution with high purity	Yaohai purification solution	1-2	
	Solution substitution	Ultrafiltration liquid exchange	1	
circRNA quality control	Concentration determination	Ultraviolet spectrophotometry (UV)	0.5	CoAs
	Purity testing	Agarose gel electrophoresis (AGE/E-gel)		
		HPLC-Optional	1	

Service Advantages

A variety of optional purification options

to meet different downstream application scenarios;

High purity

circRNA with a purity of more than 90%;

Stringent enzyme control specification

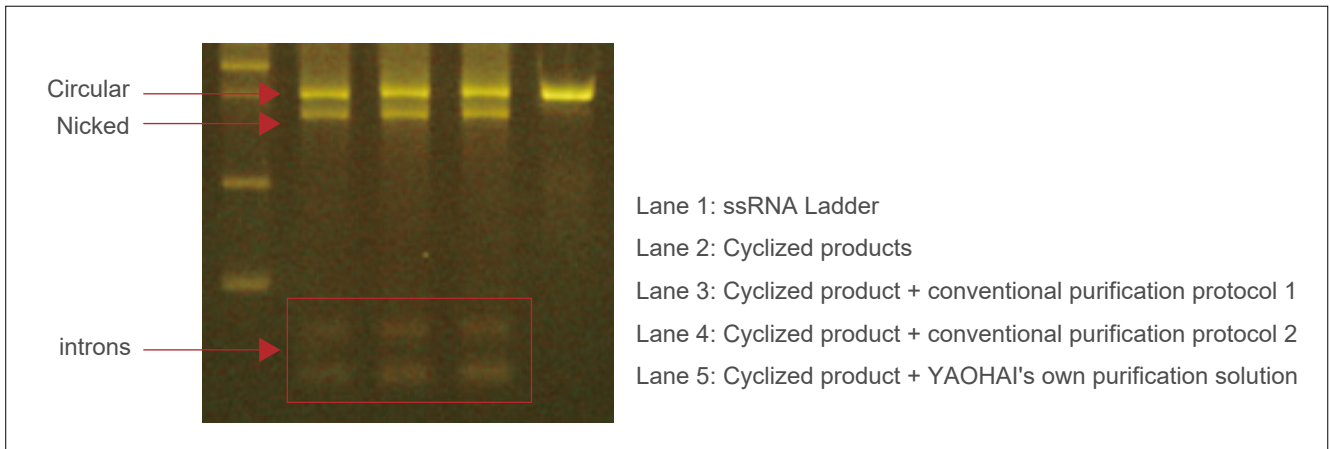
Stringent enzyme control on the test environment and consumables to effectively prevent the degradation of circRNA.

Case Studies

Yaohai Biopharmaceutical has established a mature circRNA purification solution, which can effectively remove various process-related impurities.

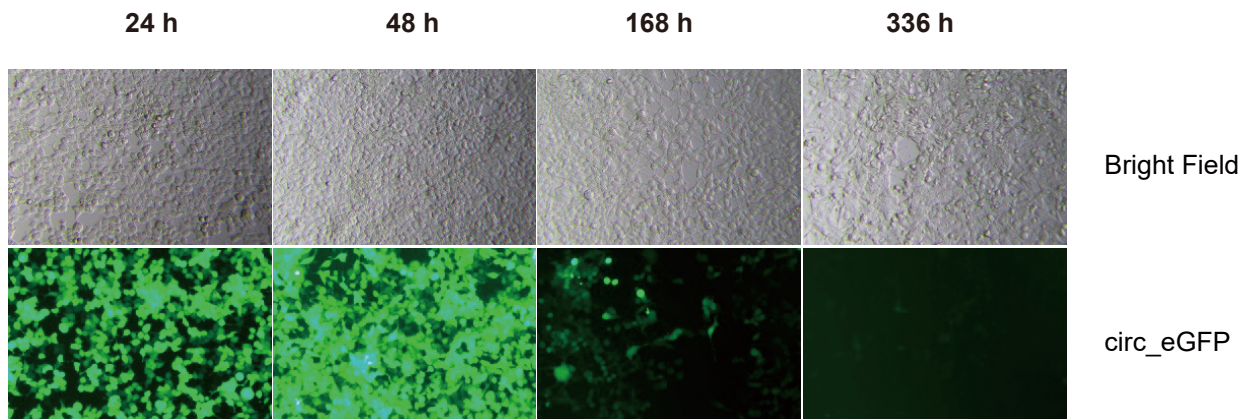
The circular RNA products purified by the conventional solution are still obviously mixed with nicked RNA and introns.

After the purification solution developed by Yaohai Biopharmaceutical, various linear RNA impurities, such as nicked RNA and introns, can be successfully removed.



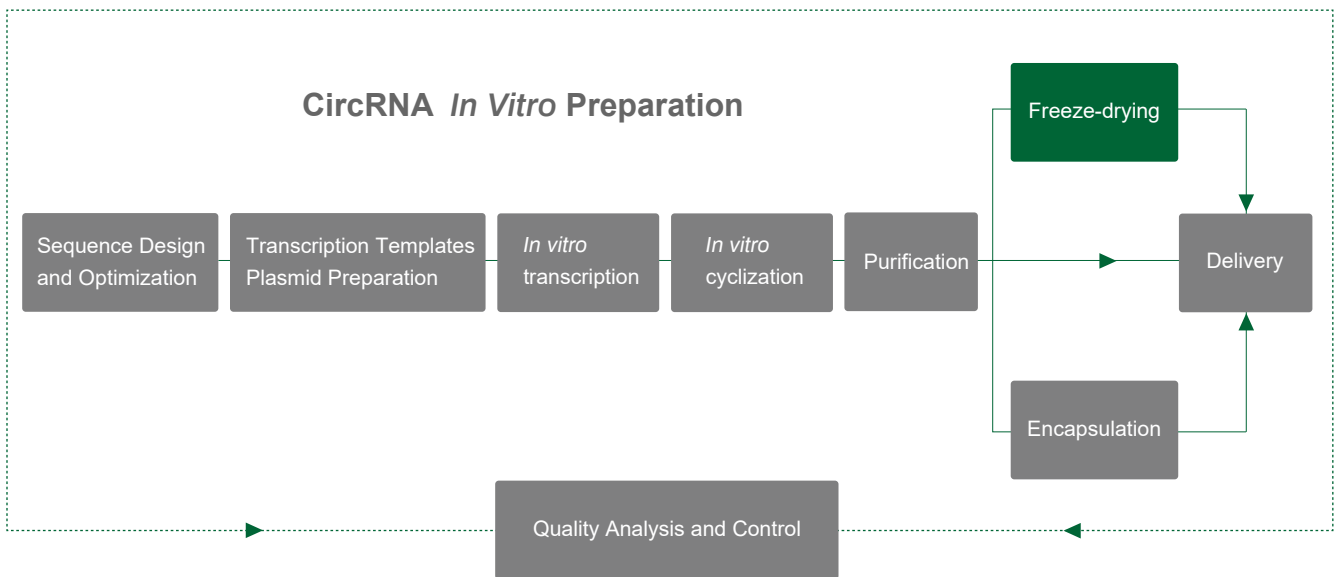
circRNA expression validation

Using a conventional liposome, purified circRNA_eGFP is transfected into 293T cells, and fluorescence signal is observed after 24 h and continued to be enhanced at 48 h. Fluorescence signal can still be detected after 7 and 14 days of transfection.



CircRNA

Lyophilization Service



In order to improve the stability of circRNA and avoid the loss in storage and transportation, YAOHAI can provide circRNA lyophilization service to freeze-dry the circRNA drug substance and store or transport in the form of lyophilized powder, which significantly reduces the degradation and loss of circRNA during storage and transportation.



Service Details

Service Items	Optional Items	Detailed steps	Delivery Period (Days)	Delivery
circRNA lyophilization	Sample dispensing	Dispensing	2-3	circRNA lyophilized powder
	Lyophilization	Pre-freezing		
		Primary sublimation		
		Secondary sublimation		
circRNA quality control	Reconstitution of lyophilized powder	Reconstitution/resuspension	-	CoAs
	Solubility of lyophilized powder	Appearance inspection	-	
	Concentration determination	Ultraviolet spectrophotometry (UV)	0.5	
	Integrity and purity testing	Agarose gel electrophoresis (AGE/E-gel)		
			HPLC-optional	

Service Advantages

Mature lyophilization process

The quality indicators of samples before and after lyophilization are consistent, with good reproducible results.

Homogeneous quality properties

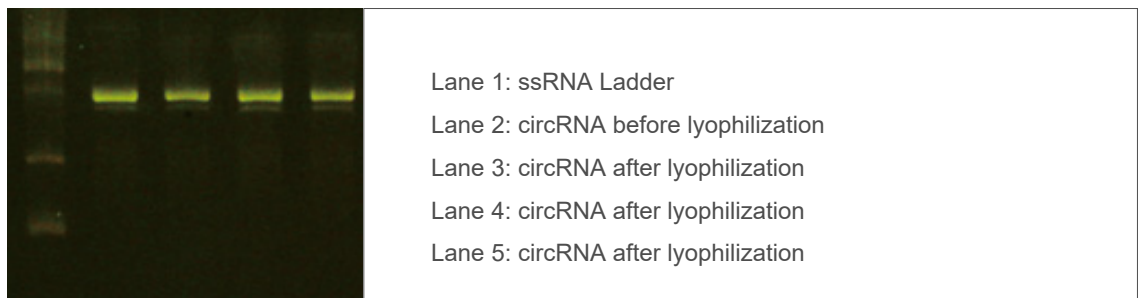
circRNA samples before and after lyophilization successfully express the target protein.

High stability

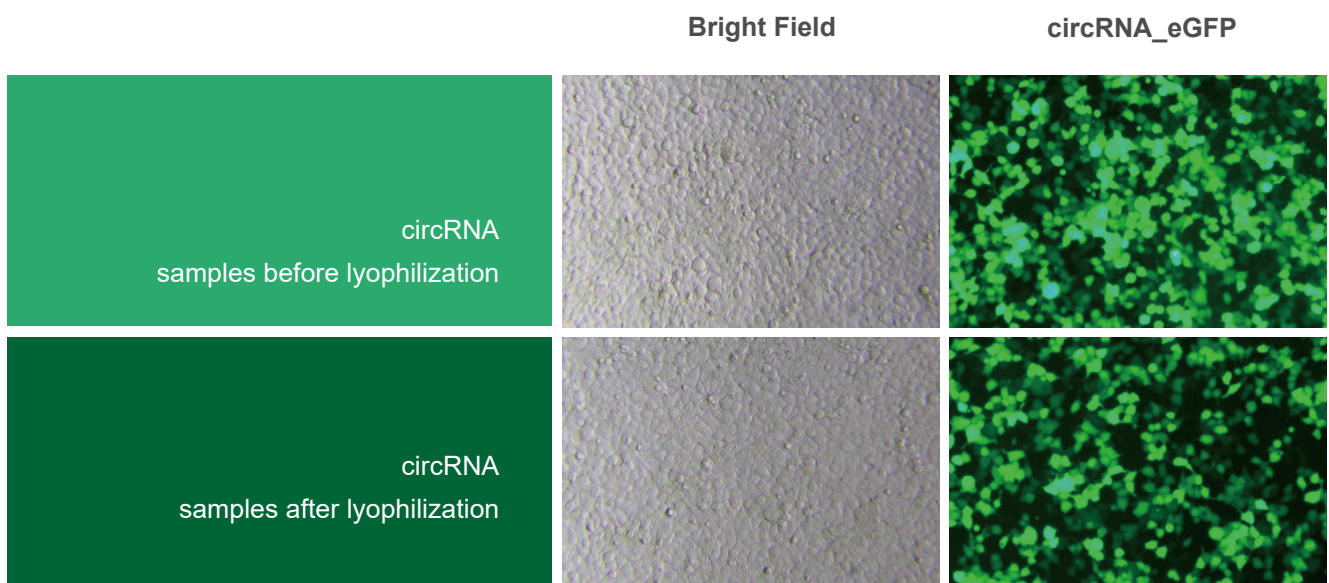
circRNA lyophilized powder is easy to store and transport.

Case Studies

E-gel electrophoresis assay is performed for circRNA before lyophilization and after reconstituted lyophilized powder to analyze the integrity and purity, respectively. The results show that there is no significant difference between the circRNA bands before and after lyophilization, and the test results after lyophilization show good reproducibility.

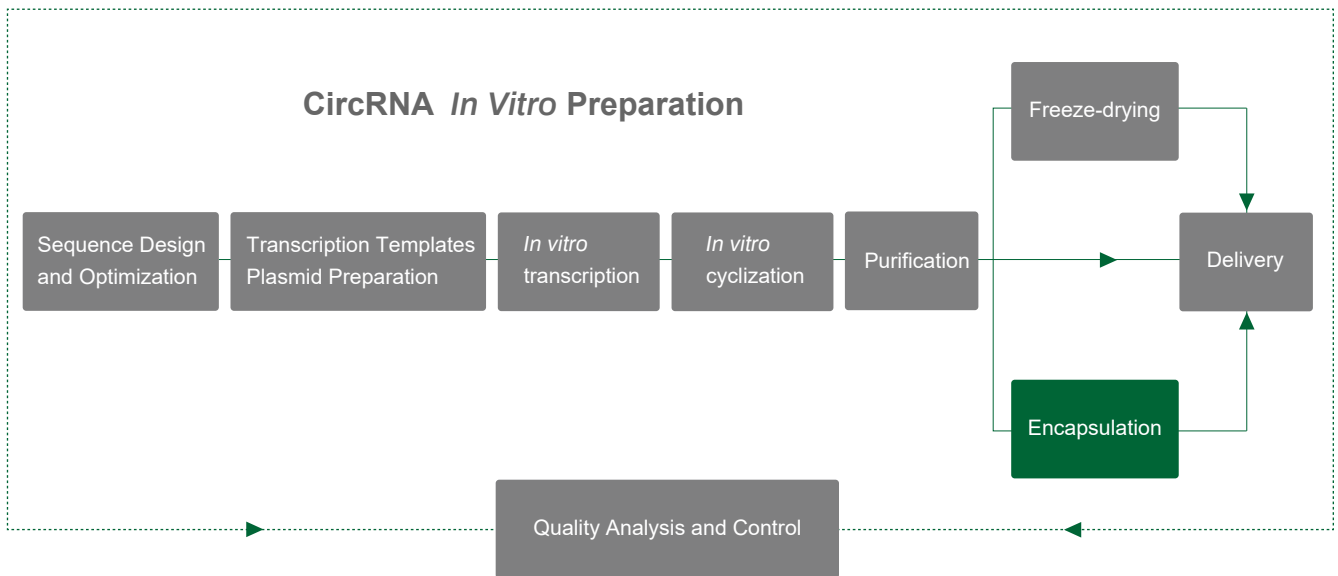


The 293T cell evaluation of circRNA samples before and after lyophilization shows that strong fluorescent signals are observed in the pre-product circRNA_eGFP samples before and after lyophilization, and the target protein is expressed efficiently *in vitro*.



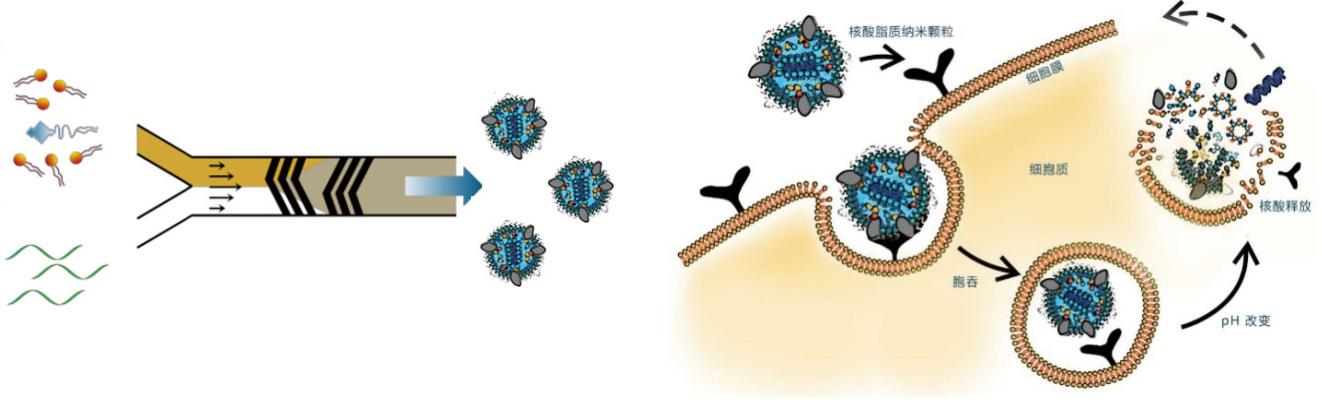
CircRNA-LNP

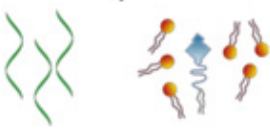
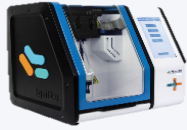


Encapsulation Service



The basis of encapsulation is the design and development of the delivery system. A well-designed delivery system allows circRNA molecules to enter the body without being degraded by RNA enzymes, and then be effectively delivered to the target site, cross the cell membrane, and be released intracellularly. Lipid nanoparticles (LNPs) are the best delivery system available, with advantages in encapsulation, in vivo expression, and in vivo safety compared to other delivery systems. Lipid nanoparticles with nucleic acid fragments are easily swallowed into cells and form intracellular bodies. Once entering the cell, the acidic environment of the intracellular body protonates and positively charges the head of the ionized lipid, thus fusing with the inner membrane of the intracellular body and releasing the target nucleic acid into the cell for action.

YAOHAI circRNA service continues to improve, and can provide circRNA-LNP encapsulation service to optimize relevant critical process parameters and improve the consistency and reproducibility of circRNA drug production.



Pre-treatment of material and liquid	Microfluidics	Tangential Flow Filtration	Sterilization filtration
 <p>Two drug substances are prepared: one for circRNA in aqueous buffer and one for lipids dissolved in ethanol.</p>	 <p>Rapid mixing of lipid, circRNA two-phase solutions using microfluidic devices, resulting in uniform LNP and high efficiency encapsulation.</p>	 <p>The drug substance is concentrated to the target concentration using a tangential flow technique and the buffer is replaced with a neutral storage solution to remove unencapsulated circRNA, excess lipids and acetic acid.</p>	 <p>Comply with sterility regulatory requirements, select terminal sterilizing filtration system and pass the validation of bacterial challenge test.</p>

Service Details

Service Items	Detailed Steps	Delivery Period (Days)	Delivery
circRNA-LNP encapsulation	Material and liquid pretreatment	2	circRNA-LNP drug product
	Microfluidic device mixing		
	Tangential flow filtration	1	
	Sterilizing filtration		
circRNA quality control	Encapsulation rate	1	CoAs
	Particle size and distribution detection		
	Surface charge detection		

Service Advantages

Formulation screening of drug product

Fast synthesis, high R&D efficiency and pre-optimized solutions;

High encapsulation rate

Encapsulation effect of up to 90% or more;

Moderate particle size

The size of lipid nanoparticles can be controlled by changing the fluid injection rate and ratio.

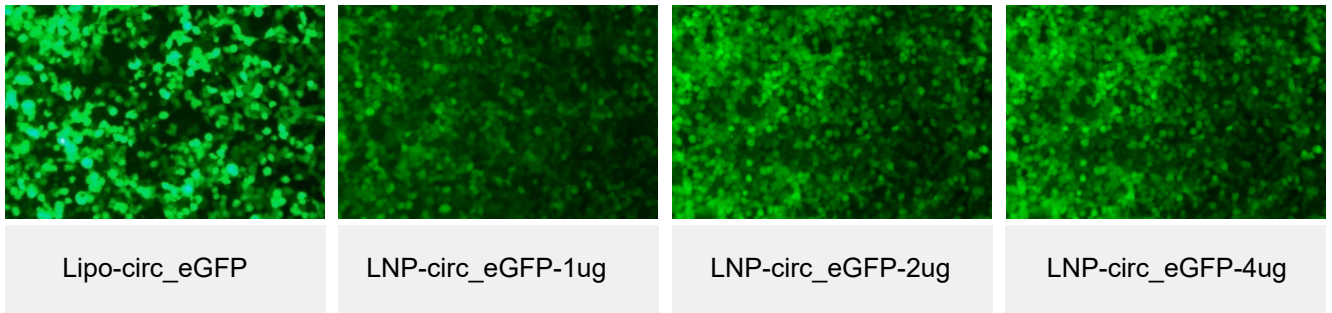
Efficient expression

circRNA-LNP pre-products are validated by in vitro cell expression and can express the target protein efficiently.

Case Studies

LNP-circRNA_eGFP samples are prepared with different levels (1ug-2ug-4ug) and directly transfected 293T cells to verify whether they can express the target protein. After transfection for 48h, a clear fluorescent signal can be observed, and there is a dose-escalation effect of fluorescence intensity.

[Note: Lipo-circ_eGFP is liposome + unencapsulated circRNA_eGFP, as transfection control]



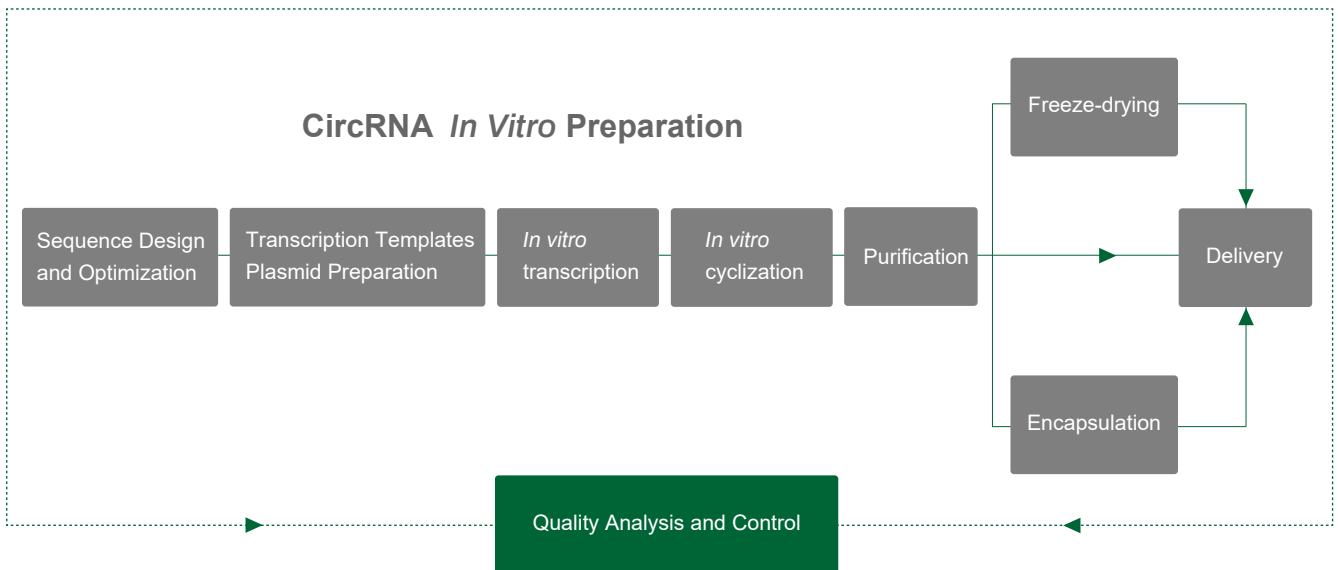
Other Services

circRNA Sequence Design And Optimization	Transcription Template Plasmid Preparation	<i>In Vitro</i> Transcription Of Linear RNA
circRNA <i>in vitro</i> cyclization	circRNA purification	circRNA-LNP encapsulation
circRNA lyophilization	circRNA quality analysis and control	<i>In vitro</i> expression validation of circRNA



CircRNA

Quality Analysis and Control Services

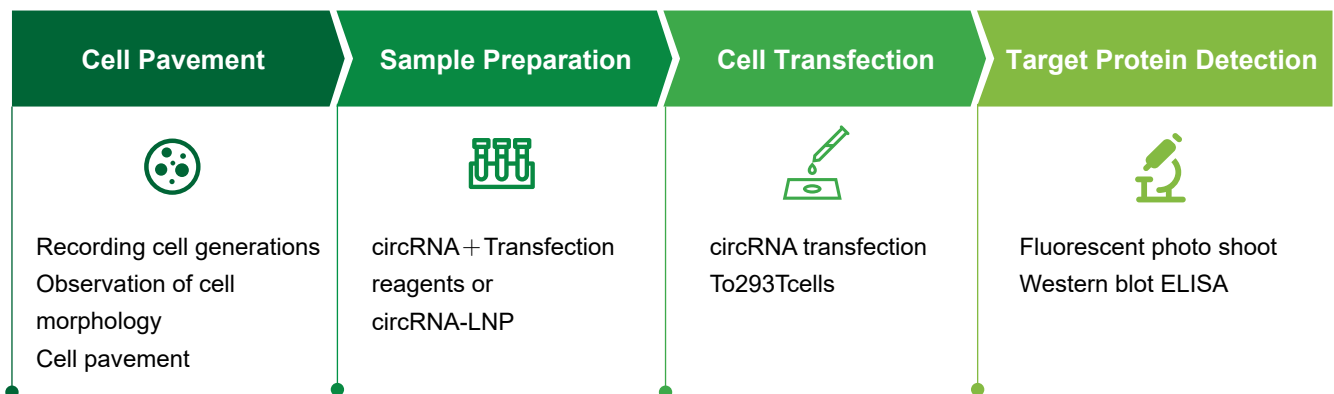


Combined with the requirements of mRNA quality analysis guidelines, YAOHAI has developed specifications for circRNA drug product and can provide quality analysis services for cyclic and linearized plasmid templates, circRNA drug substance and finished product of circRNA-LNP, and the service details are shown below:

Samples	Test Items	Testing Method	Delivery Period (Days)	Delivery Content
Cyclic plasmid DNA	Concentration and purity	Ultraviolet spectrophotometry (UV)	N/A	CoAs
	Super Spiral rate	Agarose gel electrophoresis (AGE)	0.5	
		Capillary electrophoresis (CE)	1	
Linearized plasmid DNA	Concentration and purity	Ultraviolet spectrophotometry (UV)	N/A	
	Linearization ratio	Agarose gel electrophoresis	0.5	
		Capillary electrophoresis (CE)	1	
circRNA drug substance	Concentration	Ultraviolet spectrophotometry (UV)	N/A	
	Purity	Agarose gel electrophoresis (AGE/E-gel)	0.5	
		HPLC	1	
	Cyclization rate	HPLC、qPCR	1-2	
circRNA-LNP drug product	Encapsulation rate	RiboGreen Method	1	
	Particle size and distribution	Particle size meter	1	
	Surface charge	Particle size meter	1	
Validation of circRNA expression	293T cell evaluation	Cell transfection	4	
		Fluorescence observation	1-3	
		Western Blot/ELISA		

CircRNA *in vitro* expression validation service

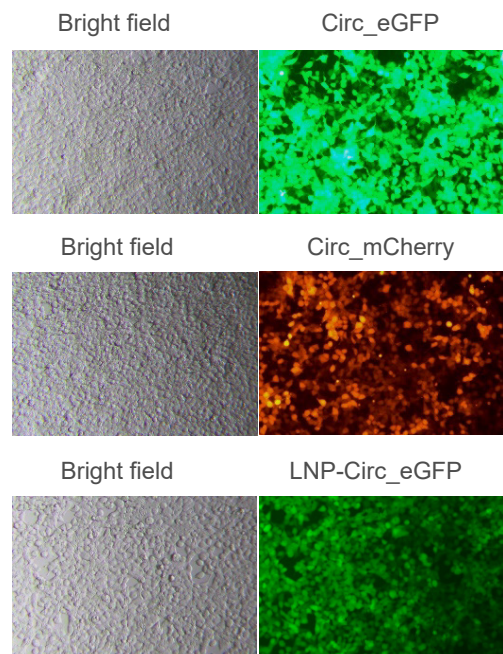
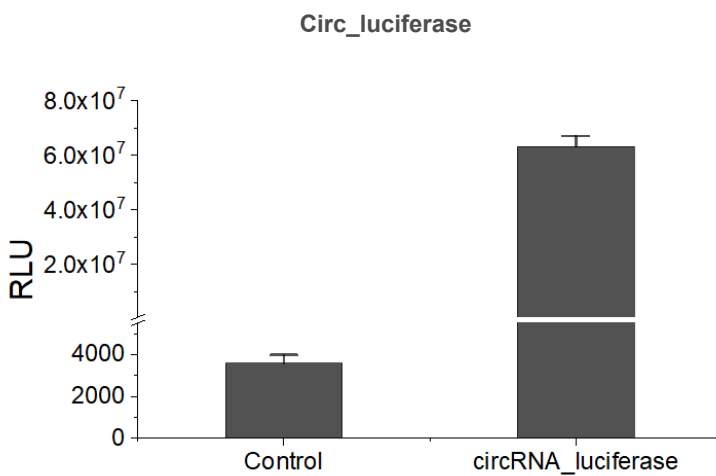
In addition to circRNA-related quality attributes, YAOHAI can provide customers with circRNA cell transfection and target protein specificity assay services based on its well-established cell culture platform. By transiently transfecting 293T cells with circRNA, we can confirm whether circRNA can successfully express the target protein in cells *in vitro*. The range of samples available for testing includes circRNA drug substance and finished product of circRNA-LNP.



Samples	Test Items	Testing Method	Delivery Period (Days)	Delivery
circRNA expression validation	293T cell evaluation	Cell pavement	4	CoAs
		Transient transfection of cells		
		Fluorescence signal observation	1-3	
		Western blot (WB)		
		ELISA		

Case Studies

Yaohai Biopharmaceutical has built a mature cell transfection platform, and the transfection samples include circRNA drug substance and finished product of circRNA-LNP; based on fluorescent signal, and enzyme-substrate reaction, strong specific signal of target protein can be detected.



CircRNA Platform

Equipment



**Phoenix PH-YGD
Fluorescence Microscope**



**Bio-Rad PCR
Instrument**



**Thermo Full Wavelength
Enzyme Labeler**



Bio-Rad Gel Imagers



Thermo qPCR instrument



Waters HPLC



PNI Microfluidic Nanoparticle Preparation System



SCIEX Capillary Electrophoresis Instrument



Cytiva AKTA Purification System

SERVE
WITH HEART &
CREATE
THE FUTURE TOGETHER

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