Yaohabio
耀海生物

PROFESSIONAL CRO SERVICES PROVIDER

- ☐ mRNA
- ☐ saRNA
- ☐ circRNA
- ☐ siRNA

R&D Direction

Raw material enzymes, plasmids, mRNA, CircRNA long chain nucleic acid drugs, single-domain antibodies (sdAbs), recombinant proteins/polypeptides and many other categories

Service Contents

- Biological raw material development
- Drug screening
- Basic technology research in the field of gene therapy
- Quality research of biological products
- Preparation process development of microbial expression drugs
- Preparation process development of cell-related drugs (biosafety at P2 level) and related quality research

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mRNA Research-Grade

Sample Preparation Services

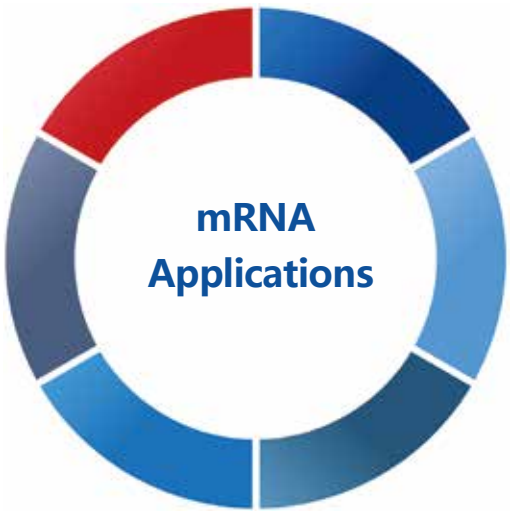
The outbreak of the COVID-19 pandemic in 2020 pushed mRNA technology to the center stage, with unprecedented heat for related research and rapid development in multiple fields such as infectious disease prevention, tumor therapy, protein replacement therapy, regenerative medicine, and cell and gene therapy (CGT).

mRNA Applications

01 Prophylactic Vaccines for Infectious Diseases

02 Therapeutic Oncology Vaccine

03 Therapeutic Oncology Drugs



06 Protein Replacement Therapy

05 Regenerative Medicine

04 Cell and Gene Therapy (CGT)

Yaohai Bio-Pharma has established a sophisticated and comprehensive "RNASci" mRNA research-grade sample preparation service platform incorporating four core modules. This platform offers a one-stop solution, encompassing sequence design and optimization, gene synthesis, recombinant plasmid construction, linearized template preparation, in vitro transcription (IVT), purification, and mRNA quality control, spanning the entire lifecycle from mRNA design to sample generation. It comprehensively supports and empowers the process of mRNA vaccine and drug development.

"RNASci"mRNA

Service Platform

PLATFORM TECHNICAL CONTENT	RNASci	RNADes	RNASyn	RNAPur	RNAQua
mRNA SERVICE PLATFORM		Platform for mRNA structural design and optimization	Platform for mRNA synthesis and modification	mRNA purification	mRNA quality analysis and control
		● 5' UTR and 3' UTR optimization design ● CDS zone optimization design ● PolyA tail optimization design	● mRNA template plasmid design and construction ● Conventional mRNA (capping structure, PolyA tail structure) ● Nucleotide modification (e.g. φ, N1ψ, 5mC mRNA synthesis)	● LiCl precipitation and purification ● Magnetic bead purification ● Self-developed chromatography column process purification	● Purity detection system [AGE (agarose gel electrophoresis), CE] ● (WB,ELISA)Translation expression detection system (WB,ELISA) ● Capping Efficiency Detection System (cooperative detection) ● PolyA distribution assay system (cooperative assay)

Features of "RNASci" mRNA Service

Highly Expressed Natural & Modified UTR

- Establishment of natural UTR library, and diversified UTR source selection can match the appropriate UTR sequence for different products;
- 5'UTR optimization for more efficient transcription of templates;
- Internationalized PolyA tail structural design strategy;
- Well-developed codon optimization methods and special optimization needs can be performed in cooperation with professional AI algorithm team.

Superior Capping Process for Efficient Transcription and Improvement of Application Activity

- Highly productive and stable capping process with a capping efficiency of >95%;
- PolyA tail integrated transcription formation, with more uniform distribution;
- Diversified mRNA modified nucleotides, effectively reducing the adverse immune response of mRNA in human;
- Flexible plasmid template design scheme for client's specific needs.

General & Self-developed Chromatography Process, Providing Diversified Purification Methods

- **Diversification:**
  A comprehensive purification solution consisting of tangential flow filtration + multiple chromatography packing can effectively remove impurities from mRNA crude products for high quality applications;
- **General & Self-developed Purification Process:**
  Well-developed and perfect LiCl precipitation + magnetic bead purification + chromatography purification solution;
  Completely self-developed, chromatography purification solution can effectively remove impurities in mRNA preparation.

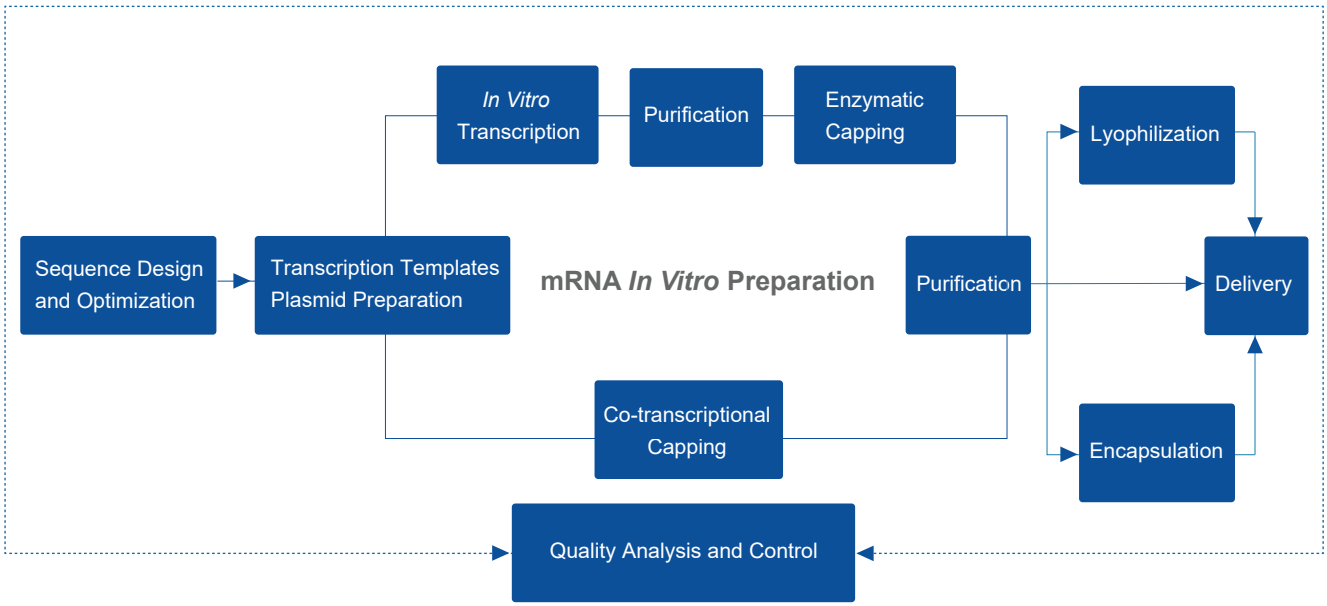
Comprehensive Quality Control Platform meets the Quality Control Needs of Each Research Phase

- Meet the general QC requirements for scientific -grade concentration and purity;
- Meet the special QC needs such as mRNA translation test, capping rate, and tail distribution, etc.

mRNA Research-Grade

Sample Preparation Services

Process Development Flow



Service Details

Service Items	Optional Items	Service Details	Delivery Period (days)	Deliverables
mRNA sequence design and optimization	Design and optimization of coding sequences	CDS sequence design and codon optimization	1-3	Sequence file
	Design and optimization of non-coding sequences	Design and optimization of UTR, polyA sequences		
Transcription template plasmid preparation	Recombinant plasmid preparation	Gene synthesis	7-10	
		Plasmid amplificationand extraction	4	
		Plasmid linearization and purification		
mRNA *in vitro* transcription	Co-transcription and capping (one-step method)	*In vitro* transcription (Clean Cap analog)	1-2	N/A
		Nucleotide modifications (UTP/CTP modifications)		
		DNA template removal (DNase I)		
	Enzymatic capping (two-step process)	*In vitro* transcription	2-3	
		Nucleotide modifications (UTP/CTP modifications)		
		DNA template removal (DNase I)		
		mRNA purification (lithium chloride/magnetic beads)		
		Enzymatic capping		
mRNA purification	Conventional purification solutions	Lithium chloride precipitation	1	mRNA drug substance
		Magnetic bead purification		
	Chromatography column purification solution	Combination of multiple chromatography methods	1-2	
	Solution exchange	Ultrafiltration and liquid exchange	1	
mRNA lyophilization	Lyophilization	Pre-freezing	2-3	mRNA lyophilized powder
		Primary drying (sublimation)		
		Secondary drying (desorption)		
mRNA encapsulation	LNP encapsulation	LNP encapsulation	2-3	mRNA-LNP drug product
		Concentration and liquid exchange		
mRNA quality analysis	mRNA drug substance/ lyophilized powder	Concentration, purity	1	Test Report
		Integrity, capping rate, polyA tail distribution	2-5	
	mRNA-LNP preparation	Encapsulation rate	1	
		Particle size and distribution detection		
		Surface charge detection		
mRNA expression validation	293T cell evaluation	Cell plating	4	
		Transient transfection of cells		
		Fluorescence signal observation	1-3	
		Western blot/ELISA		

Catalog mRNA Products

Classification of coded proteins	Product Name	Optional Modified Nucleotides	Delivery Form	Product Specification
Reported genes	mRNA_mCherry-eGFP	• No modification • Pseudouracil (Ψ) • N1methyl pseudouracil (N1Ψ) • N5methylcytosine (5mC) • Other modifications	• Lyophilized powder • Drug substance (500 ng/μL)	• 10μg • 50μg • 100 μg • 1 mg • 10 mg
	mRNA_eGFP			
	mRNA_mCherry			
	mRNA_luciferase			
Viral antigens	mRNA_Spike protein (COVID-19)			
Cytokines	mRNA_IL-2			
	mRNA_IL-4			
	mRNA_IL-22			
Immunogen	mRNA_OVA			
Nucleases	mRNA_Cas9			

Service Advantages

Integrated Service Flow

Provide a series of services from front-end sequence design to back-end mRNA preparation, quality control and expression validation.

International Cutting-edge Sequence Design and Optimization

Professional mRNA sequence design and optimization facilitates efficient mRNA expression.

Diversified Nucleotide Modifications

Effectively increase mRNA expression and reduce mRNA adverse immune responses.

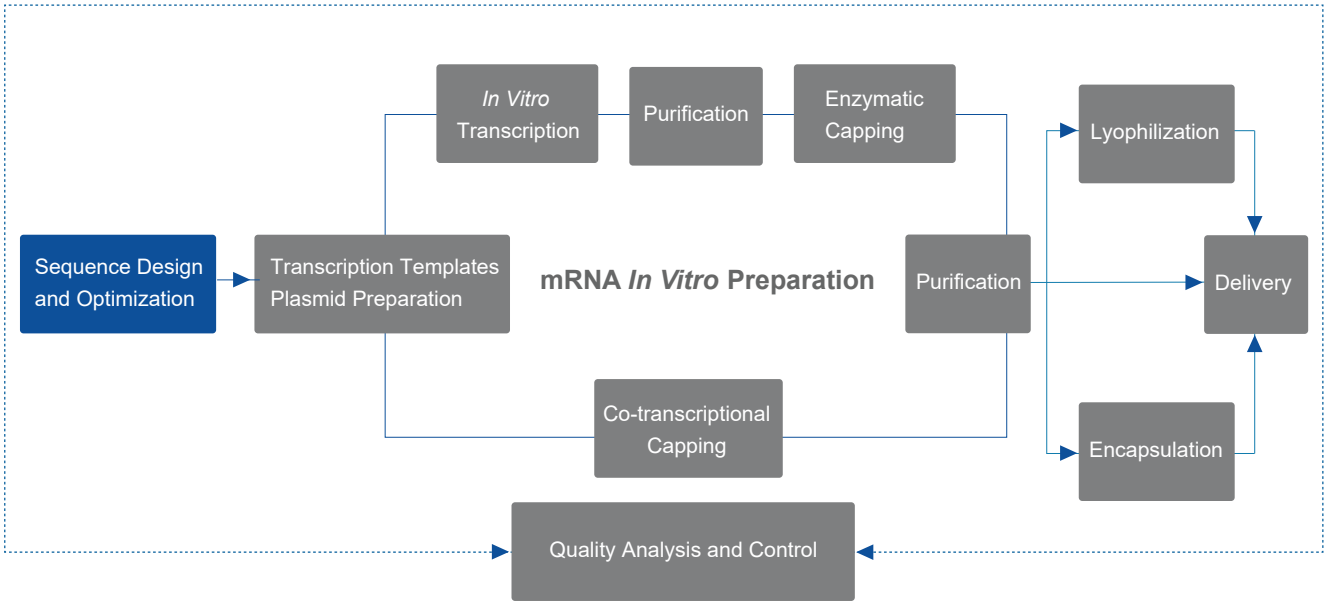
Mature Purification

A combination of general & self-developed purification process provides high purity mRNA samples. Complete QC platform: Enrich QC options to meet the requirements of routine tests such as concentration, A260/280 purity and integrity, as well as high quality control requirements such as capping rate/polyA distribution.

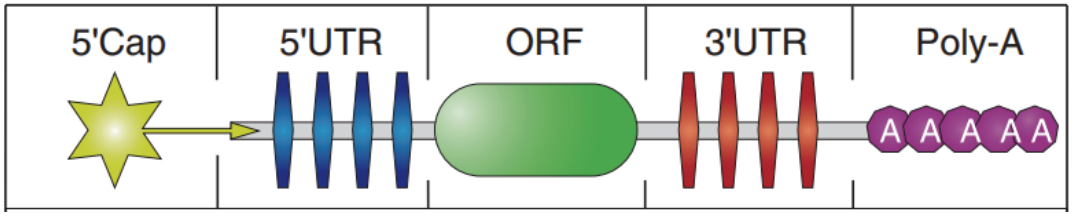
Fast Delivery

Same-day shipment of mRNA pre-products.Customized mRNA can be delivered within 7 days except for outsourced sequence synthesis.

mRNA Sequence Design and Optimization Services



According to the central dogma, messenger RNA (mRNA) is the bridge for the transmission of genetic material from DNA to proteins. mRNA plays a biological role by encoding proteins in vivo, and mature mRNA in eukaryotic organisms consists of **five components**: 5' Cap (cap structure), 5' UTR (non-coding region), the ORF (open reading frame), 3' UTR, and 3' polyA tail (polyadenylate tail).



Schematic diagram of mRNA structure

Please refer to the following for the functions and optimization strategies of each component of mRNA:

mRNA components	Biological Functions	Optimization Strategies
5’ Cap	Protect mRNA from degradation by exonucleases and act in concert with the polyA tail at the 3' end, polyA binding protein and translation initiation factor protein to initiate protein translation.	The natural Cap1 structure avoids pattern recognition receptor and thus reduces the natural immune response, which can be achieved by one-step co-transcription capping or two-step enzymatic capping [see mRNA enzymatic capping and co-transcription capping for details].
5’ UTR	The 5’ UTR can be recognized by ribosomes, regulate the translation of mRNA and affect the stability of mRNA	The 5' UTR contains Kozak sequences that do not have a highly stable second-ary structure. Natural UTRs of highly expressed genes are preferred for synthetic mRNAs such as α- and β-bead protein gene sources.
CDS	Protein-coding regions, and coding sequences for antigens, antibodies or other functional proteins.	Codon optimization increase the level of translation, noting that certain non-optimal codons may play a role in protein folding.
3’ UTR	Regulate mRNA translation and stability.	Natural UTRs of highly expressed genes are preferred for synthetic mRNAs, such as α- and β-bead protein gene sources.
3’ polyA tail	Regulate protein expression and protect cap structure from degradation.	Adequate length (100-150 bp) is required; encoding poly(A) tail on the transcription template plasmid ensures a more defined polyA tail length.

[1] Linares-Fernández S, et al. Trends Mol Med. 2020;26(3):311-323.

Service Details

Service Items	Optional Items	Detailed Steps	Delivery Period (Days)
mRNA sequence design and optimization	Design and optimization of coding sequences	• CDS sequence matching • CDS codon optimization	1
	Design and optimization of non-coding sequences	• 5' UTR sequence design and optimization • 3' UTR sequence design and optimization • polyA sequence design and optimization	1-2

Service Advantages

Diversified TR Source Selection

Multiple sources of highly expressed natural & modified UTR libraries, and mature UTR modification strategy;

Cutting-edge CDS Optimization Team

Cooperate with professional AI algorithm team to complete the optimization of codons.

Homogeneous PolyA tail Distribution

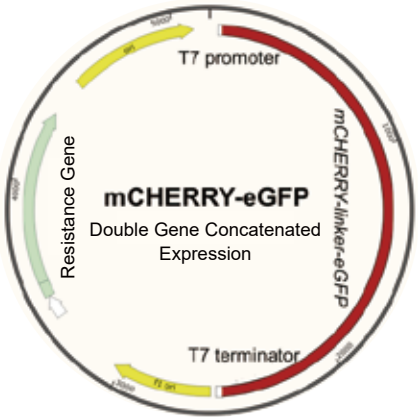
Integrated transcription formation of PolyA tail, with more homogeneous distribution.

Diversified Optimization Combination

Achieve efficient expression of mRNA, with low immunogenicity.

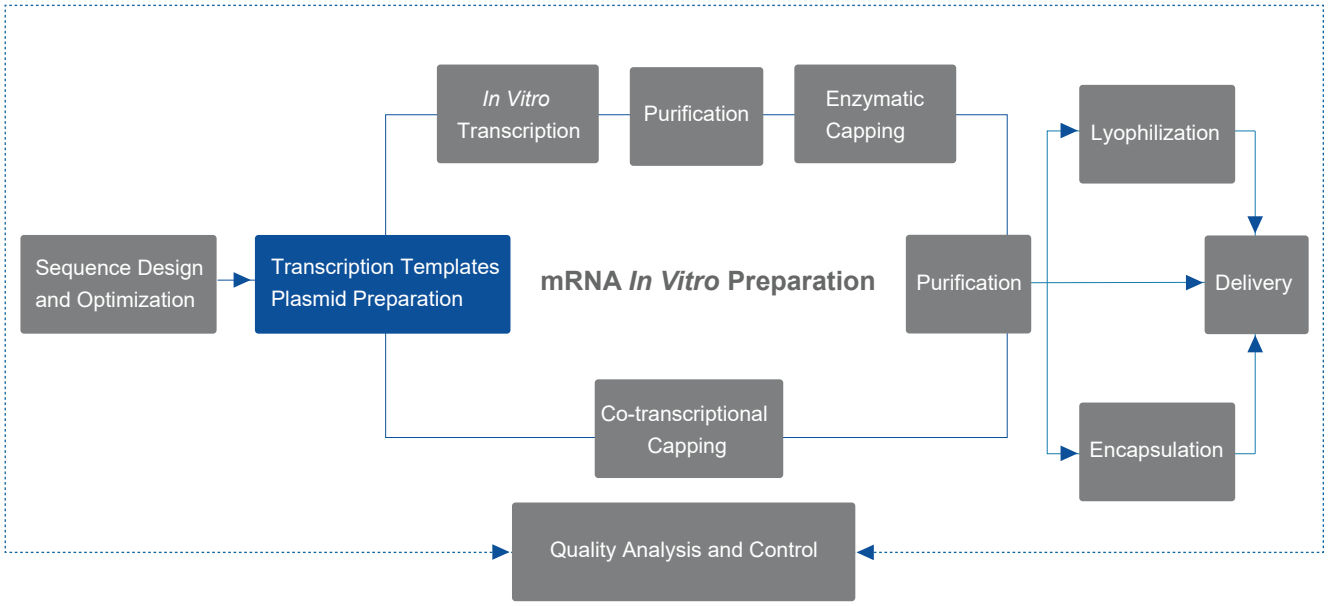
Case Study

Yaohai Bio-Pharma's mRNA service continues to be upgraded with the design and optimization of a double reporter gene tandem sequence, which allows co-expression of dual genes. Using a standard transfection reagent, the double gene tandem sequence mRNA_mCherry-eGFP is efficiently introduced into 293T cells. Subsequently, after 48 hours, two distinct fluorescent signals from mCherry (red) and eGFP (green) are simultaneously detected, with their combined visualization highlighted in yellow on the stacked graph.

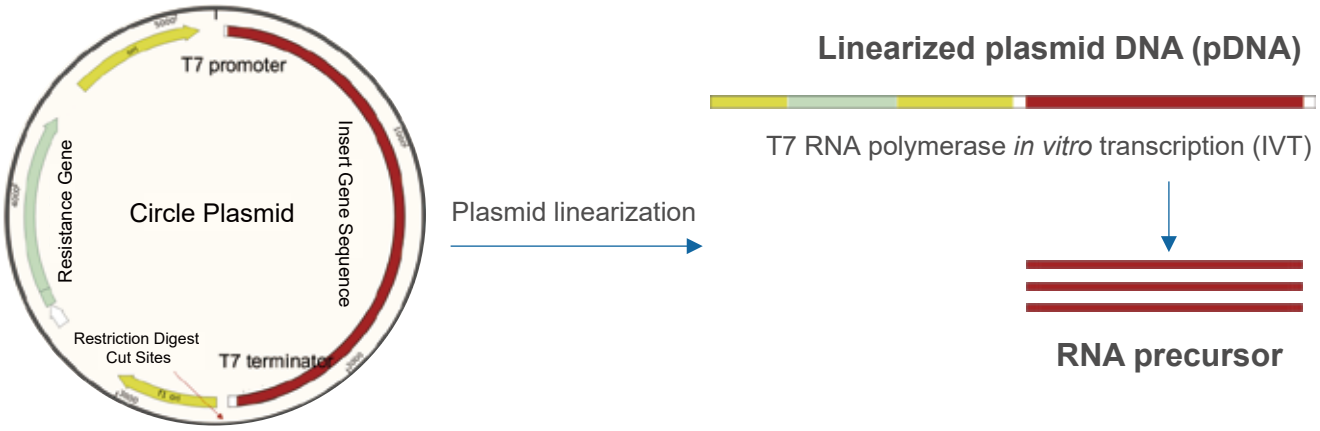


Sequence design and in vitro expression validation of circRNA_eGFP

mRNA
Transcription Template Plasmid Service



In the process of *in vitro* mRNA preparation, linearized plasmid DNA (pDNA) is required as the transcription template for *in vitro* transcription (IVT) with the help of T7 RNA polymerase. High-quality plasmid DNA is crucial for downstream IVT. Based on the mature plasmid preparation service platform, a linearized pDNA preparation service of high purity and high standard can be provided to achieve efficient downstream IVT.



Schematic diagram of IVT using linearized pDNA 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
		Purification of linearized products	
Plasmid DNA (pDNA) quality control	Concentration purity	Ultraviolet (UV) spectrophotometry	1-2
	Plasmid conformation	Agarose gel electrophoresis (AGE)	
		Capillary electrophoresis (CE)-Ooptional	
	Plasmid integrity	Restriction enzyme identification/AGE	

Service Advantages

Freecut Template Plasmids

Flexible plasmid template design options can satisfy the specific customization needs.

High Recovery

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

Mature Technology

Provide plasmid preparation and quality control services of high standard and high efficiency to meet the needs of downstream tests.

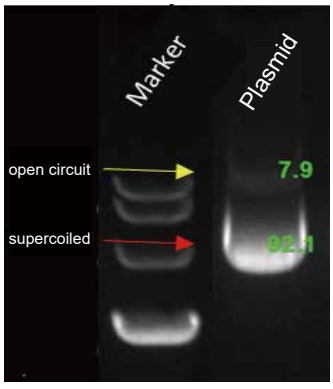
Stringent Specification

Plasmid samples for research with a superhelical conformation ratio of >70%.

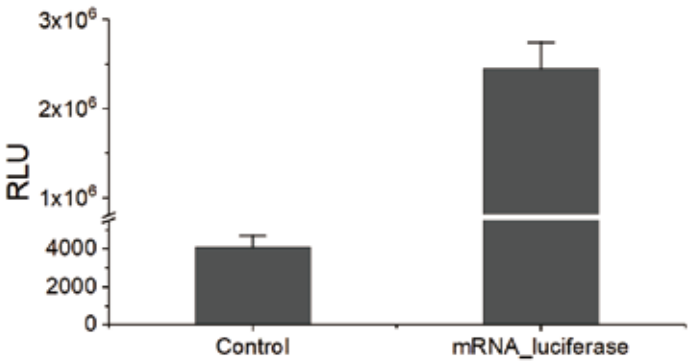
Case Study

Taking Yaohai Bio-Pharma mRNA_luciferase product as an example, the transcription template plasmid sample (research grade) has a superhelical ratio of more than 90%, a linearization ratio close to 100%, and a subsequent transcription ratio up to 1:200 (linearized pDNA: mRNA).

The mRNA_luciferase obtained through the preparation of a linearized plasmid as a template is transfected into 293T cells, and the enzyme-substrate reaction activity is evaluated 24 h after transfection. An obvious strong luciferase activity signal can be detected, i.e., the luciferase protein is expressed efficiently, suggesting the purity of the transcription template, which can fully satisfy the requirement of high-quality mRNA preparation.

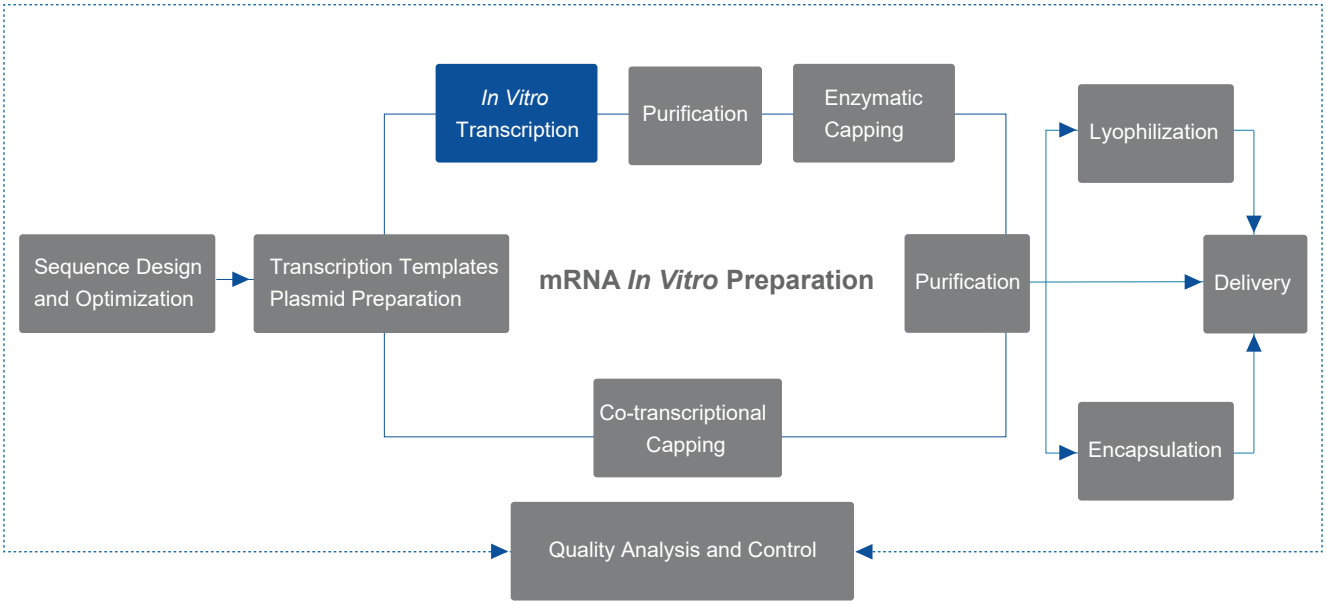


Plasmid Superhelix Ratio Assay



Validation of mRNA-mCherry Expression *In Vitro*

mRNA
In Vitro Transcription Service



Regarding the preparation of mRNA in batches, *in vitro* transcription (IVT) is a more efficient and mature method. The IVT reaction adopts linearized plasmid DNA (pDNA) containing T7 promoter as template and mRNA is synthesized with nucleoside triphosphates (NTPs) as substrate in the presence of T7 RNA polymerase.

Nucleotide modification is a major breakthrough in the exploration of drug formulation of mRNA, where unmodified mRNA molecules are recognized by intracellular RNA sensors to activate innate immunity. For considerations of mRNA *in vivo* immunogenicity and translation efficiency, the IVT process usually employs certain kinds of modified NTPs, and common modified nucleotides are pseudouridine (Ψ), N1-methyl-pseudouridine (N1Ψ), and 5-methylcytosine (5mC).

In Vitro Transcription (IVT) Process of mRNA

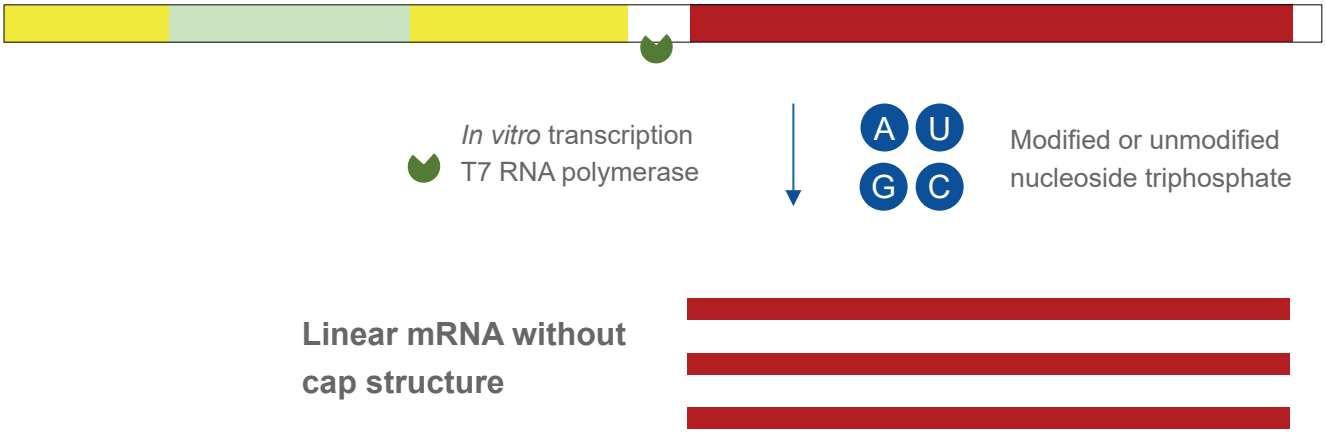


Figure: IVT reaction diagram

Service Details

Service Items	Service Details	Delivery Period (Days)
***In vitro* transcription (IVT)**	Reaction system confirmation	1
	IVT	
	Nucleotide modifications (Ψ/N1Ψ/5mC)	
	DNA template removal (DNase I)	
IVT condition optimization - optional	Reaction system design and optimization	2-5

Service Advantages

Improve mRNA stability and protein expression levels *in vivo*.

Diversified Nucleotide Modification Strategies

mRNA fragment preparation up to 10kb can be achieved.

Rigorous Test Design and Optimization

By optimizing *In vitro* transcription (IVT) reaction conditions, high efficiency transcription is achieved with a transcription ratio of up to 1:200.

Efficient Transcription

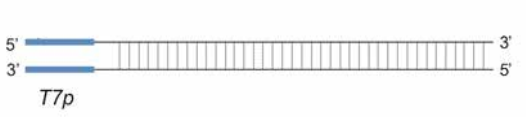
Stringent enzyme control through experimental environment and consumables can effectively prevent mRNA degradation.

Stringent Enzyme Specification

Case Study

The current *In vitro* transcription (IVT) reaction system is roughly optimized for synthetic systems with a length of about 100 nt, not for mRNAs of arbitrary length. The longer the mRNA sequence, the more difficult to transcribe and the more prone to degradation.

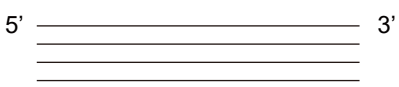
To prepare customized mRNA sequences of approximately 10 kilobases in length, Yaohai Bio-Pharma has achieved remarkable success. Through meticulous experimental design, continuous refinement of reaction conditions, and strict management of RNase, we have been able to transcribe 1 microgram of linearized plasmid in vitro, resulting in a high transcription ratio of 1:135. This process has yielded 135 micrograms of both crude and purified mRNA products, ensuring the quality and integrity of our customized samples.



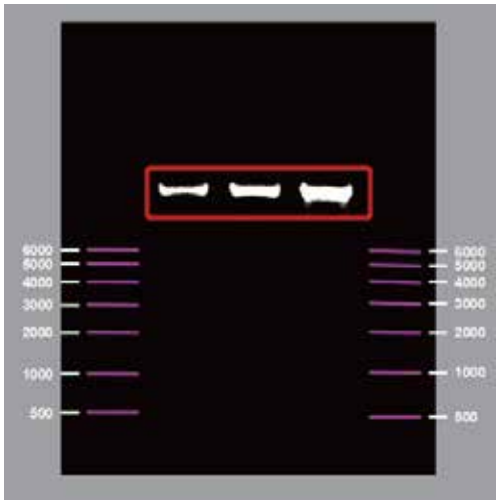
DNA template **1 μg**

High transcription ratio

In vitro transcription (IVT) reaction
Lithium chloride crude purification



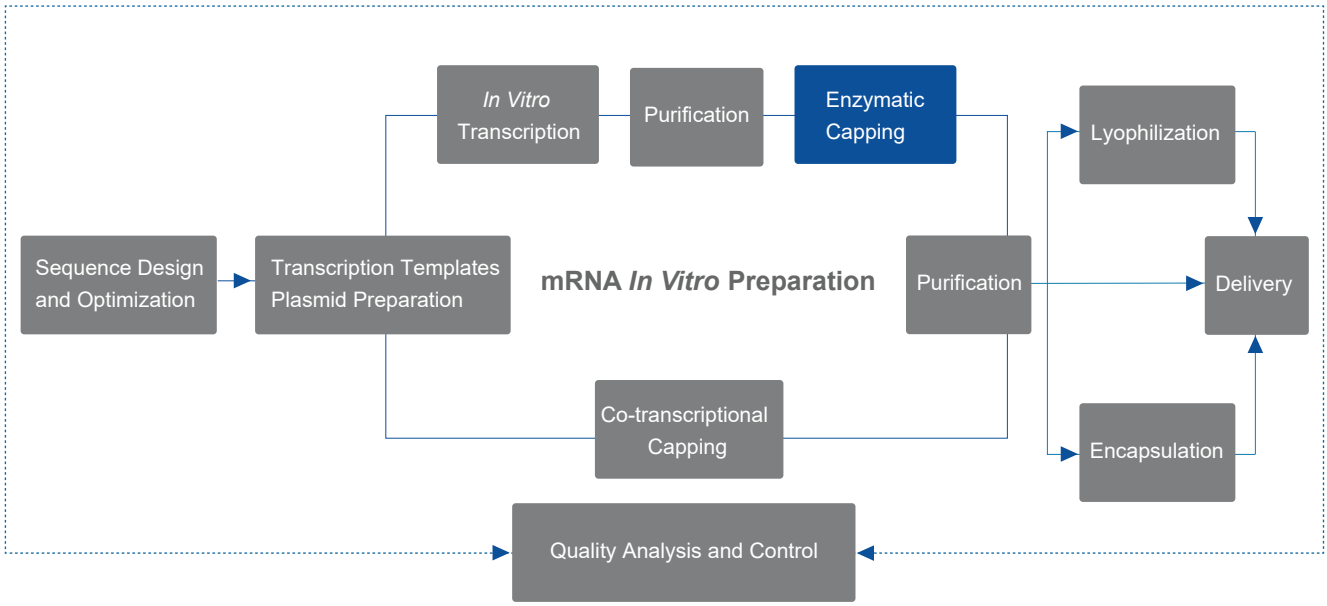
ImRNA product after crude purification **135 μg**



mRNA length and purity assay

mRNA

Enzymatic Capping Service



5'-end capping is an essential modification of mRNA. mRNAs with cap structures, especially Cap1 cap structures, facilitate mRNA evasion of innate immune responses *in vivo*, resulting in efficient protein translation.

Enzymatic capping (two-step method) is the conventional method of mRNA capping, similar to the capping process in eukaryotic organisms. Under the action of a series of enzymes, 7-methylguanine (m7G) is linked to the 5'-end of mRNA through a 5'-5' triphosphate bond and undergoes methylation modification to form the cap structure Cap 1 (m7GpppN).

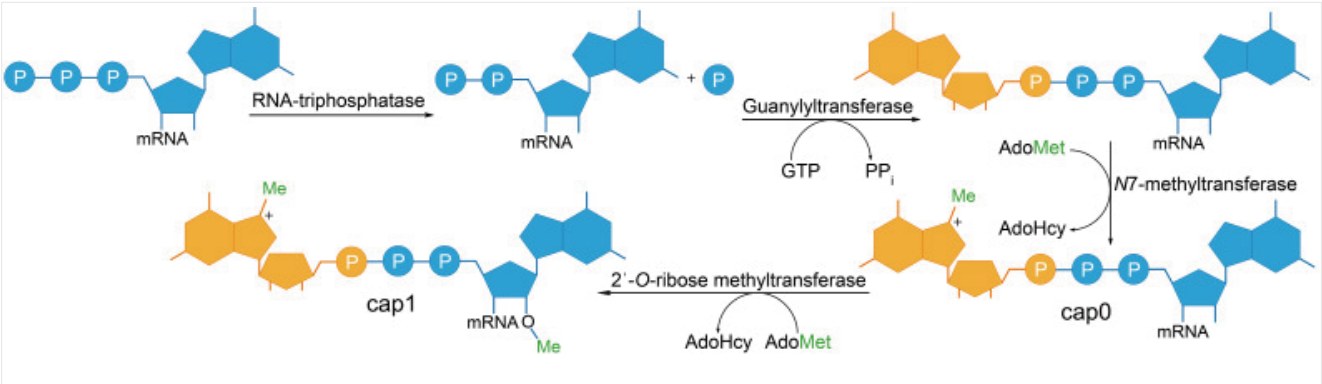


Figure: Diagram of natural cap structure formation

The enzymatic capping reaction flow is as follows: Linearized plasmid DNA (pDNA) is used as a template for *in vitro* transcription (IVT) in the presence of T7 polymerase, and mRNA with a 5' end-cap structure is formed after a one-step purification using vaccinia capping enzyme and 2'-O-methyltransferase.

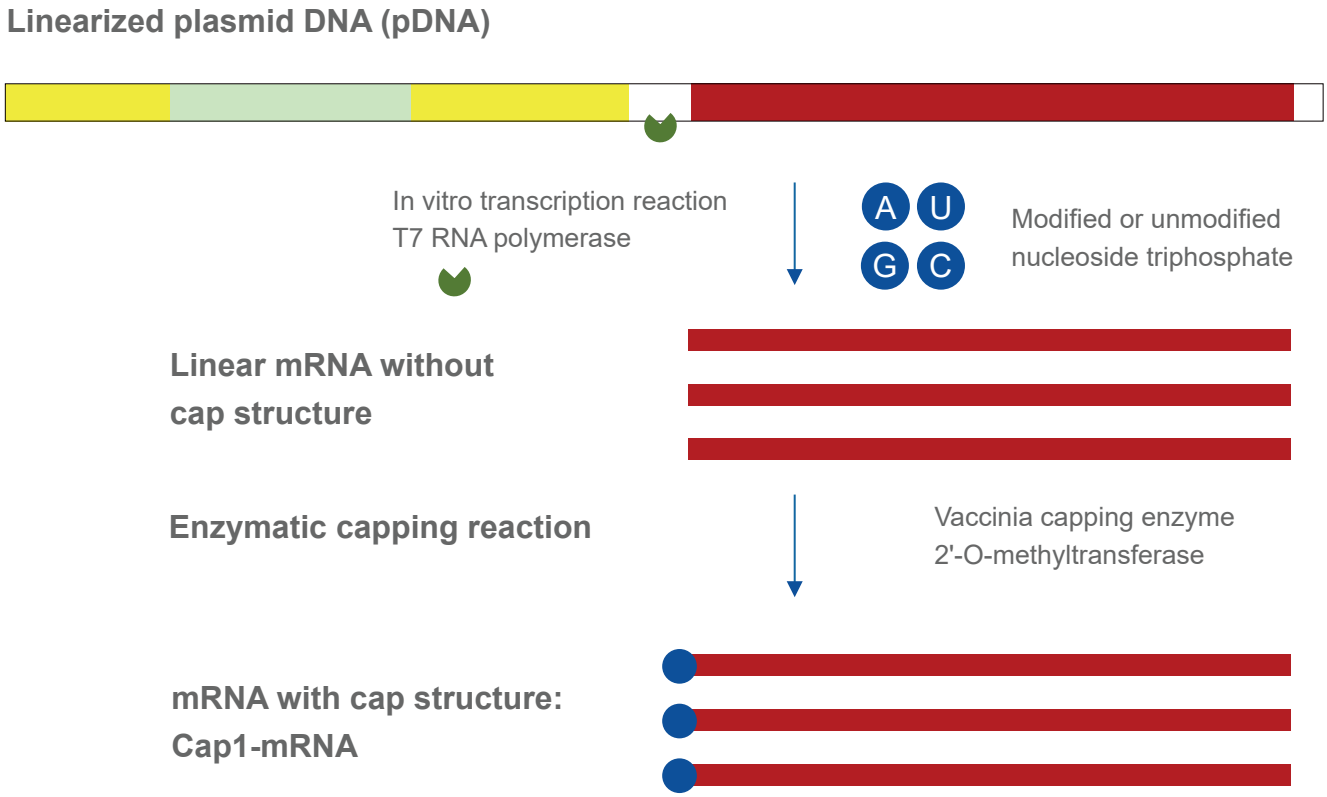


Figure: Diagram of mRNA enzymatic capping reaction

Service Details

Service Items	Service Details	Delivery Period (Days)
mRNA enzymatic capping	Reaction system verification	1
	Enzymatic capping reaction	
Capping response optimization - optional	Reaction system design and optimization	3-7

Service Advantages

Design and Optimization of the Capping Reaction System

The *in vitro* transcription (IVT) reaction system is adjusted and the mRNA transcription product is greatly enhanced.

In Vitro Expression Verification

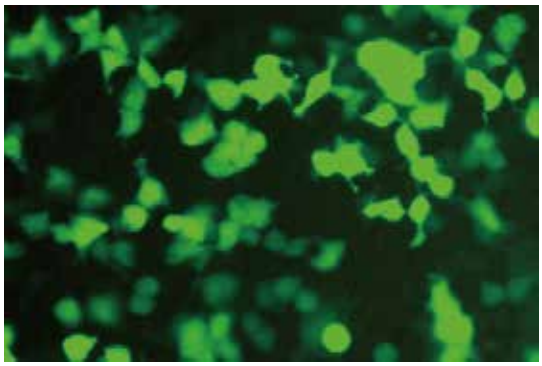
The capped mRNA is transfected into 293T cells, and the expression of the target protein can be detected.

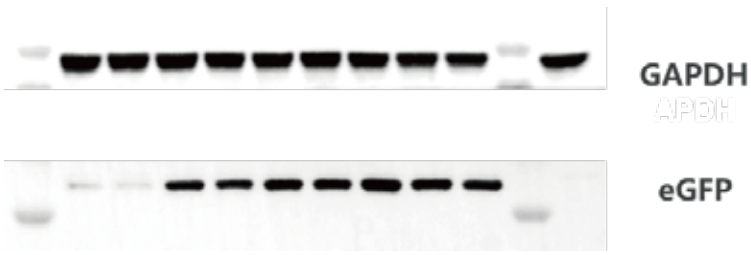
Stringent Enzyme Specification

Through stringent enzyme control on experimental environment and consumables, mRNA degradation is effectively prevented.

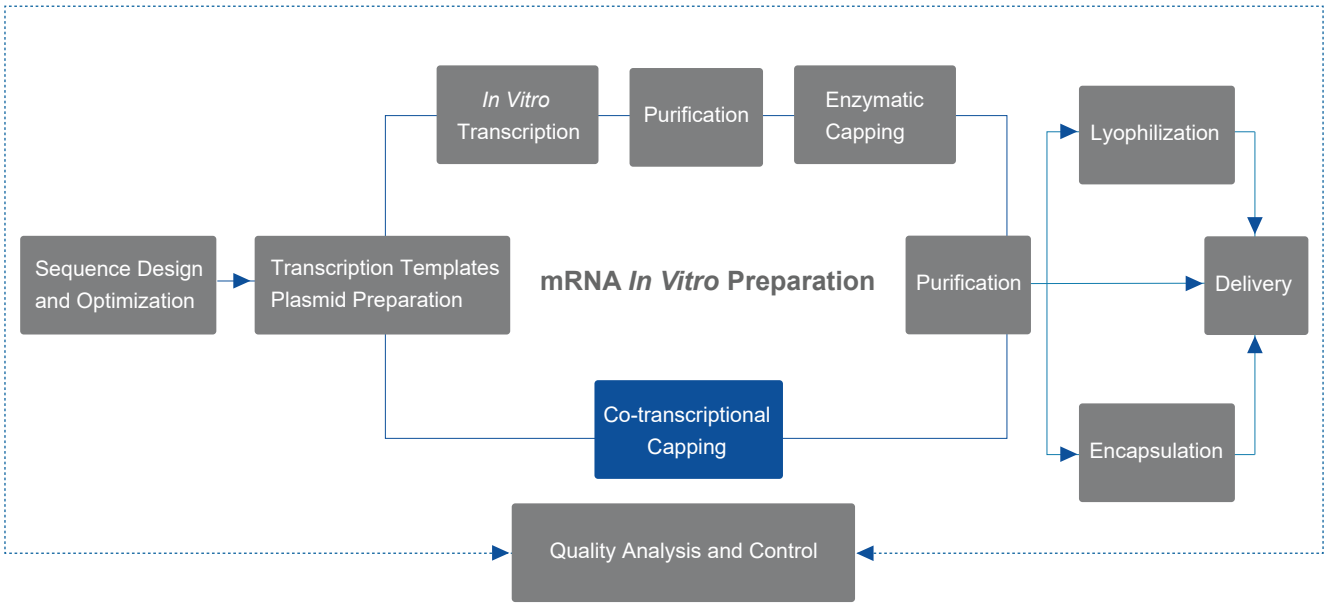
Case Study

Yaohai Bio-Pharma's mRNA platform has built a perfect capping reaction system. mRNA_eGFP is the mRNA product prepared by enzymatic capping. A high level of eGFP fluorescence signal can be detected by Western Blot(WB) after transfecting 293T cells for 24 h. It demonstrates that the target protein eGFP can be efficiently expressed *in vitro*.





mRNA
Co-transcription Capping Service



Compared with the two-step enzymatic capping method, the one-step co-transcription capping method can significantly reduce the process flow. The method is result-oriented, and by adding cap analogs to the *in vitro* transcription reaction system, cap analogs can be introduced at the start of transcription, and mRNA with cap structure can be obtained upon completion of transcription. Current third-generation cap analogs can avoid reverse capping and directly add Cap 1 cap structure to the transcription product.

For considerations of mRNA *in vivo* immunogenicity and translation efficiency, the *in vitro* transcription (IVT) process often adopts certain kinds of modified NTPs, and common modified nucleotides are pseudouridine (Ψ), N1-methyl-pseudouridine (N1Ψ), and 5-methylcytosine (5mC).

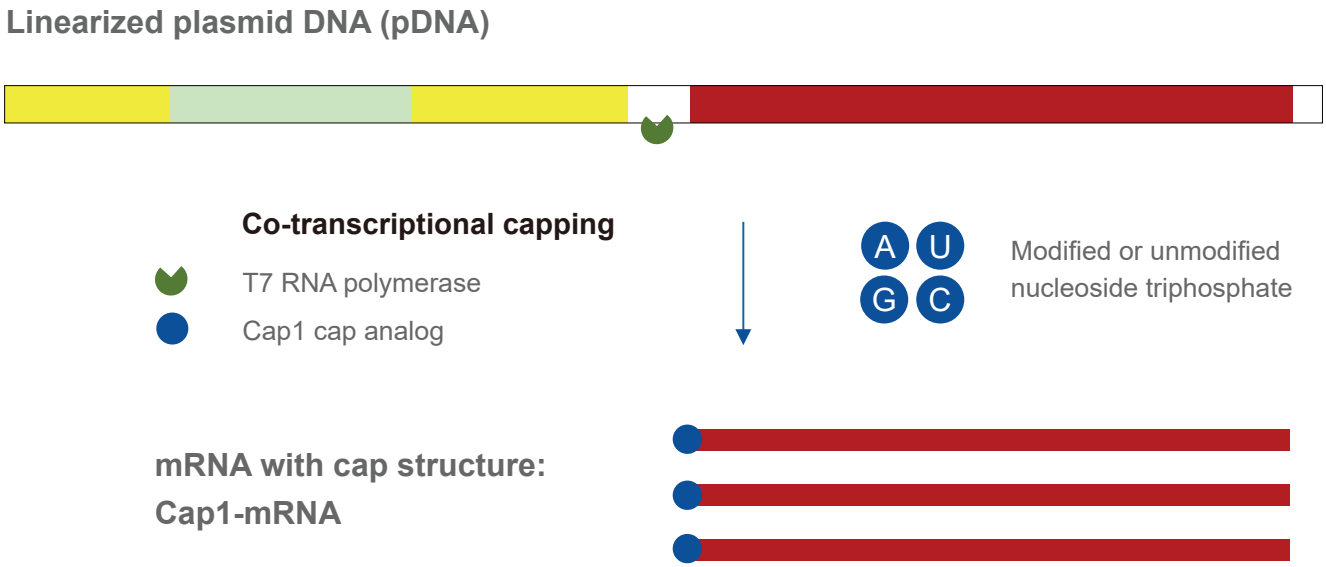


Figure: Diagram of mRNA Co-transcriptional and capping reaction

Service Details

Service Items	Service Details	Delivery Period (Days)
Co-transcriptional capping	Reaction system verification	1-2
	In vitro transcriptional response (Clean Cap analog)	
	Nucleotide modifications (Ψ/N1Ψ/5mC)	
	DNA template removal (DNase I)	
***In vitro* transcription (IVT) condition optimization - optional**	Reaction system design and optimization	3-7

Service Advantages

Multiple optional nucleotide modification strategies can improve protein expression.

Diversified Nucleotide Modification Strategies

Achieve a high transcription ratio and a high capping rate.

Optimized Reaction System

Achieve a capping rate of more than 95%.

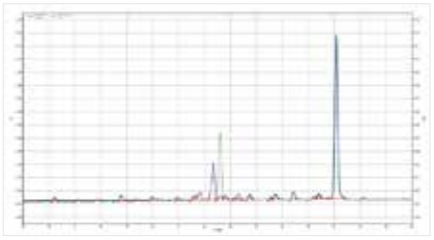
Stable Capping Process

Prevent mRNA degradation effectively through stringent enzyme control on experimental environment and consumables.

Stringent Enzyme Specification

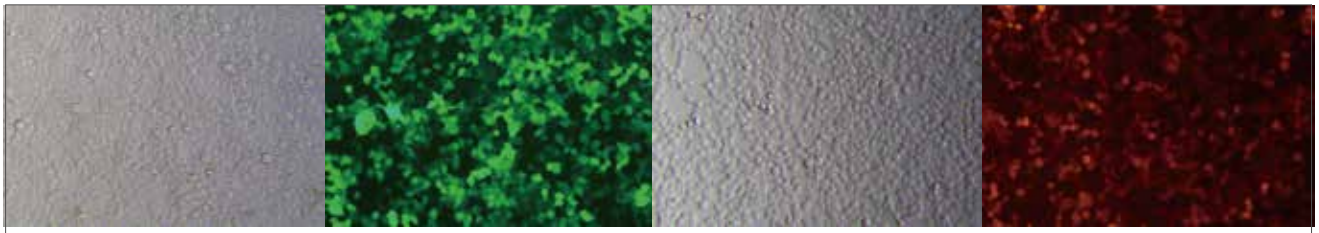
Case Study

Yaohai Bio-Pharma has developed a mature Co-transcriptional capping process platform, utilizing Clean Cap analogs to directly incorporate the Cap1 cap structure and prevent reverse capping. Following standardized sample pre-treatment and capillary electrophoresis (CE) detection, the capping rate of the mRNA_eGFP product can exceed 95%.



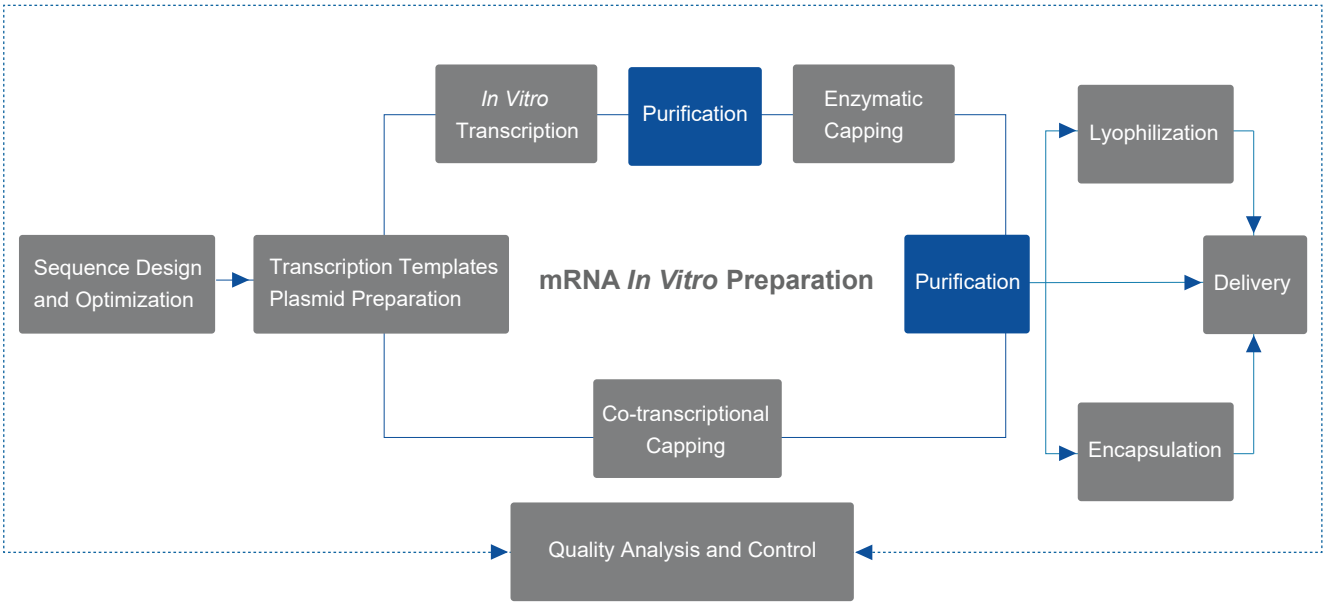
A mRNA_eGFP capping rate of more than 95%

The mRNA_eGFP and mRNA_mCherry products prepared by co-transcriptional capping are transfected into 293T cells, respectively, and a strong fluorescent signal is observed after 48h, suggesting that the mRNA is efficiently expressed in 293T cells.



Bright Field	mRNA_eGFP	Bright Field	mRNA_mCherry

mRNA
Purification Service



The mRNA prepared by *in vitro* transcription (IVT) and capping reaction requires further purification to remove the immunogenic unconsumed substrates and reaction by-products from IVT and capping reaction to ensure the efficacy and safety of the mRNA drug.
Yaohai Bio-Pharma can provide mature solutions for LiCl precipitation, magnetic bead purification and chromatography purification, which can effectively remove multiple impurities and prepare high-purity mRNA.

LiCl Precipitation Method

Simplified purification solution of small amounts of mRNA for cell transfection and some animal experiments;
For the purification of pre-capped samples after *in vitro* transcription(IVT).

Oligo dT Magnetic Bead Purification Method

Purification solutions of small amounts of mRNA for cell transfection and some animal experiments;
For the purification of pre-capped samples after *in vitro* transcription(IVT).

Chromatography Purification Method

Purification solutions with multiple chromatography compositions such as affinity, ion exchange and hydrophobic chromatography;
Meet the downstream application scenarios with higher quality requirements, such as cell transfection, and Lipid nanoparticle (LNP) encapsulation, etc.

Service Details

Service Items	Optional Items	Detailed Steps	Delivery Period (Days)	Delivery
mRNA purification	Conventional purification solution	Lithium chloride precipitation	1	mRNA drug substance (DS)
		Magnetic bead purification		
	High purity purification solutions	Affinity chromatography or multiple chromatography combinations	2	
	Buffer exchange	Ultrafiltration and buffer exchange	1	
mRNA quality control	Concentration measurement	Ultraviolet (UV) spectrophotometry	0.5	Test report
	Integrity and purity testing	Agarose gel electrophoresis		
		Capillary electrophoresis (CE)-optional	1	

Service Advantages

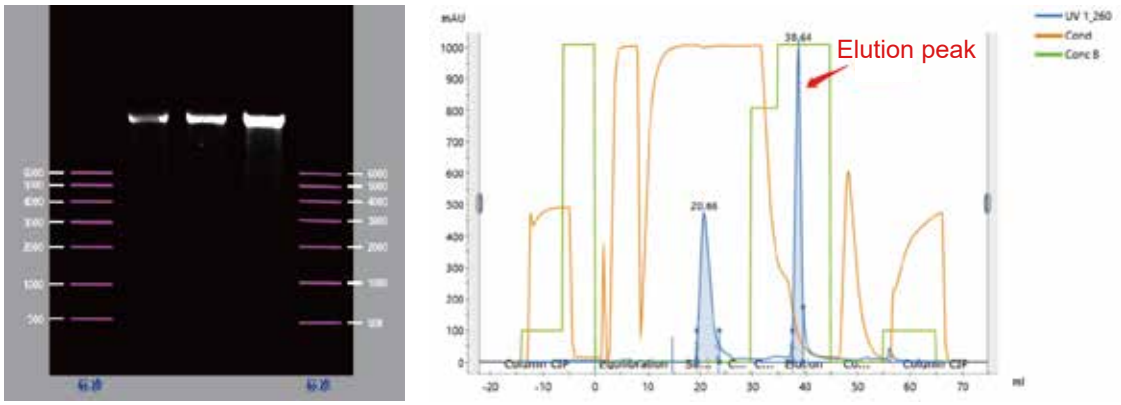
A variety of optional purification solutions can meet different downstream application scenarios.

The purity of mRNA can routinely reach more than 95%, with the highest purity reaching 100%.

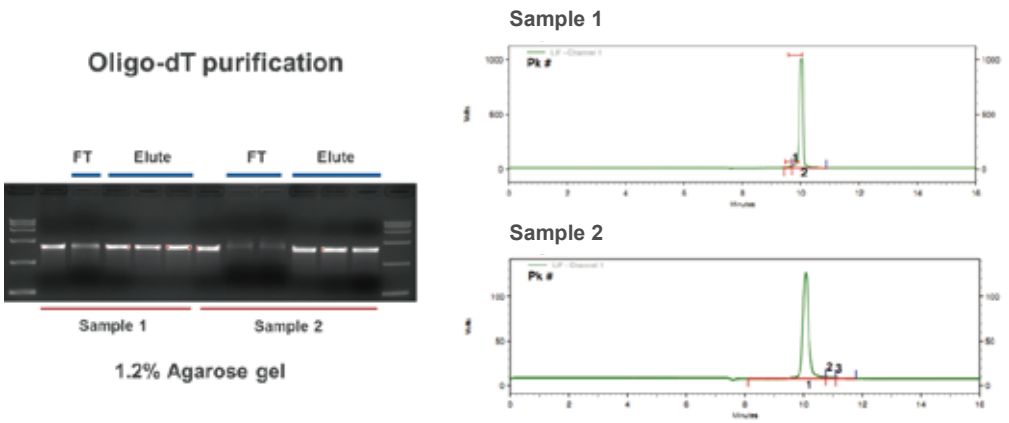
Stringent enzyme specification can prevent mRNA degradation through stringent enzyme control of experimental environment and consumables.

Case Study

Yaohai Bio-Pharma can provide mature mRNA purification solutions, which can effectively remove various small molecule process-related impurities.
The purity of mRNA samples prepared by chromatography can reach more than 95% as detected by capillary electrophoresis, and the content of dsRNA is less than 0.06% as detected by enzyme-linked immunosorbent assay (ELISA) kit, which meets the demand of downstream application of mRNA with high quality.

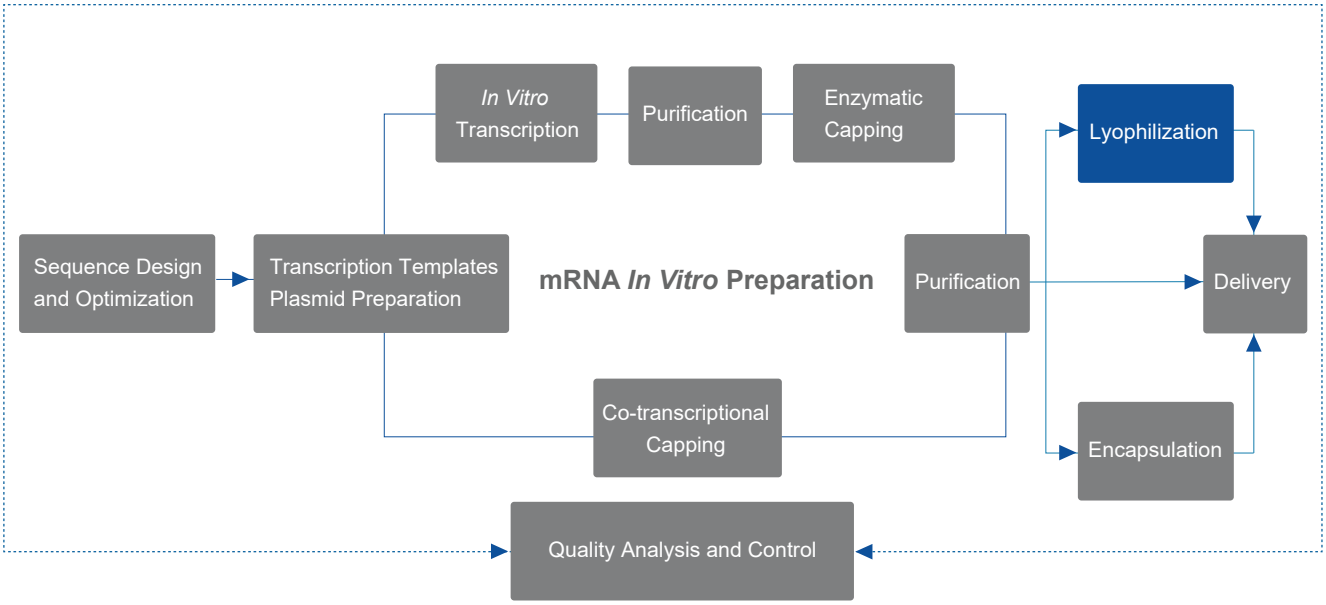


Customized purification of mRNA (>9kb)

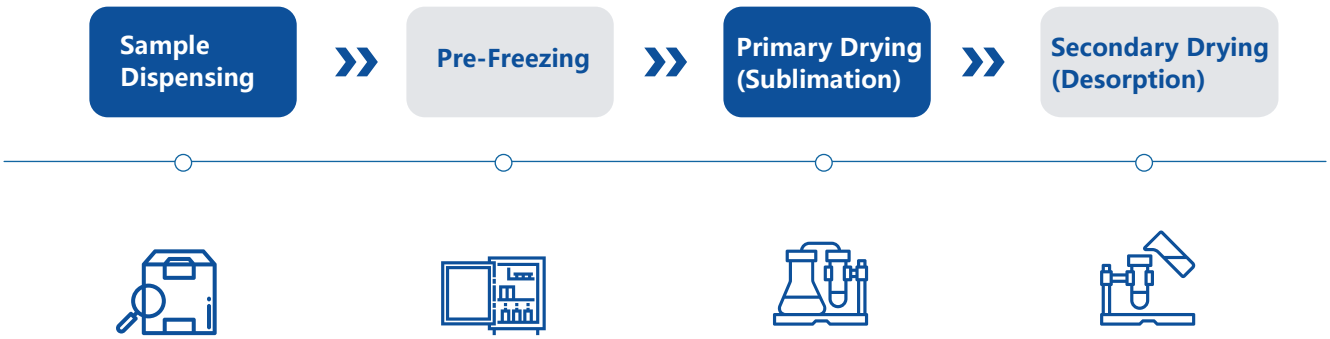


The purity of mRNA_spike protein product (new coronavirus) is more than 95%.

mRNA
Lyophilization Service



In order to improve the stability of mRNA and avoid loss in storage and transportation, Yaohai Bio-Pharma can provide mRNA lyophilization service for client to freeze-dry the mRNA drug substance (DS). In this way, DS can be stored or transported in the form of lyophilized powder, which can significantly reduce the degradation and loss of mRNA.



Service Details

Service Items	Optional Items	Detailed Steps	Delivery Period (Days)	Deliverables
mRNA lyophilization	Sample dispensing	Dispensing	2-3	mRNA lyophilized powder
	Lyophilization	Pre-freezing		
		Primary drying (sublimation)		
		Secondary drying (desorption)		
mRNA quality control	Reconstitution of lyophilized powder	Reconstitution / Resuspension	-	Test report
	Solubility of lyophilized powder	Appearance inspection	-	
		Ultraviolet (UV) spectrophotometry	0.5	
	Concentration measurement	Agarose gel electrophoresis		
	Integrity and purity testing	Capillary electrophoresis (CE)-optional	1	

Service Advantages

Mature Lyophilization Process

Lyophilization has no effect on mRNA integrity.

Homogeneous Quality Properties

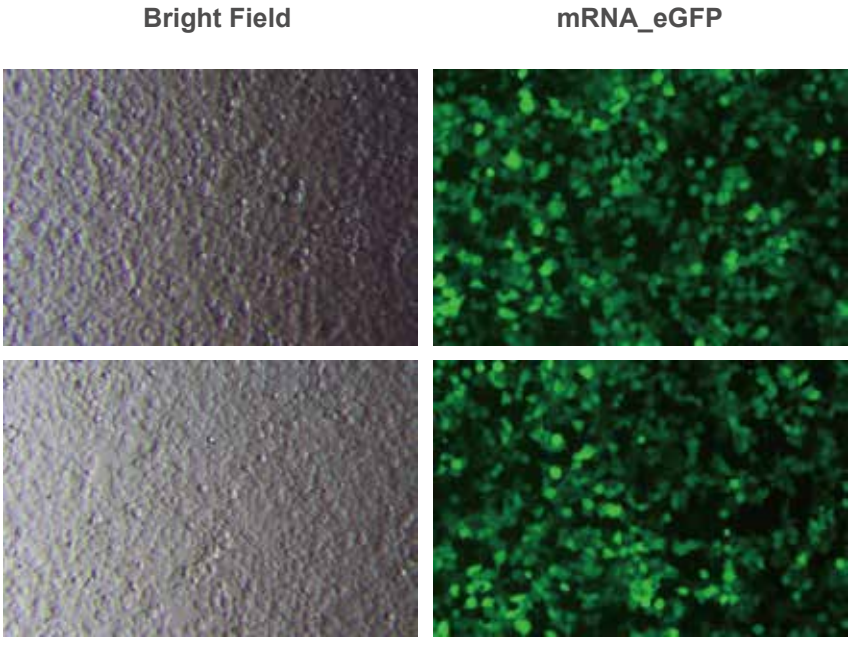
mRNA samples before and after lyophilization can successfully express the target protein.

High Stability

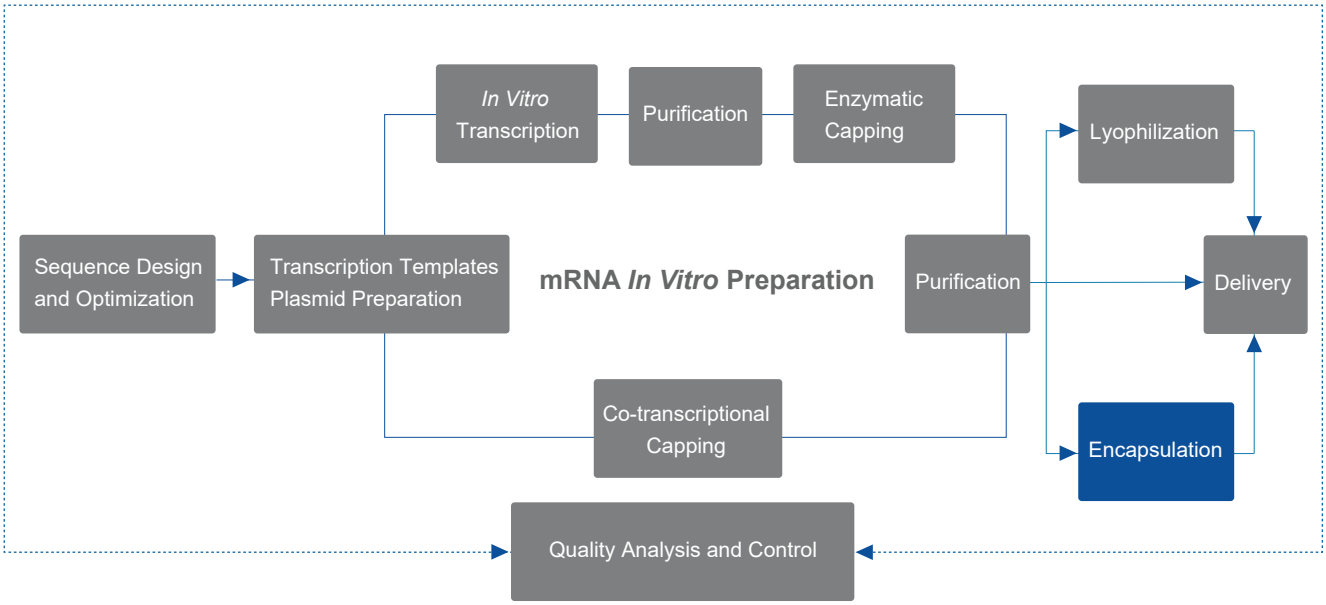
mRNA lyophilized powder is easy to store and transport.

Case Study

Using conventional liposomes, mRNA samples before and after lyophilization are transfected with 293T cells for cellular evaluation. The results show that strong fluorescence signals are observed before and after the lyophilization of mRNA_eGFP sample products, which can express the target protein efficiently *in vitro*.

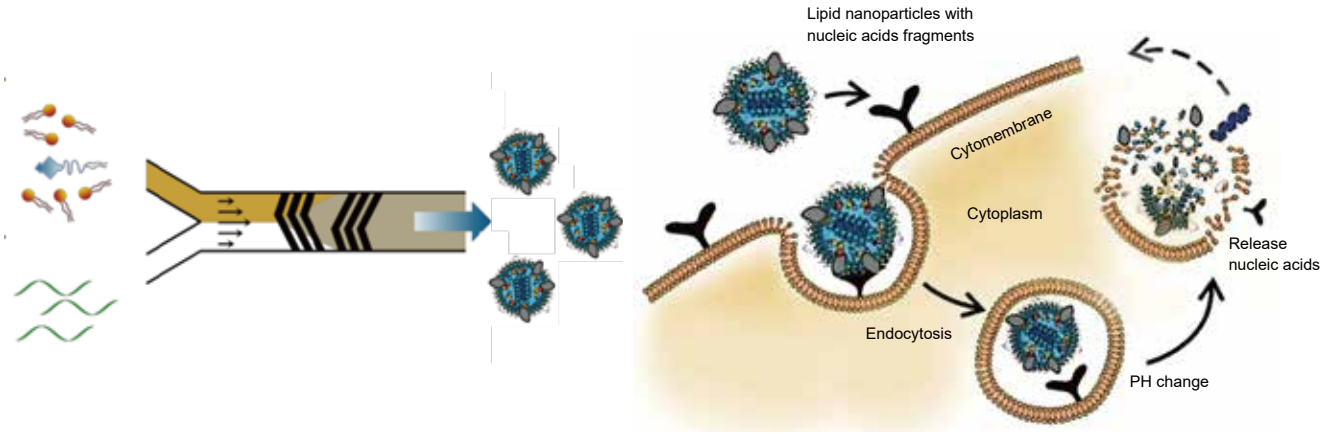


mRNA-LNP
Encapsulation Service



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

Yaohai Bio-Pharma mRNA service continues to improve and now can provide mRNA-LNP encapsulation service, optimize relevant critical process parameters, and improve the consistency and reproducibility of mRNA drug production (DP).



Material and Liquid Pretreatment	Microfluidics	Tangential Flow Filtration	Sterilization Filtration
			
Two strands of bulk are prepared: one for mRNA in aqueous buffer and one for lipids dissolved in ethanol.	Rapid mixing of lipid, mRNA two-phase solutions using microfluidic devices, resulting in uniform lipid nanoparticle (LNP) and high efficiency encapsula-tion.	The bulk is concentrated to the desired target concentration of the drug product (DP) through the process of ultrafiltration. Following this, the buffer is exchanged with a neutral storage solution in order to effectively remove any unencapsu-lated mRNA, excess lipids, and acetic acid.	Comply with the sterility regulations, the terminal sterilizing filtration system is selected, and the bacterial challenge test passes.

Service Details

Service Items	Detailed Steps	Delivery Period (Days)	Delivery
mRNA-LNP encapsulation	Material and liquid pretreatment	2	**mRNA-LNP drug product (DP)**
	Microfluidic device mixing		
	Ultrafiltration concentration	1	
	Sterilizing filtration		
mRNA-LNP quality control	Encapsulation rate	1	**Test report**
	Particle size and distribution detection		
	Surface charge detection		
	mRNA-LNP expression validation	5-7	

Service Advantages

Mature Manufacturing Technology

Fast synthesis, efficient R&D services and pre-optimized solutions.

High Encapsulation Rate

mRNA-LNP encapsulation rate can reach more than 90%.

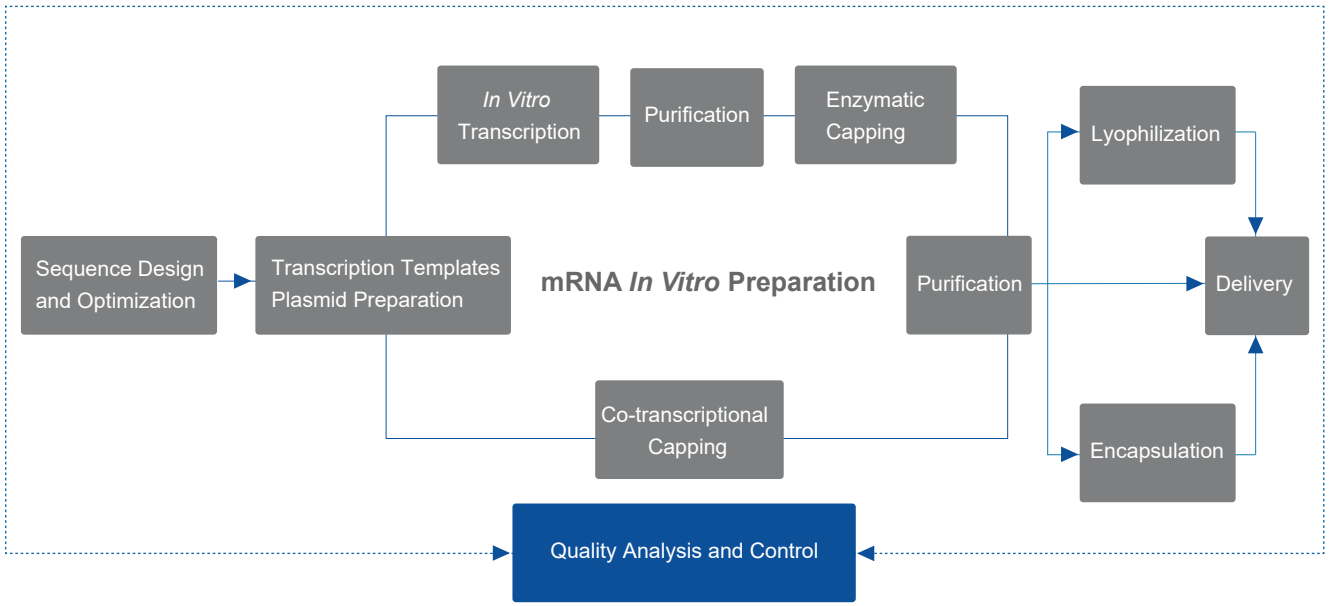
Nanoparticle Size

Lipid nanoparticle (LNP) size can be effectively controlled by changing the fluid injection rate and ratio.

Efficient Expression

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

mRNA
Quality Analysis and Control Service



According to the Technical Guidelines for Pharmacological Studies of Novel Coronavirus Prophylactic mRNA Vaccines issued by the NMPA in 2020, it is recommended to implement quality control measures for the DNA template, mRNA drug substance (DS), and finished mRNA-LNP.

Yaohai Bio-Pharma can provide quality analysis services for cyclic and linearized plasmids, mRNA drug substance (DS) and finished LNP-mRNA to meet client project needs in all aspects.

Service Details

Samples	Test Items	Testing Method	Delivery Period (Days)	Deliverables
Cyclic plasmid DNA	Concentration/Purity	Ultraviolet (UV) spectrophotometry	N/A	Test report
	Superhelix ratio	Agarose gel electrophoresis (AGE)	0.5	
		Capillary electrophoresis (CE)	1	
Linearized plasmid DNA (pDNA)	Concentration/Purity	Ultraviolet (UV) spectrophotometry	N/A	
	Linearized rate and integrity	Agarose gel electrophoresis (AGE)	0.5	
		Capillary electrophoresis (CE)	1	
mRNA drug substance (DS)	Concentration/Purity	Ultraviolet (UV) spectrophotometry	N/A	
	Integrity	Agarose gel electrophoresis (AGE)	0.5	
		Capillary electrophoresis (CE)	1	
	Capping rate	Capillary electrophoresis (CE)	3	
	PolyA distribution	Capillary electrophoresis (CE)	3	
mRNA-LNP drug product (DP)	dsRNA	Enzyme-linked immunosorbent assay (ELISA)	1	
	Encapsulation rate	RiboGreen method	1	
	Particle size and distribution	Particle size meter	1	
	Surface charge	Particle size meter	1	
mRNA expression validation	293T cell evaluation	Cell transfection	4	
		Fluorescence observation	1-3	
		Western blot (WB)/Enzyme-linked immunosorbent assay (ELISA)		

mRNA
In Vitro Expression Validation Service

In addition to mRNA-related quality attributes, based on the mature cell culture platform, Yaohai Bio-Pharma can provide clients with specificity assay services of mRNA cell transfection and target proteins to transiently transfect 293T cells with mRNA to verify whether mRNA can successfully express the target protein in cells *in vitro*. The range of samples that can be tested includes mRNA drug substance (DS) and finished mRNA-LNP.

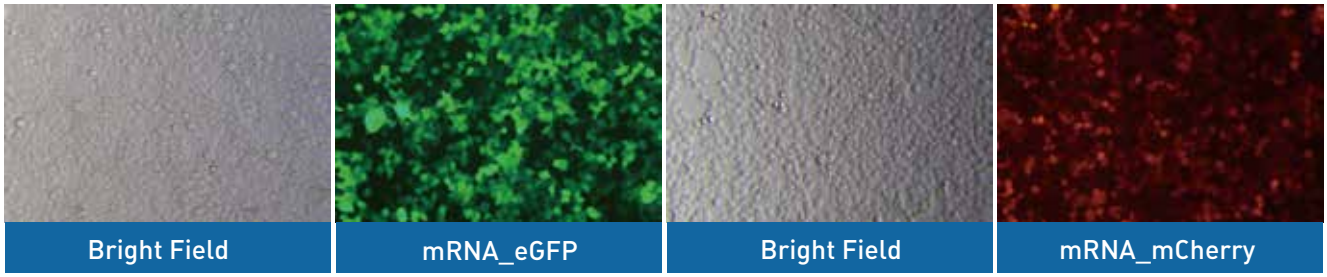
Cell Plating	Sample Preparation	Cell Transfection	Target Protein Detection
Recording cell generations Observation of cell morphology Cell plating	mRNA + Transfection reagents or mRNA-LNP	mRNA Transfected to 293T cells	Fluorescent photo shoot Western blot (WB) Enzyme-linked immunosorbent assay (ELISA)

Service Details

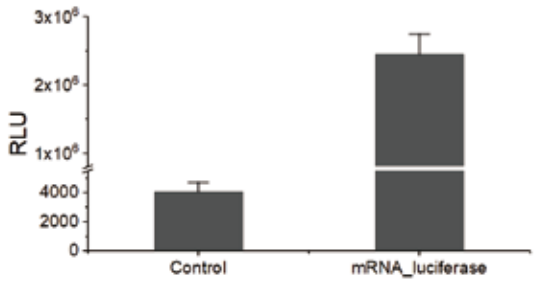
Samples	Test Items	Testing Methods	Delivery Period (Days)	Deliverables
mRNA expression validation	293T cell evaluation	Cell plating	4	Test report
		Transient transfection of cells		
		Fluorescence signal observation	1-3	
		Western blot (WB)		
		Enzyme-linked immunosorbent assay (ELISA)		

Case Study

Yaohai Bio-Pharma has built a mature platform for cell culture, cell transfection and protein specificity assays, which can verify the *in vitro* expression of target proteins based on fluorescence signal, Western Blot (WB) / enzyme-linked immunosorbent assay (ELISA) or substrate - enzyme reaction signal.



Bright Field | mRNA_eGFP | Bright Field | mRNA_mCherry



GAPDH

The full length of the novel coronavirus sprotein

mRNA_luciferase | mRNA_Spike protein(Newcrest)

mRNA Platform

Equipment

Bio-Rad Gel Imagers

Cytiva AKTA Purification System

Bio-Rad PCR Machine

Thermo qPCR Machine

SCIEX Capillary Electrophoresis Instrument

Waters HPLC

Thermo Full Wavelength Enzyme Labeler

Fluorescence Microscope

PNI Microfluidic Nanoparticle Preparation System

Yaohabio 耀海生物

CircRNA Innovative Therapeutic

CRO Services

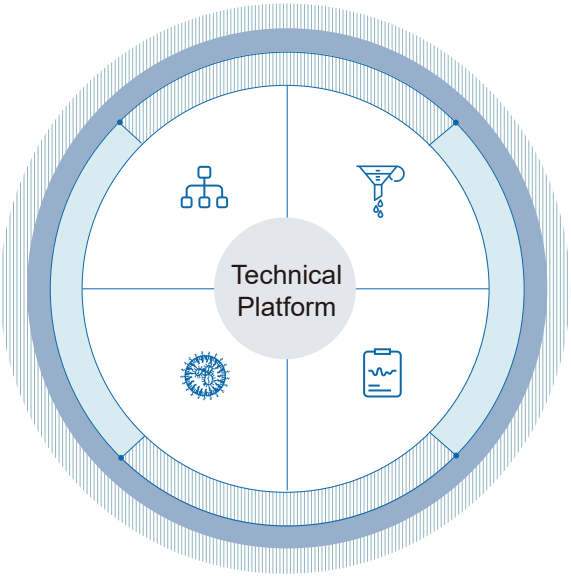
Technical Platform

RNADes

circRNA structure design and optimization

RNASyn

circRNA synthesis and modification



RNAPua

circRNA purification

RNAQua

circRNA quality analysis and control

Yaohai Bio-Pharma has built a mature CRO service platform for circRNA innovative therapeutics,which can realize efficient preparation and purification of circRNA. We also 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

Cutting-edge "PIE" loop-forming technology, efficient intron and exon combination

CDS, internal ribosome entry site (IRES) optimization design

CircRNA Synthesis and Modification

circRNA template plasmid design and construction

circRNA synthesis solution with a loop formation rate of >80%

CircRNA Purification

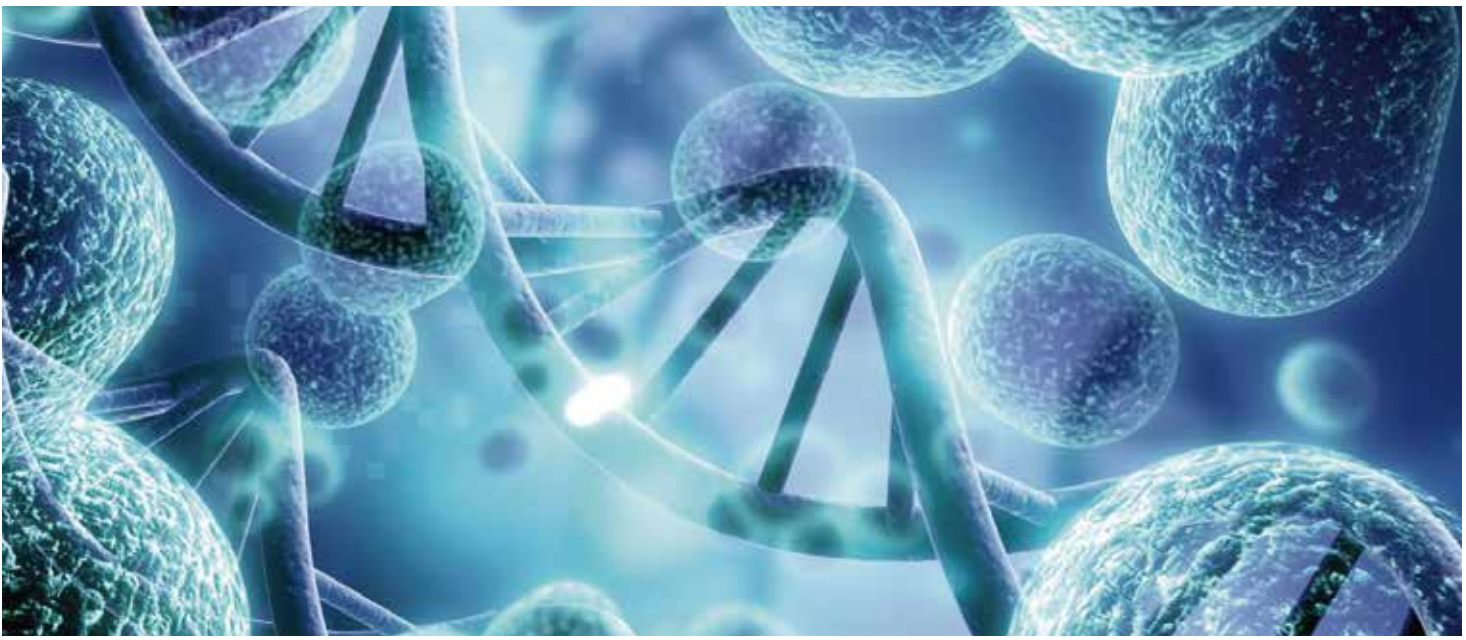
Conventional purification solution of trial grade

Self-developed chromatography column purification process

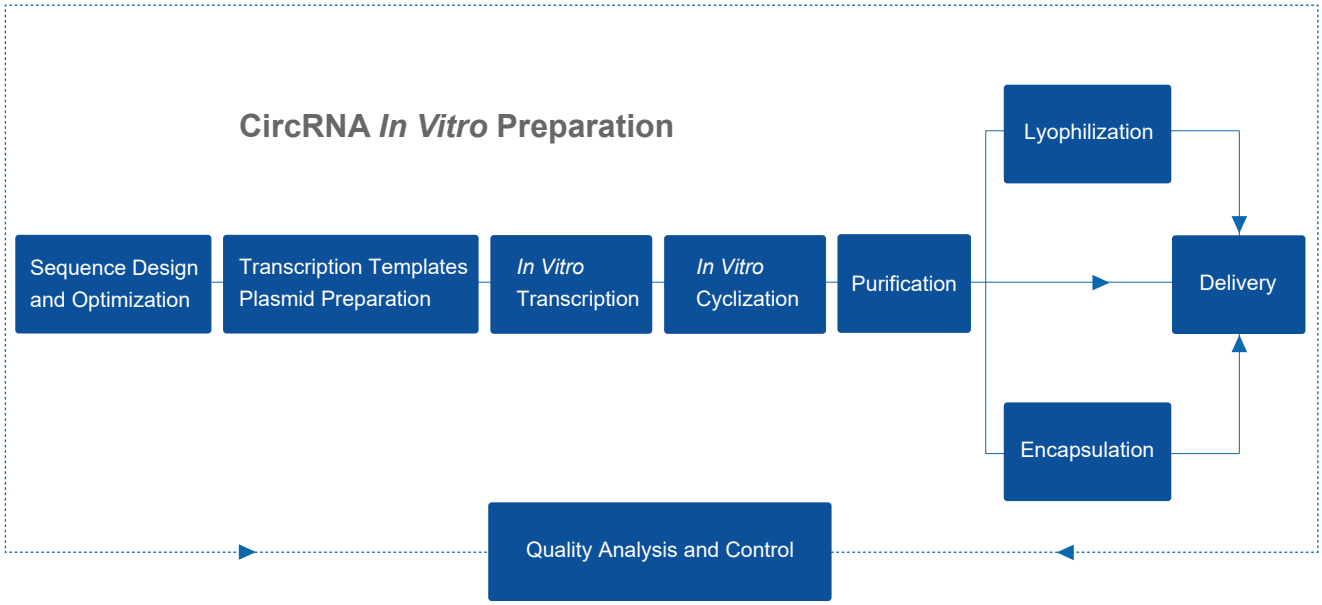
CircRNA Quality Analysis and Control

Multiple purity assays

High-performance loop formation rate assay



Process Development Flow



Service Details

Service Items	Optional Items	Service Details	Delivery Period (days)	Deliverables
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 Internal ribosome entry site (IRES), intron		
circRNA transcription template plasmid service	Plasmid DNA preparation	Gene synthesis	7-10	
		Plasmid amplification and extraction	4	
		Plasmid linearization and purification		
circRNA *in vitro* transcription and cyclization	*In vitro* transcription	*In vitro* transcription (IVT)	2-3	N/A
	In vitro cyclization	RNA cyclization based on PIE system		
		circRNA enrichment		
circRNA purification	Conventional purification solutions	Lithium chloride precipitation	1	circRNA drug substance (DS)
		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 drying (sublimation)		
		Secondary drying (desorption)		
circRNA encapsulation	Lipid nanoparticle (LNP) encapsulation	Lipid nanoparticle (LNP) encapsulation	2-3	circRNA-LNP drug product (DP)
		Concentration liquid exchange and filtration		
circRNA quality analysis	circRNA drug substance (DS)/lyophilized powder	Concentration, purity	1	Test report
		Cyclization rate	2-3	
	circRNA-LNP drug substance (DS)	Encapsulation rate	1	
		Particle size and distribution detection		
		Surface charge detection		
circRNA expression validation	293T cell evaluation	Cell plating	4	Test report
		Transient transfection of cells		
		Fluorescence signal observation	1-3	
		Western blot (WB) / Enzyme-linked immunosorbent assay (ELISA)		

CircRNA Products

Product Name	Test Uses	Delivery Form	Product Specification
circRNA_eGFP circRNA_mCherry circRNA_luciferase circRNA_OVA circRNA_IL-2 circRNA_Cas9	• Reference standard • *In vitro* or *in vivo* tests	• Lyophilized powder • Drug substance (DS) (500 ng/μL)	• 100 μg • 1 mg • 10 mg

Service Advantages

Process Robustness

Cyclization of RNAs from 50-4000 nt in length;

Stability

Rigorous quality control methods, with successful *in vitro* expression of products in cells;

All-round

From front-end sequence design to back-end circRNA cyclization, purification, quality control and expression validation;

High Efficiency

With a validated cyclization rate of NLT 80%;

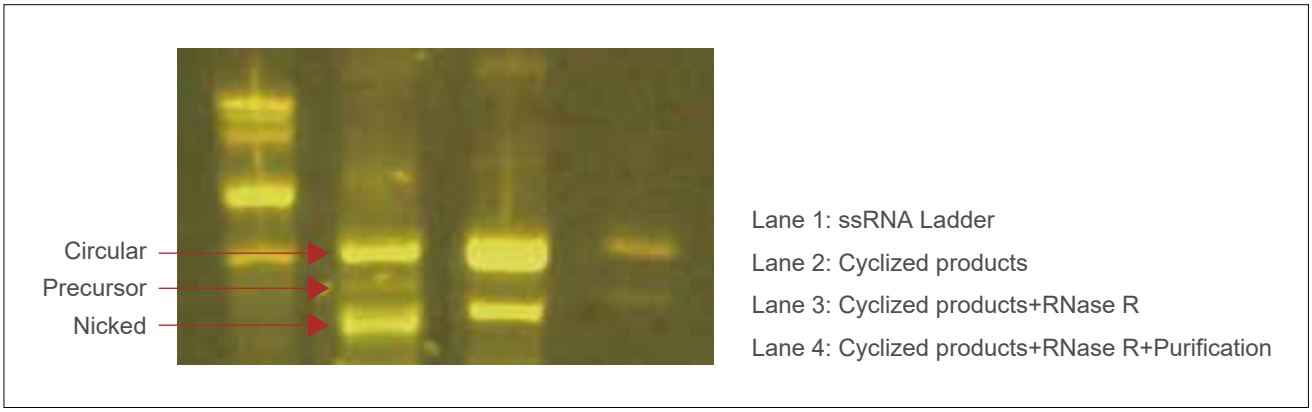
Flexibility

Diversified purification methods meet different downstream test needs;

Case Study

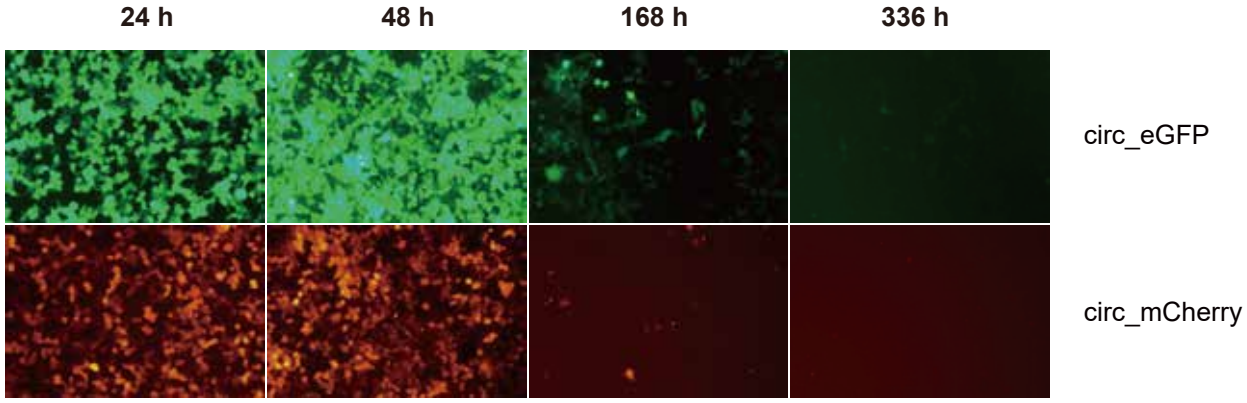
circRNA Enrichment and Purification

To enrich circRNA, PIE cyclized products are treated with RNase R. The electrophoresis results show that most of the nicked circRNA can be removed after linear RNA precursors are digested and further purified.* The purification solution is self-developed by Yaohai Bio-Pharma circRNA platform.



circRNA Expression Validation

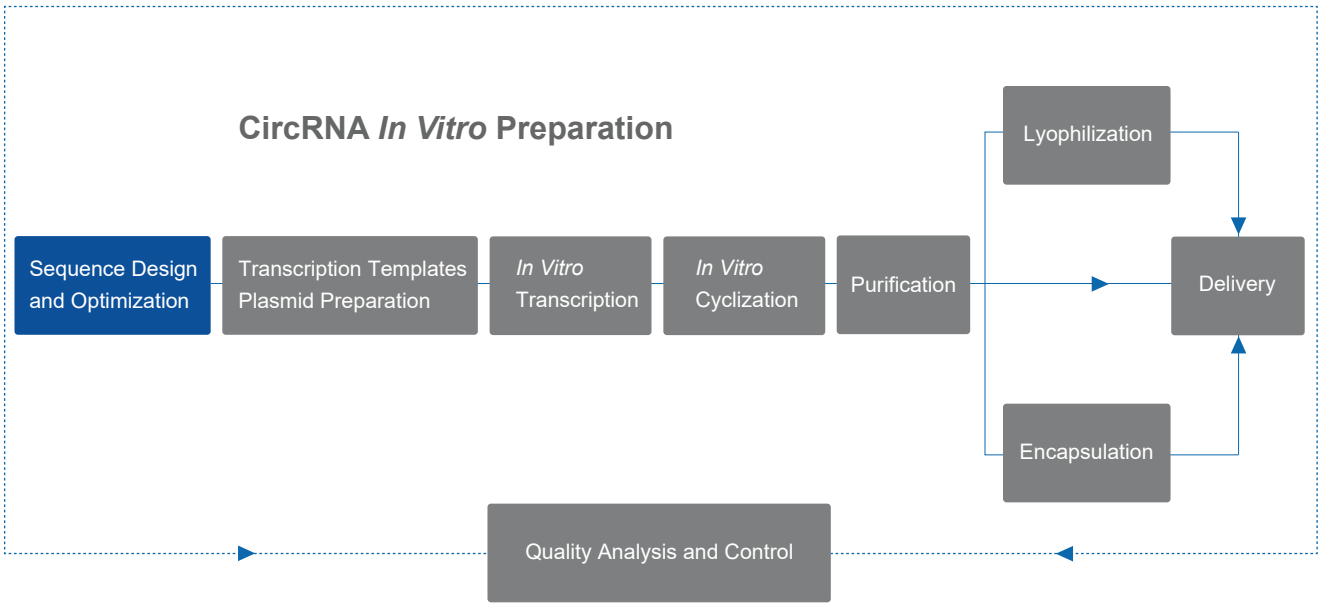
The purified circRNA eGFP are transfected with circRNA Cherry into 293T cells, and fluorescence signal can be observed after 24 h, which will continuously enhanced after 48 h. The fluorescence signal can still be observed after 7 days and 14 days of transfection.



CircRNA

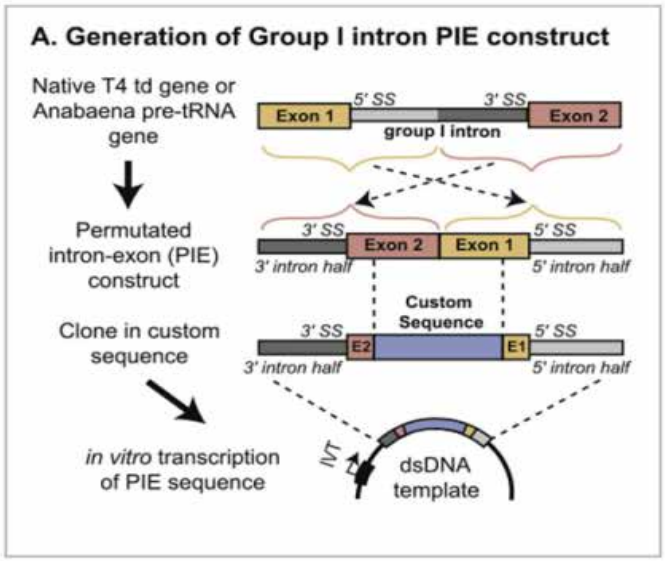
Sequence Design and Optimization Service

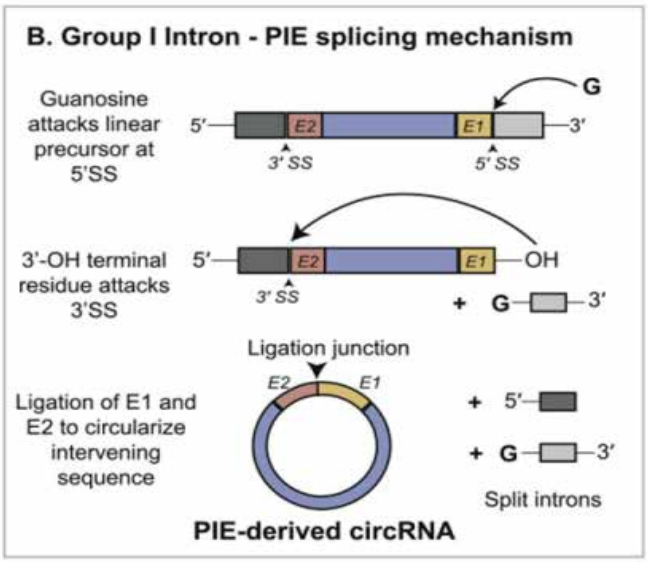
Process Development Flow



Yaohai Bio-Pharma 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 Bio-Pharma 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 fish oil tRNA precursor gene.
Coding	IRES	Internal ribosome recognition site that regulates the translation of circRNA.	Screening of internal ribosome entry site (IRES) sequences from different viral sources, e.g. EMCV, CVB3 sources.
	CDS	Protein-coding regions, sequences coding for antigens, single-domain antibodies (sdAbs) 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	• CDS sequence matching • CDS codon optimization	1
	Design and optimization of non-coding sequences	• 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 a professional AI algorithm team to complete the optimization of CDS region codons;

Mature Manufacturing Technology

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

Case Study

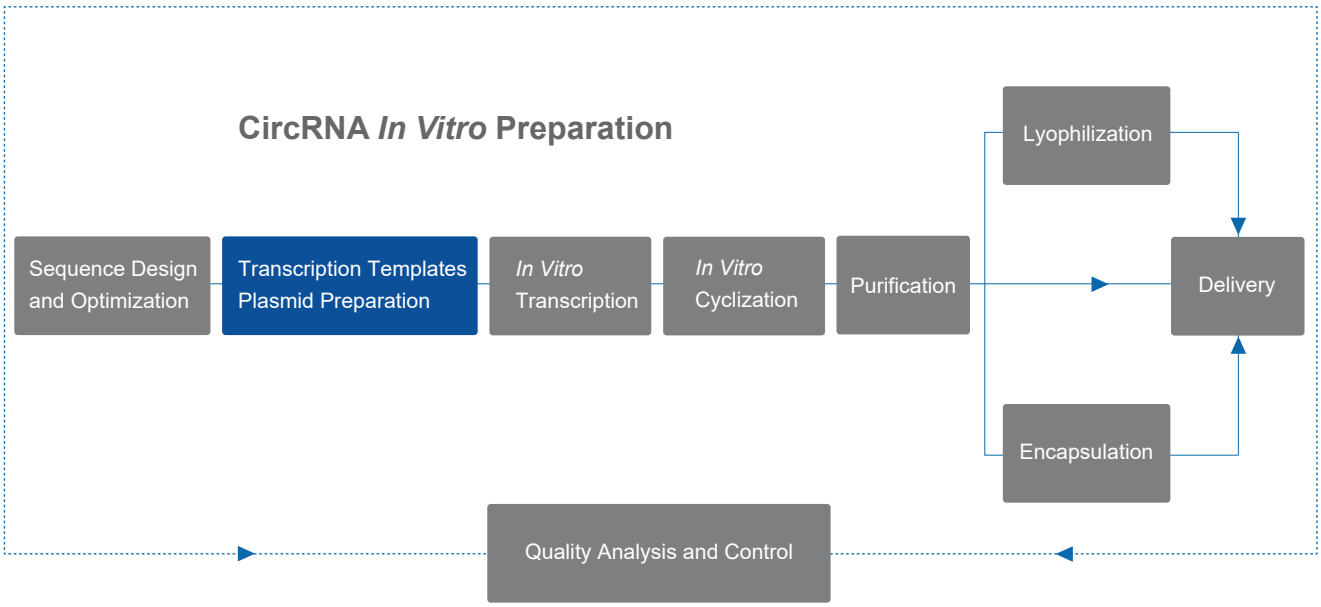
Yaohai Bio-Pharma launched the control product circRNA_eGFP, which is based on the PIE system to achieve the cyclization of RNA. Using a conventional transfection reagent, circRNA_eGFP is transfected with 293T cells, and eGFP (green) fluorescent signal can be detected after 24h, which will be enhanced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

Sequence design and *in vitro* expression validation of circRNA_eGFP

CircRNA

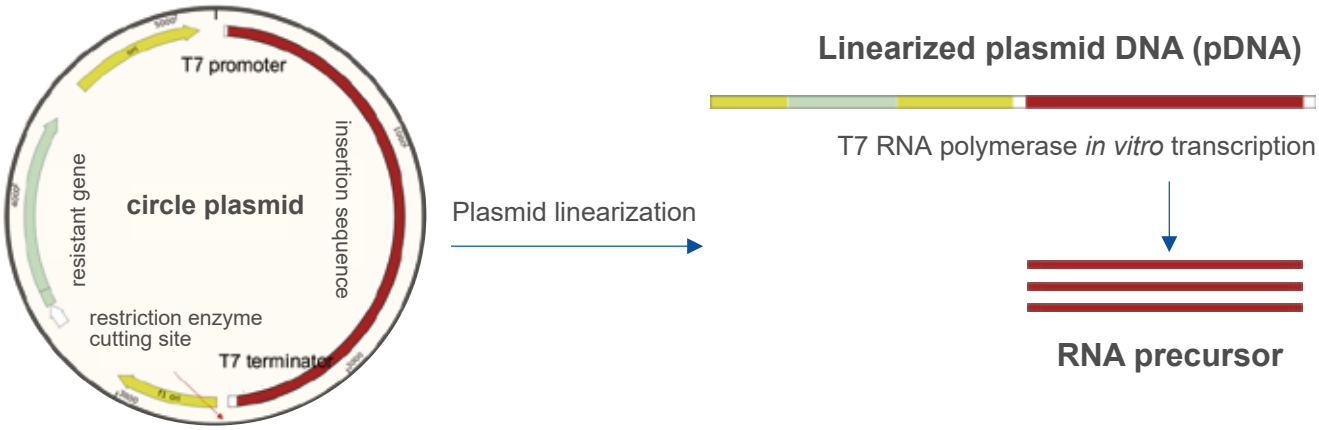
Transcription Template Plasmid Service

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 (pDNA) as a transcription template and transcribed in vitro with the help of T7 RNA polymerase.

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



Schematic diagram of *in vitro* transcription (IVT) using linearized plasmid DNA (pDNA) 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
		Purification of linearized product	
Plasmid DNA quality control	Concentration purity	Ultraviolet (UV) spectrophotometry	1-2
	Plasmid conformation	Agarose gel electrophoresis (AGE)	
		Capillary electrophoresis (CE)-optional	
	Plasmid integrity	Restriction enzyme identification/AGE	

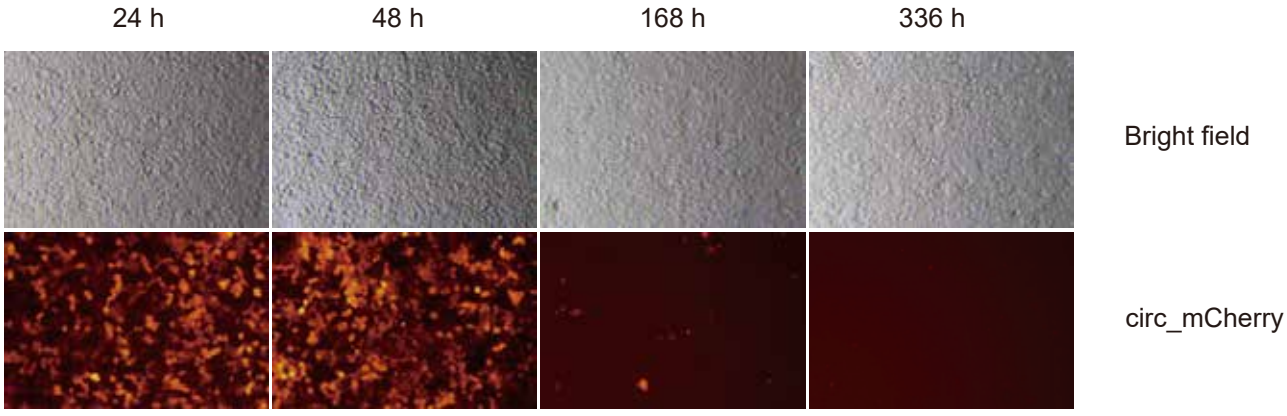
Service Advantages

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 Study

Take Yaohai Bio-Pharma's circRNA_mCherry product 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%.

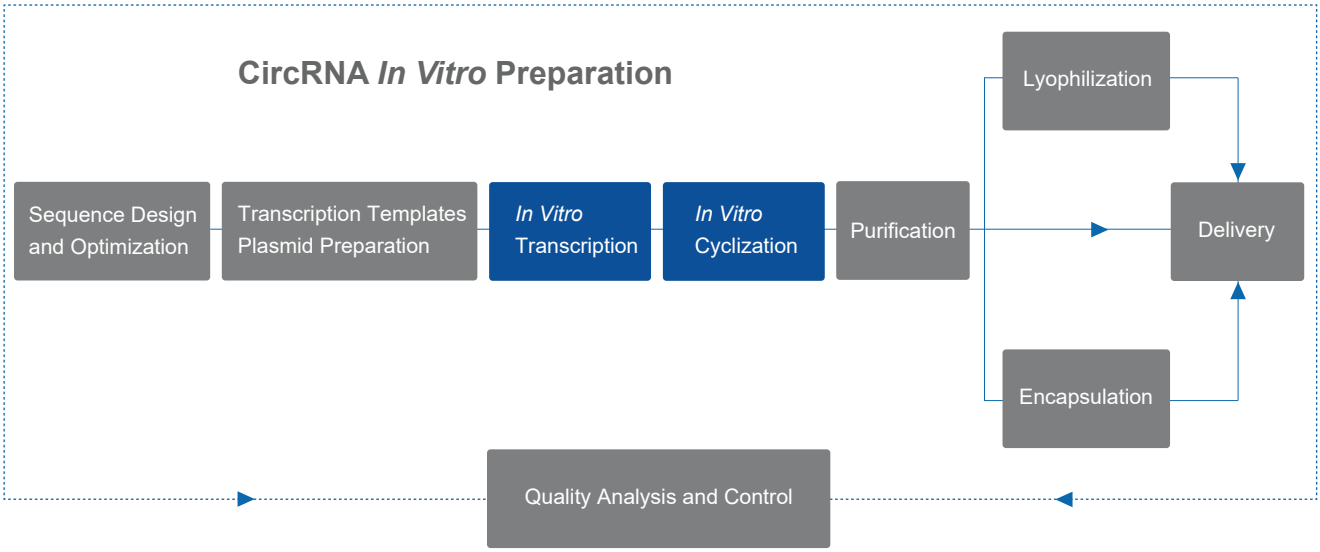
CircRNA_mCherry is prepared with linearized plasmid as template and transfected into 293T cells. A high level of fluorescence expression (red fluorescence) is detected after 24 hours of transfection, which will be continuously enhanced after 48 hours, and can still be detected after 7 and 14 days of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



***In vitro* expression validation of circRNA-mCherry**

CircRNA

In Vitro Transcription and *In Vitro* Cyclization Service



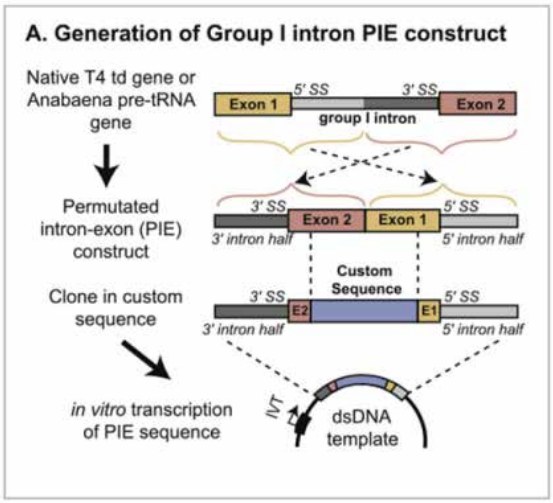


Figure: PIE sequence diagram

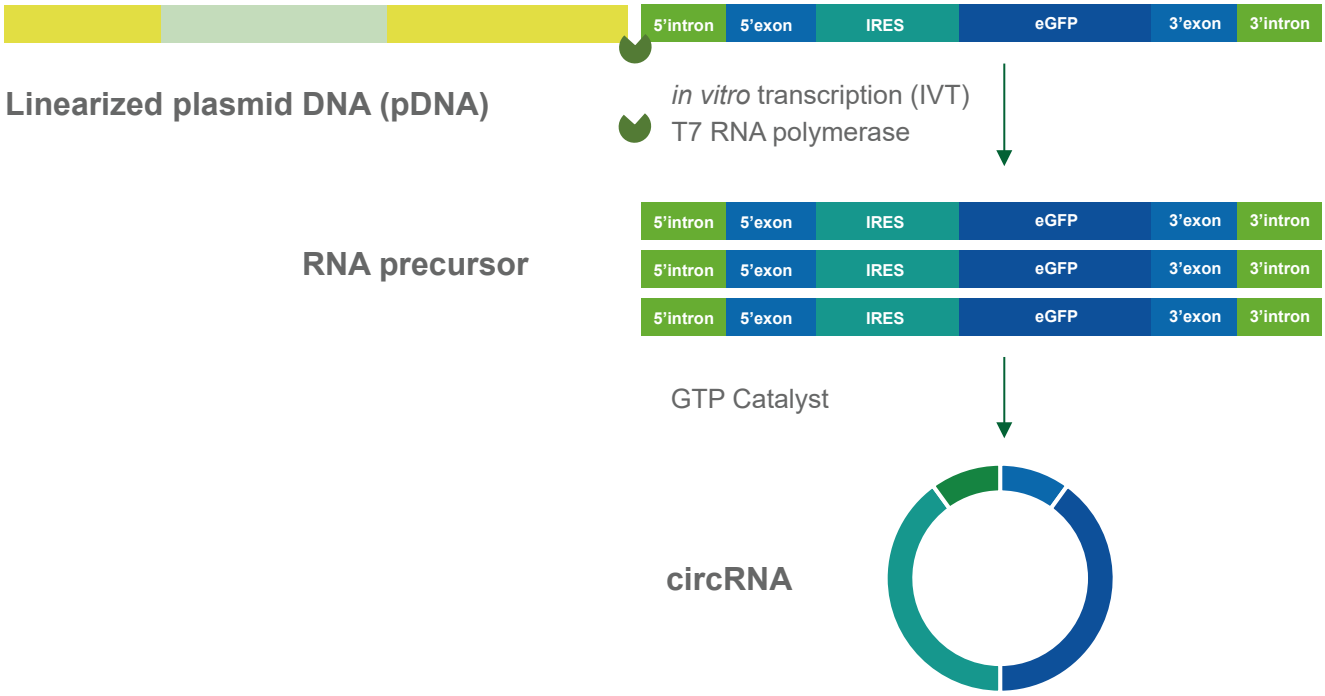
Regarding the batch preparation of RNA precursor, the commonly used method is *in vitro* transcription (IVT). Its reaction uses linearized plasmid DNA (pDNA) containing T7 promoter as a template and synthesizes RNA precursor with nucleoside triphosphates (NTPs) as a substrate in the presence of T7 RNA 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 Bio-Pharma 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:

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



Service Details

Service Items	Detail Steps	Delivery Period (Days)
circRNA *in vitro* transcription and *in vitro* cyclization	Reaction system confirmation	1-2
	In vitro transcription (IVT) 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.

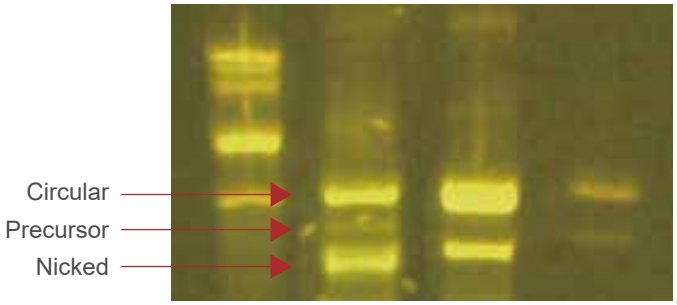
Stringent Enzyme Control Specification

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

Case Study

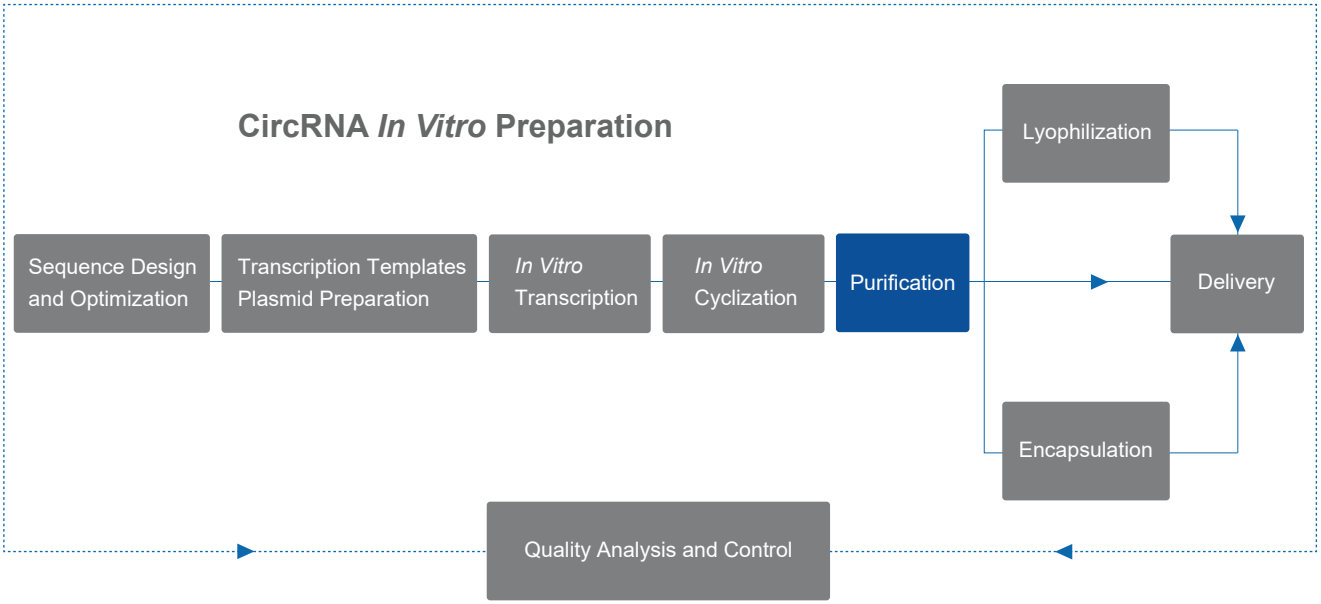
In Vitro Cyclization and Enrichment of circRNA

Based on the PIE system, Yaohai Bio-Pharma has optimized the sequence of circRNA, with a cyclization rate of more than 80% by agarose gel electrophoresis (AGE). By using RNase R to enrich circRNA, E-gel electrophoresis results show that most of the nicked circRNA can be removed after the linear RNA precursor is digested and further purified. The purification solution is self-developed by the circRNA platform of Yaohai Bio-Pharma.



Lane 1: ssRNA Ladder
Lane 2: Cyclized products
Lane 3: Cyclized products +RNase R
Lane 4: Cyclized products +RNase R+ Purification

CircRNA
Purification Service



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

Yaohai Bio-Pharma 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 used in 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 Bio-Pharma's 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)	Deliverables
circRNA purification	Conventional purification solution	Lithium chloride precipitation	1	circRNA drug substance (DS)
		Magnetic bead purification		
	Purification solution with high purity	Yaohai Bio-Pharma's purification solution	1-2	
	Solution substitution	Ultrafiltration liquid exchange	1	
circRNA quality control	Concentration determination	Ultraviolet (UV) spectrophotometry	0.5	Test report
	Purity testing	Agarose gel electrophoresis (AGE)/E-gel		
		HPLC-optional	1	

Service Advantages

A Variety of Optional Purification Choices

Meeting different downstream application scenarios;

High Purity

More than 90% purity of circRNA;

Stringent Enzyme Control Specification

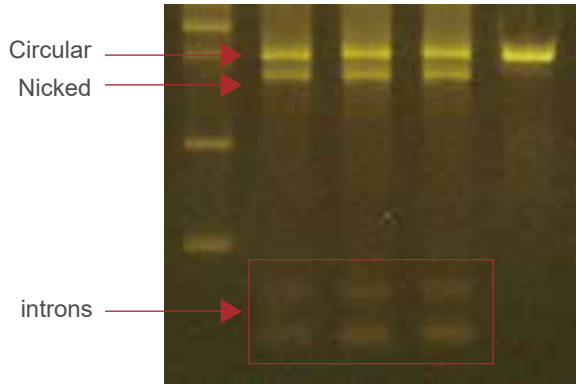
Stringent enzyme control on the test environment and consumables to effectively prevent the degradation of circRNA.

Case Study

Yaohai Bio-Pharma 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.

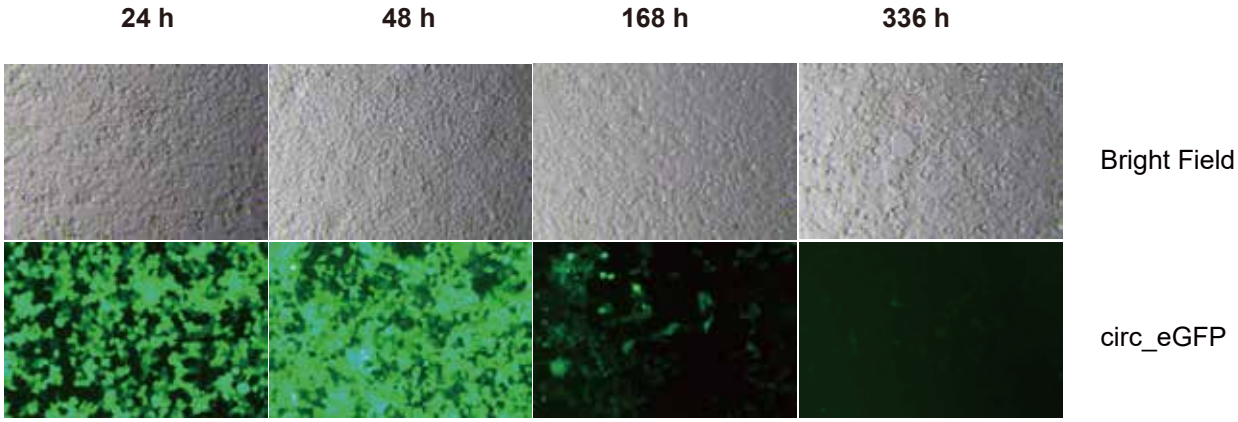
After the purification solution developed by Yaohai Bio-Pharma, various linear RNA impurities, such as nicked RNA and introns, can be successfully removed.



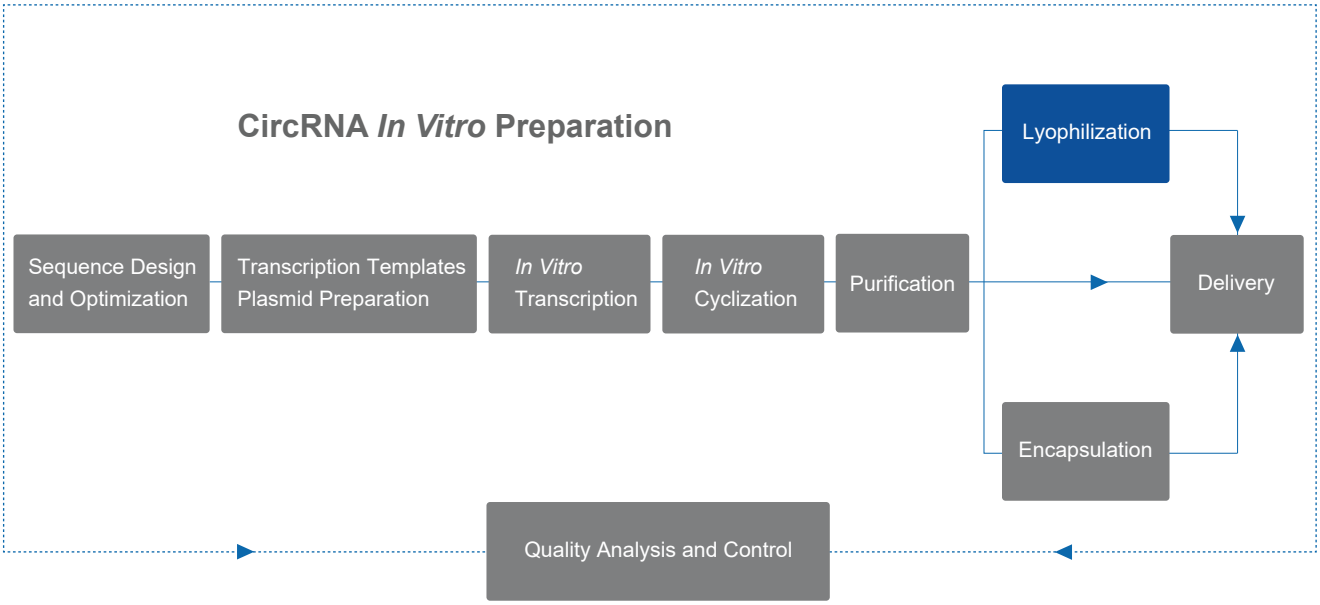
Lane 1: ssRNA Ladder
Lane 2: Cyclized products
Lane 3: Cyclized product + conventional purification protocol 1
Lane 4: Cyclized product + conventional purification protocol 2
Lane 5: Cyclized product + Yaohai Bio-Pharma's own purification solution

circRNA Expression Validation

By 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 loss in storage and transportation, Yaohai Bio-Pharma can provide circRNA lyophilization service to lyophilize the circRNA drug substance (DS) and store or transport it in the form of lyophilized powder, which significantly reduces the degradation and loss of circRNA during storage and transportation.

Sample Dispensing » **Pre-Freezing** » **Primary drying (sublimation)** » **Secondary drying (sublimation)**

Service Details

Service Items	Optional Items	Detailed Steps	Delivery Period (Days)	Deliverables
circRNA lyophilization	Sample dispensing	Dispensing	2-3	circRNA lyophilized powder
	Lyophilization	Pre-freezing		
		Primary drying (sublimation)		
		Secondary drying (desorption)		
circRNA quality control	Reconstitution of lyophilized powder	Reconstitution/resuspension	-	Test report
	Solubility of lyophilized powder	Appearance inspection	-	
	Concentration determination	Ultraviolet (UV) spectrophotometry	0.5	
	Integrity and purity testing	Agarose gel electrophoresis (AGE)/E-gel		
		HPLC-optional	1	

Service Advantages

Mature Lyophilization Process

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

Homogeneous Quality Properties

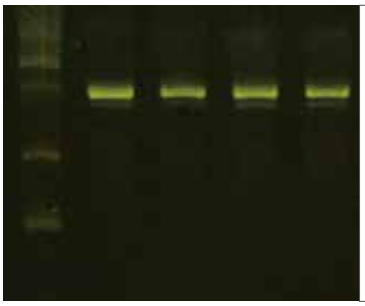
circRNA samples before and after lyophilization successfully express the target protein.

High Stability

circRNA lyophilized powder is easy to store and transport.

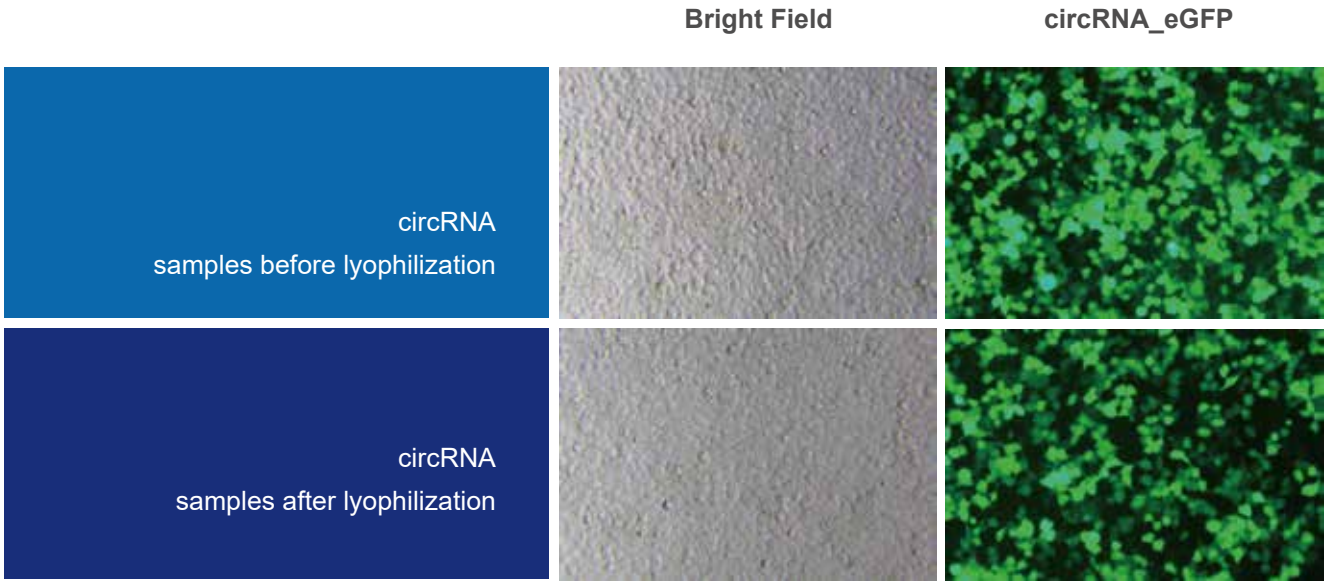
Case Study

E-gel electrophoresis assay is performed for circRNA before lyophilization and after reconstituted lyophilized powder to analyze its 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.

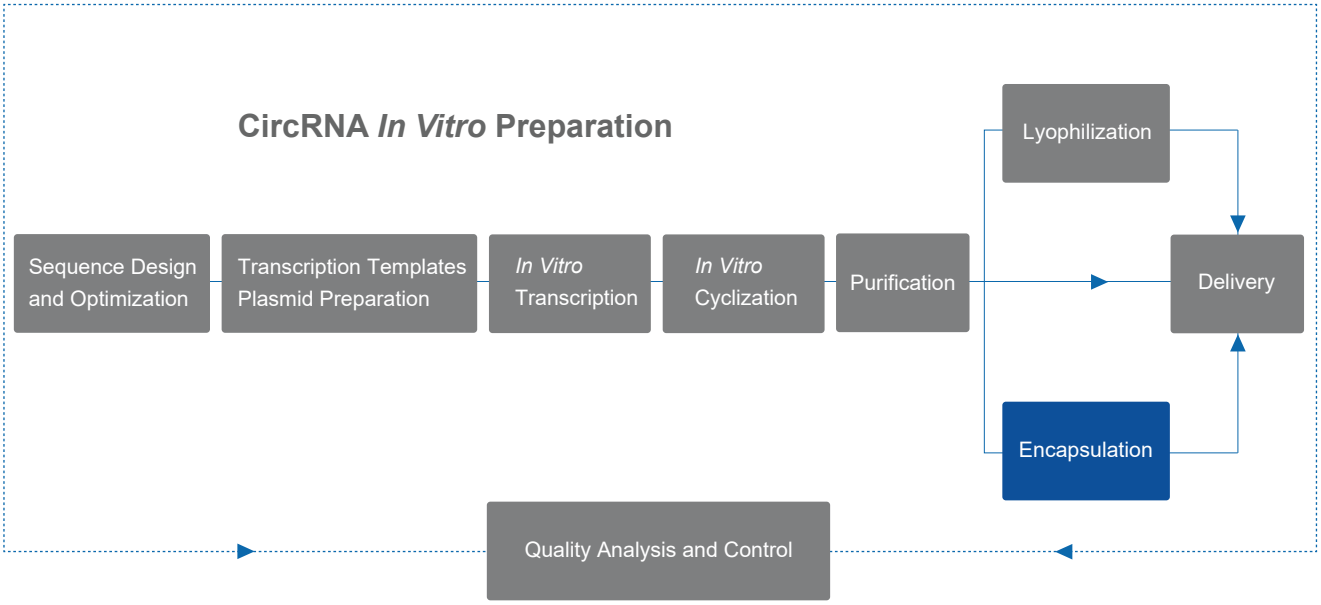


Lane 1: ssRNA Ladder
Lane 2: circRNA before lyophilization
Lane 3: circRNA after lyophilization
Lane 4: circRNA after lyophilization
Lane 5: circRNA after lyophilization

The 293T cell evaluation of circRNA samples before and after lyophilization show 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*.

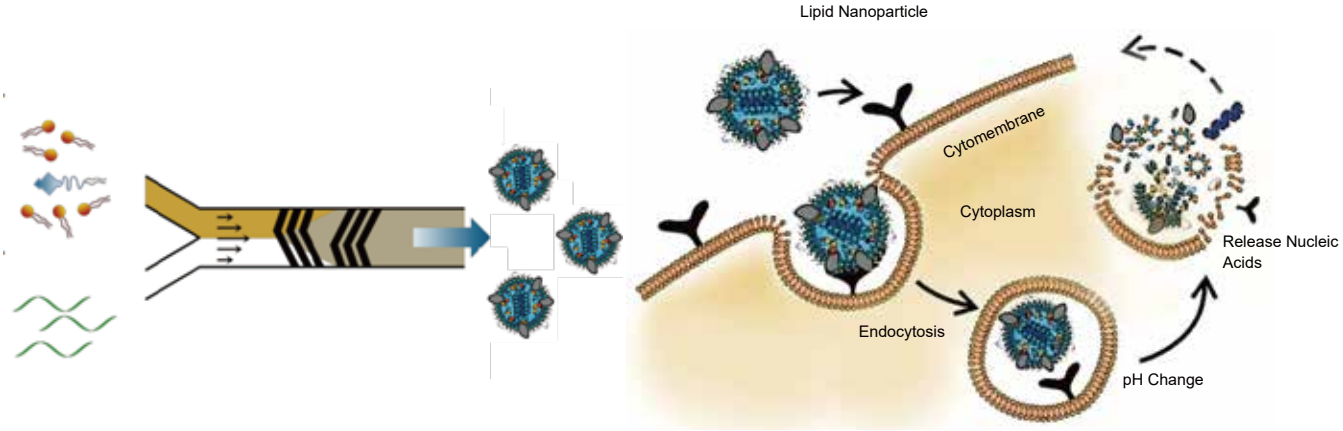


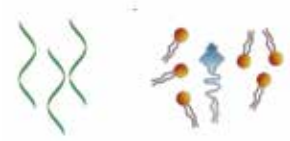
CircRNA-LNP
Encapsulation Service



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. LNPs 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 Bio-Pharma 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
			
Two drug substances are prepared: one for circRNA in aqueous buffer and one for lipids dissolved in ethanol.	Rapid mixing of lipid, circRNA two-phase solutions using microfluidic devices, resulting in uniform LNP and highly efficient encapsulation.	The drug substance (DS) is concentrated to the target concentration using a tangential flow technique. The buffer is replaced with a neutral storage solution to remove unencapsulated circRNA, excess lipids and acetic acid.	Comply with sterility regulatory requirements, select terminal sterilizing filtration system and pass the validation of bacterial challenge test.

Service Details

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

Service Advantages

Formulation Screening of Drug Product (DP)

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

High Encapsulation Rate

Encapsulation rate up to 90% or more;

Moderate Particle Size

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

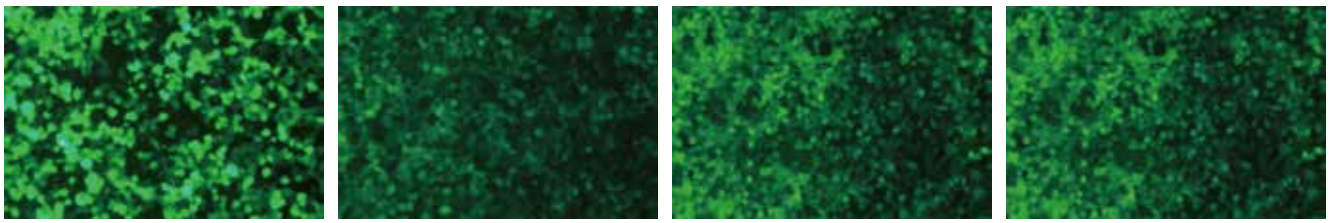
Efficient Expression

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

Case Study

LNP-circRNA_eGFP samples are prepared with different levels (1 ug, 2 ug, and 4 ug) and directly transfected 293T cells to verify whether they can express the target protein. After transfection for 48 hours, 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]



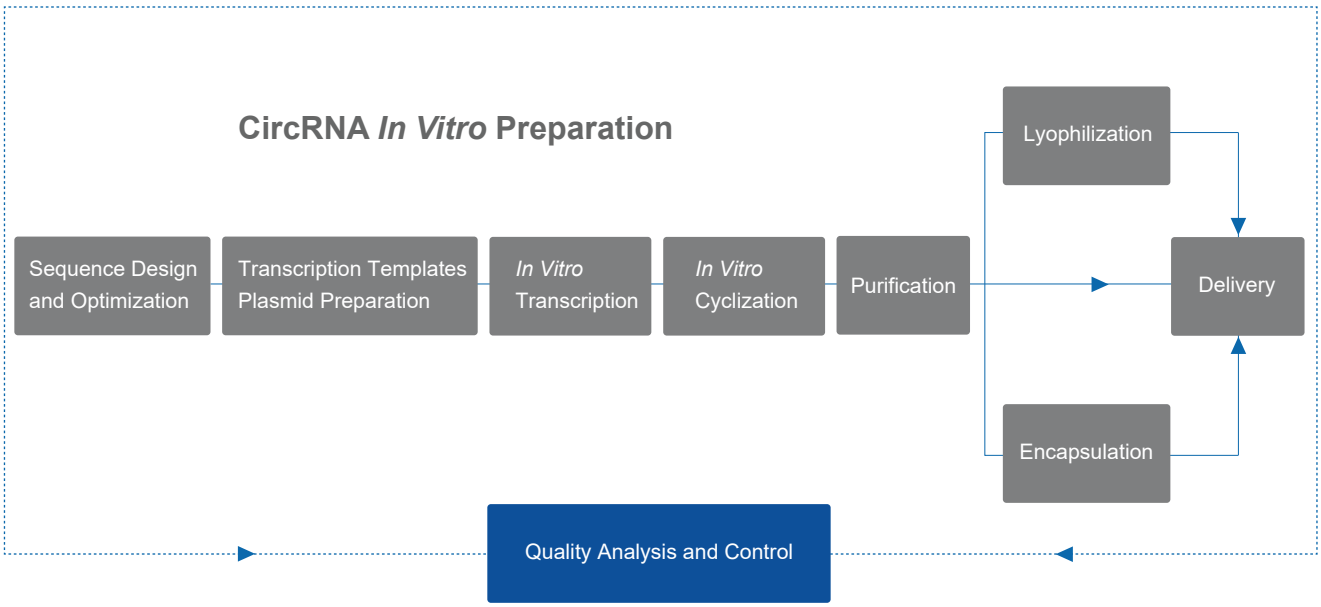
Lipo-circ_eGFP	LNP-circ_eGFP-1ug	LNP-circ_eGFP-2ug	LNP-circ_eGFP-4ug

Other Services

circRNA Sequence Design and Optimization	Transcription Template Plasmid Preparation	*In Vitro* Transcription of Linear RNA
circRNA *in vitro* cyclization	circRNA purification	circRNA-LNP encapsulation
circRNA lyophilization	cricRNA quality analysis and control	*In vitro* expression validation of circRNA

CircRNA
Quality Analysis and Control Service

Process Development Flow



In adherence to the stringent guidelines for mRNA quality analysis, Yaohai Bio-Pharma has formulated comprehensive specifications for circRNA drug products (DPs). We offer comprehensive quality analysis services encompassing both cyclic and linearized plasmid templates, circRNA drug substances (DSs), as well as the final product of circRNA-LNP. The detailed scope of these services is outlined below:

Samples	Test Items	Testing Methods	Delivery Period (Days)	Deliverables
Cyclic plasmid DNA	Concentration and purity	Ultraviolet (UV) spectrophotometry	N/A	Test Report
	Supercoiling ratio	Agarose gel electrophoresis (AGE)	0.5	
		Capillary electrophoresis (CE)	1	
Linearized plasmid DNA (pDNA)	Concentration and purity	Ultraviolet (UV) spectrophotometry	N/A	
	Linearization ratio	Agarose gel electrophoresis (AGE)	0.5	
		Capillary electrophoresis (CE)	1	
circRNA drug substance (DS)	Concentration	Ultraviolet (UV) spectrophotometry	N/A	
	Purity	Agarose gel electrophoresis (AGE)/E-gel	0.5	
		HPLC	1	
	Cyclization rate	HPLC/qPCR	1-2	
circRNA-LNP drug product (DP)	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 (WB)/Enzyme-linked immunosorbent assay (ELISA)		

CircRNA *In Vitro* Expression Validation Service

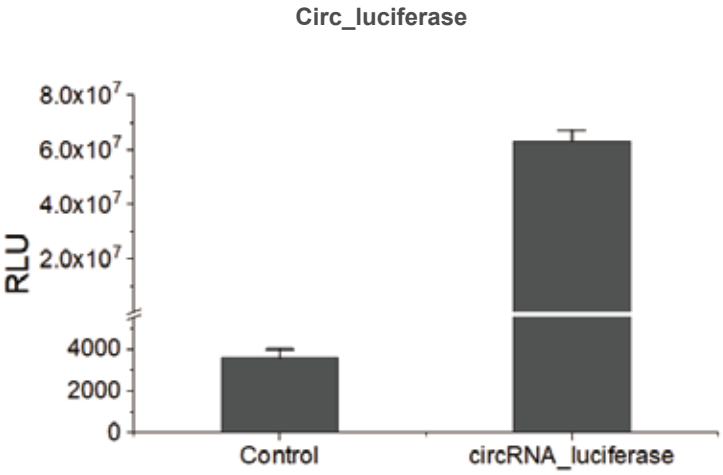
In addition to circRNA-related quality attributes, Yaohai Bio-Pharma can provide clients with circRNA cell transfection and target protein specificity assay services based on its well-established cell culture platform. By transiently transfecting 293T cells with circRNA, we can confirm whether circRNA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

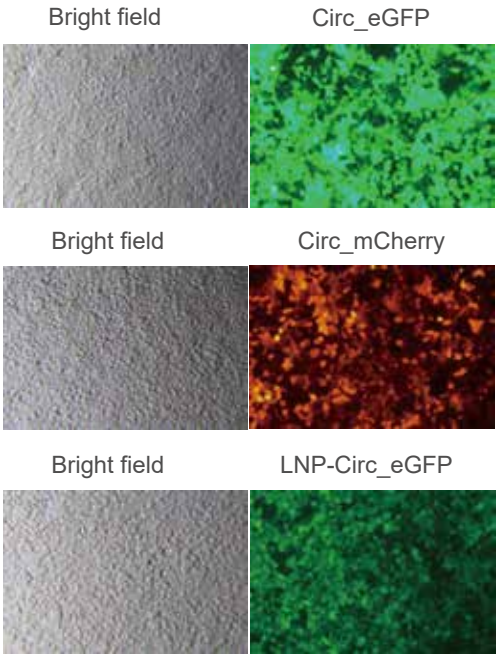
Cell Plate	Sample Preparation	Transfection	Target Protein Detection
			
Recording cell generations Observation of cell morphology Cell plate	circRNA＋Transfection reagents or circRNA-LNP	circRNA transfection to 293Tcells	Fluorescent photo shoot Western blot (WB)/Enzyme-linked immunosorbent assay (ELISA)

Samples	Test Items	Testing Methods	Delivery Period (Days)	Deliverables
circRNA expression validation	293T cell evaluation	Cell plate	4	Test report
		Transient transfection of cells		
		Fluorescence signal observation	1-3	
		Western blot (WB)		
		Enzyme-linked immunosorbent assay (ELISA)		

Case Study

Yaohai Bio-Pharma has built a mature cell transfection platform, and the transfection samples include circRNA drug substance (DS) and the finished product of circRNA-LNP. Based on the fluorescent signal and the enzyme-substrate reaction, a strong specific signal of the target protein can be detected.





CircRNA Platform

Equipment

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

Single-domain Antibodies (sdAbs) CRO Service Platform

Yaohai Bio-Pharma's single-domain antibodies (sdAbs) CRO service platform is dedicated to providing clients with one-stop sdAbs R&D and production services from strain construction, multifunctional sdAbs expression and purification to large-scale production, which are efficient and flexible to meet clients' different experimental or project needs.

Full ecological recombinant expression system

E.coli prokaryotic expression system
Yeast eukaryotic expression system
Mammalian cell expression system

Diversified single-domain antibodies (sdAbs) types

Monodomain single-domain antibodies (sdAbs)
Bivalent single-domain antibodies (sdAbs)
Multivalent single-domain antibodies (sdAbs)

Well-developed and perfect purification platform

Complete purification platforms
Combination purification methods
Highly efficient and flexible

From μg to kg

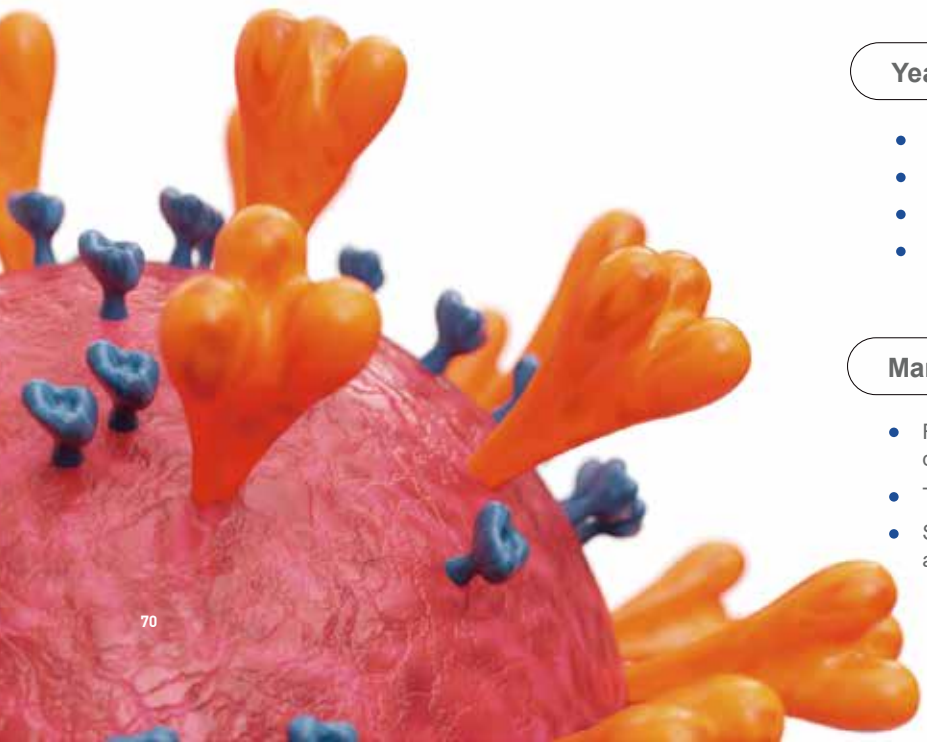
single-domain antibodies (sdAbs) with a high
expression of 10g/L
Production at a scale of 7-2000L

Full Ecological Recombinant Expression System

At present, Yaohai Bio-Pharma has established a full ecological recombinant expression system for single-domain antibodies (sdAbs). The existing expression systems include: *E.coli* prokaryotic expression system, yeast expression system (*pichia pastoris*), and mammalian cell expression system, and are skilled in using a variety of expression host strains to provide sdAbs with high quality according to the actual needs of clients.

E.coli prokaryotic expression system

- Development experiences of 20+ products
- Flexible selection of different *E.coli* hosts
- Efficient selection of different expression vectors



Yeast expression system *(pichia pastoris)*

- Development experiences of 20+ products
- Flexible selection of different *pichia pastoris* hosts
- PAOX1 methanol induced expression system
- PGAP constitutive expression system

Mammalian cell expression system

- Rich experience in single-domain antibodies (sdAbs) development of 5+ products
- Transient expression single-domain antibodies (sdAbs)
- Stable transformation strain expression single-domain antibodies (sdAbs)

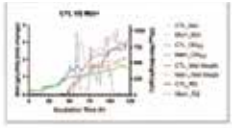
Single-domain Antibodies (sdAbs) Expression CRO Services

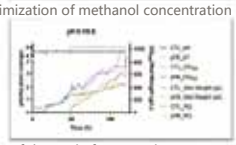
The client provides the gene sequence (or amino acid sequence) of the single-domain antibodies (sdAbs), selects the expression host cells. Yaohai Bio-Pharma provides one-stop gene synthesis to sdAbs expression, purification and production of a full range of customized sdAbs services.

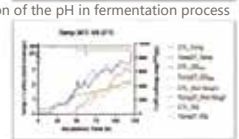
Steps	Service Content	Period	Deliverables
Construction of single-domain antibodies (sdAbs)-expressed engineering strains (selection from *B.aeruginosa, E.coli or* mammalian cells)	*E. coli* expression system (cytoplasmic or periplasmic space expression) *P. pastoris* expression systems (either methanol-induced expression system PAOX1 or constitutive expression system PGAP) Mammalian cell expression system (single-domain antibodies (sdAbs) expression by transient or stable transfection)	1 week - 2 weeks for *E.coli* (excluding gene synthesis period) 2 weeks-3 weeks for P. *pastoris* 1 week - 2 weeks for transient mammalian cells, and about 2 months for stable transformation strains	Purification of the obtained single-domain antibodies (sdAbs), purification antibody assay report SDS-PAGE, SEC-HPLC (optional), RP-HPLC (optional) and CE-SDS (optional) purity analysis methods
Lab scale expression purification (labeling)	For the single-domain antibodies (sdAbs) expression of the constructed engineering strains, purify the protein for an expression volume of 1 L (labeling is recommended).	2 weeks - 3 weeks	
Large-scale expression purification	Large-scale fermentation of single-domain antibodies (sdAbs)samples, along with the expression purification of the fermented nanobodies.	5 weeks - 8 weeks	

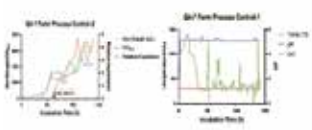
Production process development » **Large-scale production** » **Purification system** » **Quality analysis system**

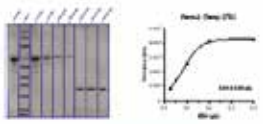
- To achieve optimal fermentation conditions through single-factor optimization of methanol concentration, fermentation temperature, and pH in the fermenter.
- To achieve higher yields of expressed single-domain antibodies (sdAbs) fermentation liquid by optimizing the combination of optimal conditions for nanobodies fermentation.
- Rich experience in purification of single-domain antibodies (sdAbs)
- Flexible combination of various purification methods, such as affinity chromatography, ion chromatography, hydrophobic chromatography, etc.
- Quality analysis system assurance
- Diversified quality analysis methods
- Rich experience and purity assurance

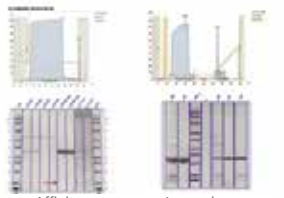

Optimization of methanol concentration


Optimization of the pH in fermentation process


Optimization of the temperature in fermentation process


Fermentation process optimization

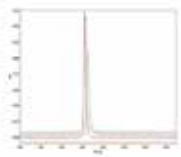

single-domain antibodies (sdAbs) fermentation yield

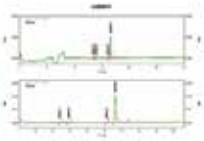

Affinity chromatography

Ion exchange chromatography


Hydrophobic chromatography


SDS-PAGE gel analysis


SEC-HPLC analysis


CE-SDS analysis

Yaohabio
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