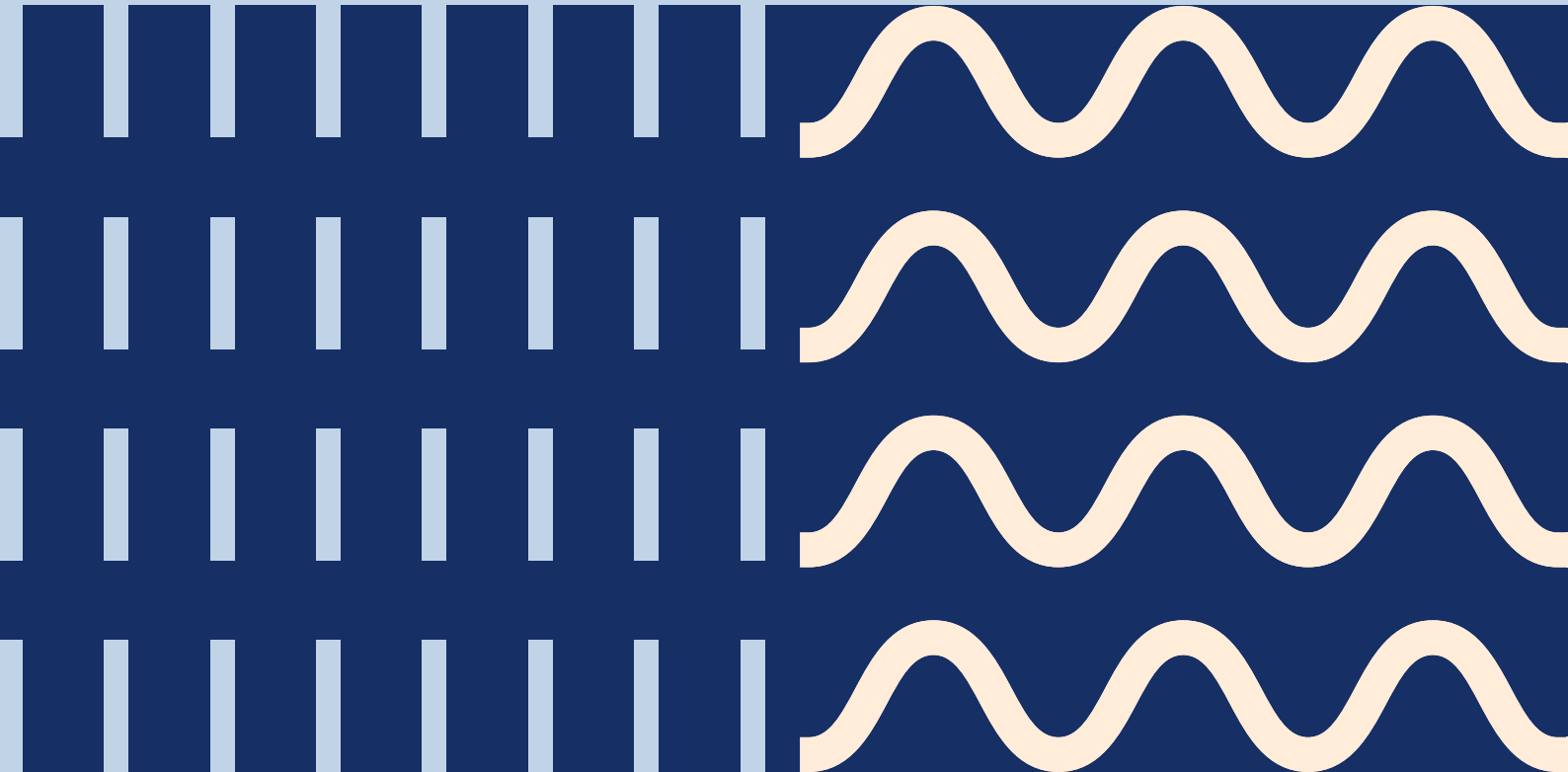
Hongene Biotech

# HiXCap™: Rational 5′ Cap Design Enabling Clinical Advancement of Next-Generation mRNA Therapeutics



# Executive Summary

HiXCap™ cap analogs represent a new generation of rationally designed 5′ cap structures that address a major limitation in the field of mRNA therapeutics: innate immune suppression via IFIT1-mediated translational inhibition. Through structure-guided engineering, the incorporation of sterically disruptive modifications - specifically 2′-*O*-ethyl at the N1 position - has enabled the development of HiXCap™ analogs with enhanced resistance to IFIT1 binding and improved translational performance.

Key findings from both in vitro (IFN α–stimulated JAWS II cells) and in vivo (LPS-induced inflammation in murine models) studies demonstrate that HiXCap™ analogs consistently outperform conventional GAG Cap1 and GAAG Cap2 structures. This is particularly evident under inflammatory conditions where IFIT1 expression is elevated. Notably:

### HiXCap™ E2

A trinucleotide cap with significantly enhanced protein expression under immune stress compared to conventional cap analogs. Recently advanced to clinical trials for cancer immunotherapy.

### HiXCap™ E3

A tetranucleotide cap which exhibits the strongest enhancement in translation efficiency and protein expression across mRNA chemistries (U and m1Ψ), supporting its broad applicability in therapeutic contexts.

Case studies in oncology and infectious disease vaccination models further validate the potential translational impact of HiXCap™ designs. In both tumor inhibition and immune priming models, HiXCap™ E2 capped mRNA vaccines produced stronger responses compared to GAG Cap1 analog, reflecting superior antigen expression and immune activation.

Taken together, these results highlight the utility of HiXCap™ analogs as advanced cap structures that overcome translational repression, support sustained protein production, and are compatible with conventional IVT and LNP workflows. Their implementation offers a strategic advantage for next-generation mRNA-based therapeutics, particularly those requiring performance in immunologically active settings.

HiXCap™ E2 capped mRNA cancer vaccines have recently advanced into clinical evaluation, with trials now underway targeting end-stage solid tumors, acute myeloid leukemia and post-surgical pancreatic cancer.

# Structures and Biological Properties of Naturally Occurring 5′ mRNA End Caps

In eukaryotic cells, the 5′ cap structure is an essential post-transcriptional modification of messenger RNA (mRNA) that has a profound influence on properties including transcript stability, translation efficiency, intracellular trafficking, and immune recognition. This modification is introduced co-transcriptionally in the nucleus, is universally conserved across eukaryotic species, and is found in many viral systems that rely on the host translation machinery.

## Structural Composition of the 5′ Cap

The canonical 5′ cap consists of a 7-methylguanosine (m7G) linked to the first transcribed nucleotide via an atypical 5′–5′ triphosphate bridge. This structure (Cap0, m7GpppN), may be methylated at the ribose 2′-*O* position of the first nucleotide (Cap1, m7GpppNm) and, in some organisms, also the second nucleotide (Cap2, m7GpppNmNm)[1]. The different methylation states of Cap0, Cap1, and Cap2 directly impact how the transcript is recognized by the innate immune system (Figure 1).

### Key Structural Elements:

#### m7G moiety:

Enhances binding affinity to the eukaryotic translation initiation factor eIF4E and protects against 5′→3′ exonuclease degradation.

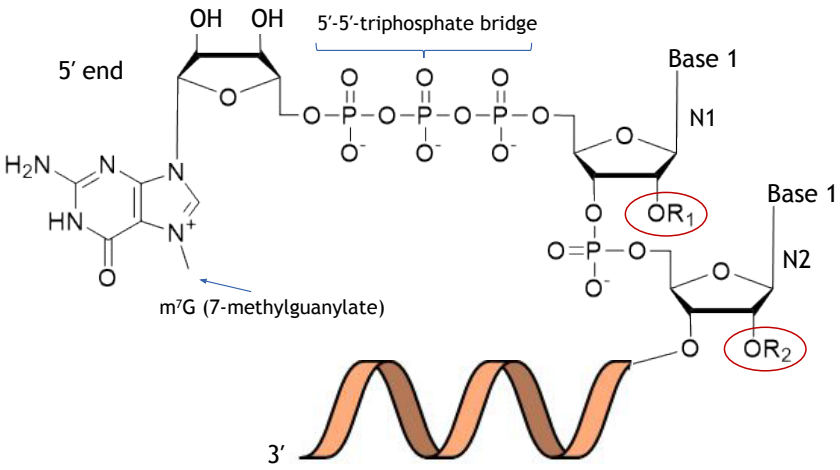
#### 5′–5′ triphosphate bridge:

Confers structural resistance to exonucleases and is critical for eIF4E recognition.

#### 2′-O-methylation:

Acts as a molecular signature of “self” RNA, reducing activation of cytosolic pattern recognition receptors (PRRs) such as RIG-I and MDA5, and modulating binding by interferon-induced proteins with tetratricopeptide repeats (IFITs) which competes with eIF4E[2].

## Figure 1. Chemical structures of naturally occurring 5′ end mRNA Caps




| 5′ cap type | Chemistry | |
|---|---|---|
| | $R_1$ | $R_2$ |
| Cap0 | H | H |
| Cap1 | Methyl | H |
| Cap2 | Methyl | Methyl |

The 5′ cap is an important regulator of self vs non-self immune system recognition

# Biological Functions of the 5′ Cap

**The 5′ cap plays multiple roles in mRNA biology[3] including:**

### 1. Promotion of Translation Initiation

The m7G cap recruits the eIF4F complex, facilitating 43S pre-initiation complex assembly and ribosomal scanning toward the AUG start codon.

### 2. Protection from Degradation

Capped mRNA is resistant to rapid decay by 5′ exonucleases. Decapping enzymes such as DCP2 initiate mRNA turnover, but the cap structure significantly delays this process.

### 3. Immune Discrimination: Self vs. Non-self

The innate immune system uses cap structure as a discriminatory marker. Cap0 RNA, characteristic of many viral genomes, binds RIG-I and activates interferon (IFN) signaling. Cap1 and Cap2 RNA structures confer reduced immune activation and IFIT binding.

### 4. Nuclear Export and Processing

The cap-binding complex (CBC) engages capped transcripts in the nucleus, promoting splicing, polyadenylation, and nuclear export.

# Rational Structure Based Design of Novel Cap Analogs

In the context of exogenously delivered in vitro–transcribed (IVT) mRNA therapeutics, innate immune recognition pathways, including as mediated by IFIT1, can severely attenuate protein expression leading to mRNA decay under certain conditions. This presents an opportunity for rational chemical design of cap analogs that retain all beneficial interactions with the host translation machinery while minimizing immune inhibitory effects.

## IFIT1 Structure and Relevance for Cap Analog Design

IFIT1 is a critical effector in the antiviral response. Upon type I interferon signaling, IFIT1 is rapidly upregulated and binds the 5′ termini of mRNAs lacking proper 2′-*O* methylation at the first and second transcribed nucleotides. This interaction, which is typical in many viral mRNA transcripts, sterically occludes the recruitment of translation initiation factors, effectively silencing the transcript.

High-resolution structural studies[4] reveal that human IFIT1 has an adaptable RNA-binding tunnel that accommodates m7GpppN structures and engages strongly with Cap0 and, to a lesser degree, Cap1 RNAs, an observation subsequently confirmed by biochemical studies[5]. Single (N1) and to an even greater extent double (N1 and N2) 2′-*O*-ribose methylations, can induce clashes with key IFIT1 residues (e.g., Y157, R187), inhibiting binding and thereby enhancing translation under immune-stressed conditions (Figure 2).

### Figure 2. Crystal structure of human IFIT1 bound to 5′-capped RNA

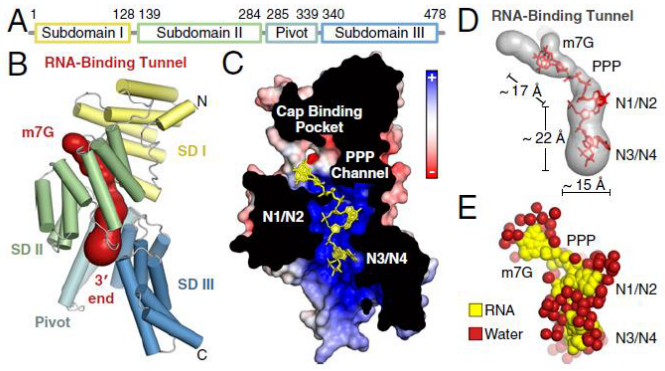



Fig. 2. Overall structure of monomeric, RNA-bound human IFIT1 (A) Schematic of IFIT1 subdomains. (B) Cartoon representation of human IFIT1 colored by subdomain (SD) and surface representation of the tunnel (dark red) determined by CAVER (50). (C) Cross-section of IFIT1 colored by surface electrostatic potential from negative (-10 kTe-′; red) to positive (+10 kTe; blue) with capped-RNA (yellow sticks). (D) Dimensions of the IFIT1 tunnel (gray surface) and capped RNA (red sticks). (E) Waters surrounding the RNA inside the tunnel.

- N1 and N2 methylations predicted to clash with protein (right)
- N1 methylation alone only partially reduces sensitivity to IFIT1

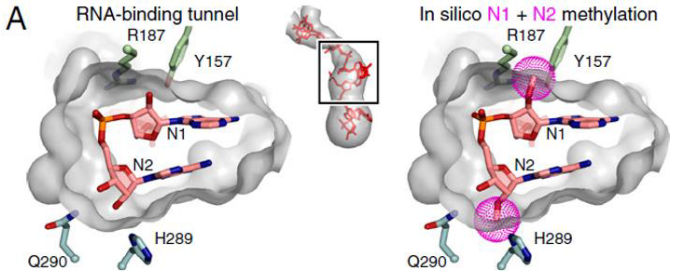



Fig. 8A. Sensing of N1 and N2 ribose 2′-O methylation by IFIT1.

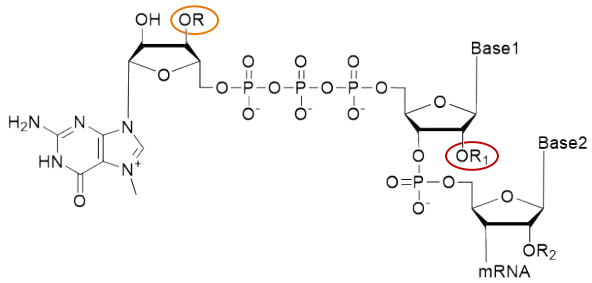
Adapted from Abbas et al., “Structure of Human IFIT1 with Capped RNA Reveals Adaptable mRNA Binding and Mechanisms for Sensing N1 and N2 Ribose 2′-O Methylations.” PNAS, 2017, E2106.

# In Silico Docking and Steric Clash Hypothesis

Leveraging the crystallographic data of human IFIT1 bound to capped RNA, we conducted in silico docking analyses to simulate the binding of cap analog variants. We used this model to predict that substitution of the 2′-O-methyl at the N1 ribose with larger functional groups, such as a 2′-O-ethyl moiety, would augment steric clash with Y157 and R187 (Figure 3). Such steric interference is hypothesized to further destabilize IFIT1–RNA complex formation, thereby preserving mRNA translation even in the presence of elevated levels of IFIT1.

**Figure 3. HiXCap™ - Rational 5′ cap design to evade IFIT1 binding**

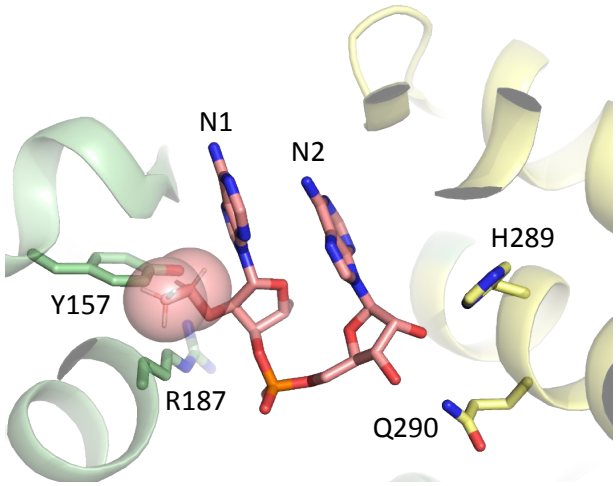
**HiXCap™**




R = H or Me

R1 = Bulky group such as Et

R2 = H (Cap1) or Me (Cap2)




Our analysis predicts a severe steric clash between N1 2′-OEthyl and Y157 and R187 of IFIT1

Hypothesis: Bulkier group at 2′ position of N1 better evades IFIT1 binding, leading to increased translation under immune stress

# Introducing HiXCap™ Designs

HiXCap™ analogs are the result of this structure-guided approach. They are chemically modified tri and tetranucleotide cap structures incorporating strategic substitutions at the N1 position to maximize IFIT1 evasion while maintaining optimal binding to eIF4E and compatibility with wild type T7 RNA polymerase–driven IVT protocols.

**Three principal HiXCap™ designs have been developed to date (Figure 4):**

### HiXCap™ E1

N1 2′-O-ethyl modification on a GAG Cap1 scaffold.

### HiXCap™ E2

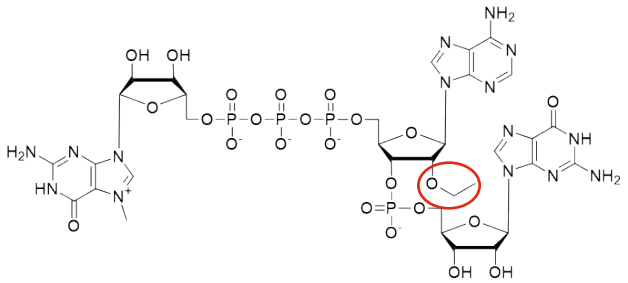
N0 3′-*O*-methyl and N1 2′-O-ethyl modification on a GAG Cap1 scaffold.

### HiXCap™ E3

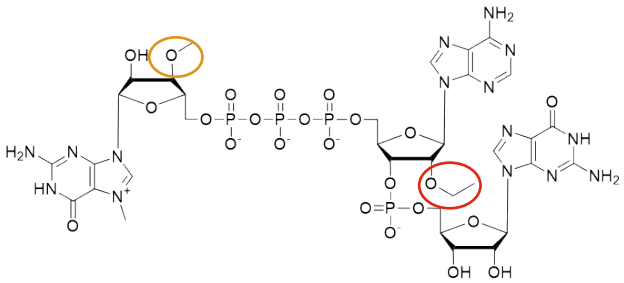
N0 3′-*O*-methyl and N1 2′-O-ethyl modification on a GAAG Cap1 scaffold.

## Figure 4. Chemical structures of HiXCap™ designs and comparators

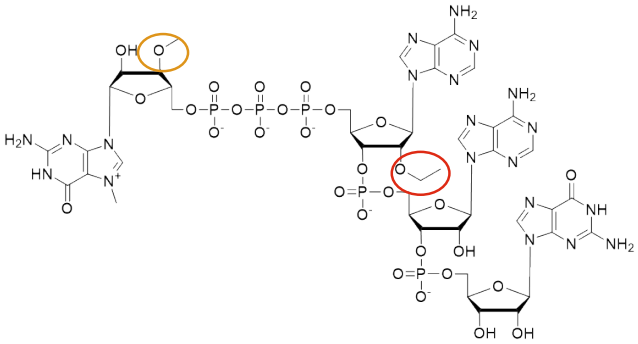
### HiXCap™ E1



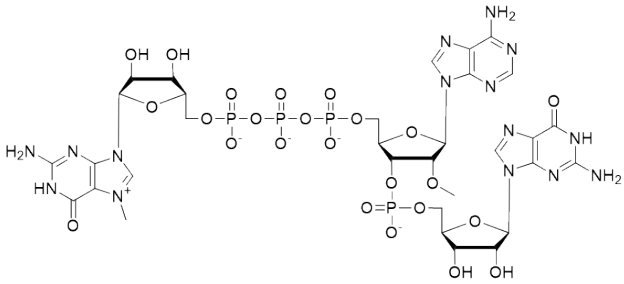
### HiXCap™ E2



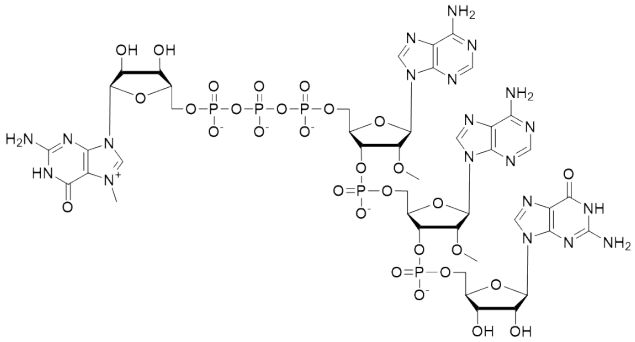
### HiXCap™ E3



### GAG Cap1



### GAAG Cap2



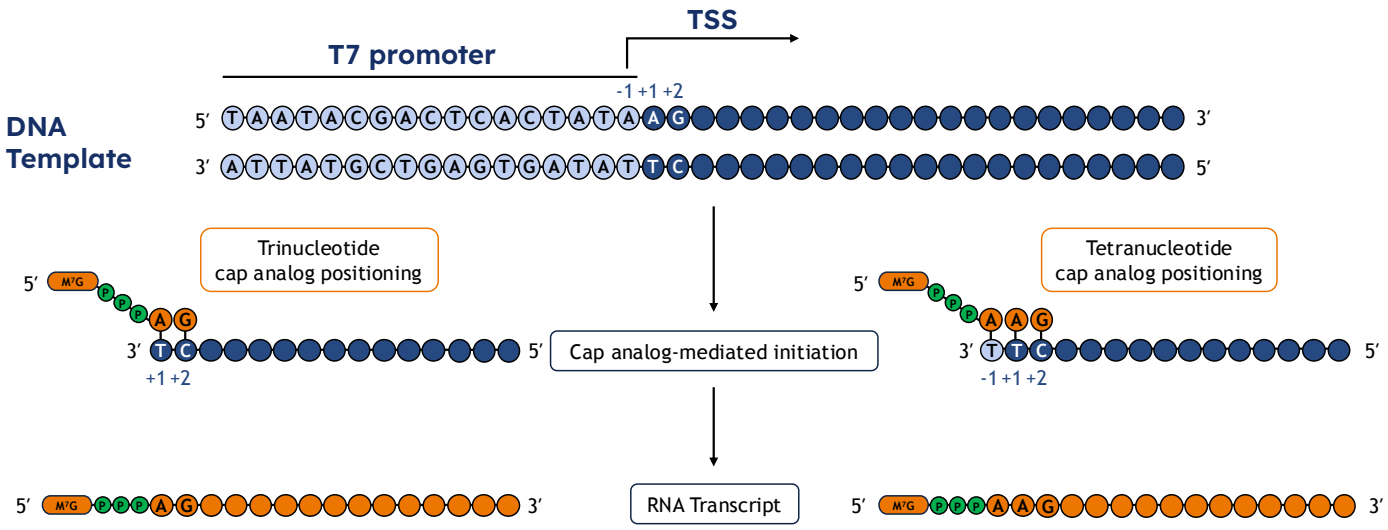
# Rationale for Inclusion of Tetranucleotide Structures

Tetranucleotide cap analogs enable chemical modifications at the N2 position, offering structural flexibility beyond that of conventional trinucleotide Cap1 analogs which can yield performance advantages. Indeed, prior studies have shown that Cap2 structures outperform Cap1 in translational activity in both cellular and in vivo contexts[6].

For certain Cap1 designs such as HiXCap™ E2, capping efficiency may be reduced due to steric hindrance during initiation. The tetranucleotide HiXCap™ E3 analog extends the structure by one nucleotide to mitigate this steric constraint. This extension improves both capping efficiency and translational output.

Importantly, tetranucleotide analogs should be precisely aligned with the transcription start site (TSS) to ensure efficient complementary base-pairing and incorporation during IVT. To facilitate this, developers can design IVT templates to initiate transcription at TSS positions –1, +1, +2, instead of the conventional +1, +2, +3[7,8] (Figure 5).

## Figure 5. TSS can overlap with T7 promoter for tetranucleotide capping




# Quality Assessment of HiXCap™ Analogs

## Synthesis and Material Attributes of Cap Analogs

All new HiXCap™ designs were synthesized at Hongene using processes that had been optimized for high chemical purity, and consistent lot-to-lot reproducibility. Analytical testing of material attributes conformed to the compositions as summarized in figure 6.

**Figure 6. Quality data for the cap analogs used in this study**

| Short name | GAG Cap1 | GAAG Cap2 | HiXCap™ E2 | HiXCap™ E3 |
|---|---|---|---|---|
| Product Name | m7G(5′)pppA(2′OMe)pG | m7G(5′)pppA(2′OMe)pA(2′OMe)pG | (3′OMe)m7G(5′)pppA(2′OEt)pG | (3′OMe)m7G(5′)pppA(2′OEt)pApG |
| Cap Structure | Cap1 | Cap2 | Cap1 | Cap1 |
| Appearance | Clear to slightly yellow solution | Clear to slightly yellow solution | Clear to slightly yellow solution | Clear to slightly yellow solution |
| ID (Mass spec) | 1145.7 (free acid) | 1488.9 (free acid) | 1173.7 (free acid) | 1502.1 (free acid) |
| ID ($^{1}$H NMR) | Conforms to structure | Conforms to structure | Conforms to structure | Conforms to structure |
| ID ($^{31}$P NMR) | Conforms to structure | Conforms to structure | Conforms to structure | Conforms to structure |
| Concentration | 98 mM | 101 mM | 103 mM | 100 mM |
| Purity (HPLC) | 99.50% | 98.4% | 99.70% | 99.5% |
| Unspecified Impurity | <1.0% | <1.0% | <1.0% | <1.0% |
| pH (22-25 ) | 6.3 | 6.3 | 6.2 | 6.2 |
| Bacterial Endotoxin | <1 EU/mL | <1 EU/mL | <1 EU/mL | <1 EU/mL |
| Salt | Ammonium, 0.33% | Ammonium, 0.69% | Ammonium, 0.48% | Sodium, 0.98% |
| Residual Solvent | MeOH:<100ppm; ACN:<100ppm | MeOH:<1000ppm; ACN<1000ppm | MeOH:<100ppm; ACN:<100ppm | MeOH:<100ppm; ACN:<100ppm |

## In Vitro Transcription (IVT) Performance

All cap analogs were tested in wild type T7 RNA polymerase–driven IVT reactions to synthesize eGFP-mRNA and Fluc-mRNA using optimized buffer conditions. The results are summarized in figure 7 and the combined data confirm that HiXCap™ analogs are generally similar in performance compared to GAG and GAAG cap analogs. HiXCap™ analogs were compatible with both uridine-containing and N1-methylpseudouridine (m1Ψ)–containing mRNA sequences, a key requirement for diverse therapeutic modalities.

### Figure 7. Performance of the cap structure in IVT and impact on mRNA quality

| mRNA seq | nt | U | Cap | Yield (mg/mL) | Integrity | Capping efficiency | Rel dsRNA | Cap:GTP/ATP |
|---|---|---|---|---|---|---|---|---|
| eGFP | 1,003 | Uridine | GAG Cap1 | 5.3 | 96.4% | 97% | <0.01% | 1:1 |
| | | | GAAG Cap2 | 5.2 | 95.7% | 99% | <0.01% | 1:1 |
| | | | HiXCap™ E2 | 5.2 | 96.0% | 94% | <0.01% | 2:1 |
| | | | HiXCap™ E3 | 5.3 | 96.2% | 97% | <0.01% | 1:1 |
| | | m1Ψ | GAG Cap1 | 5.1 | 96.6% | 98% | <0.01% | 1:1 |
| | | | GAAG Cap2 | 5.0 | 94.9% | 99% | <0.01% | 1:1 |
| | | | HiXCap™ E2 | 5.1 | 97.3% | 94% | <0.01% | 2:1 |
| | | | HiXCap™ E3 | 5.1 | 96.5% | 97% | <0.01% | 1:1 |
| Fluc | 1,939 | Uridine | GAG Cap1 | 5.6 | 91.4% | 99% | <0.01% | 1:1 |
| | | | GAAG Cap2 | 5.5 | 92.5% | 99% | <0.01% | 1:1 |
| | | | HiXCap™ E2 | 5.5 | 91.1% | 95% | <0.01% | 2:1 |
| | | | HiXCap™ E3 | 5.4 | 92.5% | 98% | <0.01% | 1:1 |
| | | m1Ψ | GAG Cap1 | 5.4 | 91.9% | 99% | <0.01% | 1:1 |
| | | | GAAG Cap2 | 5.2 | 91.9% | 99% | <0.01% | 1:1 |
| | | | HiXCap™ E2 | 5.3 | 92.9% | 95% | <0.01% | 2:1 |
| | | | HiXCap™ E3 | 5.1 | 92.7% | 98% | <0.01% | 1:1 |

IVT reaction conditions: Tris (40 mM, pH 8.0 for GAG Cap1 and GAAG Cap2) or HEPES (40 mM, pH 6.5 for HiXCap™ E2 and HiXCap™ E3 ), 20 mM Mg(OAc)2 for GAG Cap1 and GAAG Cap2 or 30 mM Mg(OAc)2 for HiXCap™ E2 and HiXCap™ E3), 10 mM DTT, 2 mM Spermidine; 5 mM each rNTP, 5 mM cap analog, 50 ug/mL Template; 15 KU/mL T7; 0.5 U/mL IPPA, 200 U/mL RNase inhibitor; 37°C; 2 h. Analysis: Integrity: Agilent fragment analyzer 5200; Capping efficiency: RNase H digestion and measurement of 5′ mRNA fragment by LCMS; dsRNA content: ELISA (K1 & K2 Ab).

# Model Systems Employed for Biological Evaluation

To study the protein translation of HiXCap™ analogs under physiologically relevant immune conditions, we selected in vitro and in vivo model systems that have been reported to recapitulate inflammatory stress.

## JAWS II In Vitro Immune Stress Model[9,10]

JAWS II murine dendritic cells respond robustly to type I interferon (IFNα) stimulation, upregulating transcription of IFIT1.

### Experimental protocol:

- Cells pretreated with IFNα1,2 to elevate IFIT1 levels.
- Transfection with 5′-capped eGFP mRNA.
- Reporter expression quantified by flow cytometry at 24 hours post-transfection.

## LPS-Induced In Vivo Acute Inflammation Model[11]

Mice dosed with lipopolysaccharide (LPS) exhibit elevated systemic cytokines and increased IFIT1 expression in multiple tissues simulating the immune stress encountered by mRNA therapeutics in inflammatory or repeat-dose scenarios.

### Experimental protocol:

- C57BL/6 mice (n=5 per group) dosed intraperitoneally with PBS or LPS (3 mg/kg).
- Four hours later, animals received intravenous LNP-formulated Fluc mRNA (10 µg) capped with GAG Cap1, GAAG Cap2, HiXCap™ E2, or HiXCap™ E3.
- Bioluminescent imaging (whole body and dissected spleen) conducted 10 hours post-mRNA delivery.

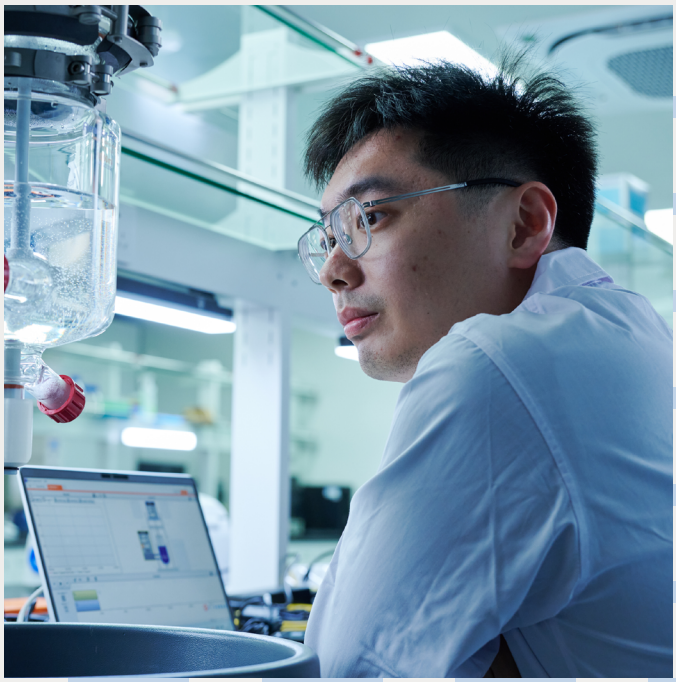
# Results and Discussion

## Study 1: Evaluation of GAG, HiXCap™ E1 and HiXCap™ E2

Study 1 evaluated the performance of HiXCap™ E1 and HiXCap™ E2 cap analogs in comparison with GAG cap analog under inflammatory conditions.

In IFNα-stimulated JAWS II cells, both HiXCap™ designs enhanced translation of eGFP mRNA as compared to GAG cap, while HiXCap™ E2 showed highest expression, suggesting improved performance under inflammatory conditions. In vivo studies in the LPS induced inflammation model using LNP-delivered Fluc mRNAs further supported these findings.

This preliminary study demonstrated that both HiXCap™ designs confer superior translational efficiency and resilience under inflammatory conditions with HiXCap™ E2 showing superior overall performance. HiXCap™ E1 was therefore dropped as a test article in Study 2.

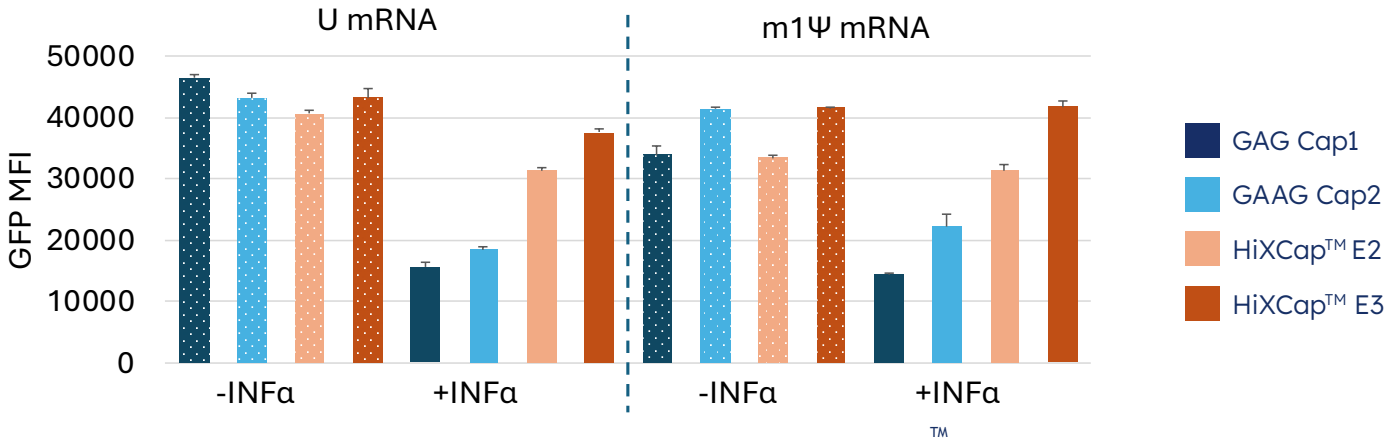


## Study 2: Evaluation of GAG, GAAG, HiXCap™ E2 and HiXCap™ E3

This study compared the performance of HiXCap™ E2 and the tetranucleotide analog HiXCap™ E3 to conventional GAG Cap1 and GAAG Cap2 structures under inflammatory conditions.

In IFNα-treated JAWS II cells, translation of eGFP mRNAs capped with GAG Cap1 and GAAG Cap2 was markedly suppressed, consistent with IFIT1-mediated inhibition. In contrast, mRNAs capped with HiXCap™ E2 and HiXCap™ E3 maintained high levels of protein expression, consistent with enhanced evasion of IFIT1 binding. Notably, HiXCap™ E3 preserved translation levels comparable to those observed under non-inflammatory conditions (-INFα), highlighting its superior performance in this experiment (Figure 8).

## Figure 8. HiXCap™ enhances mRNA translation in IFNα–stimulated JAWS II cells



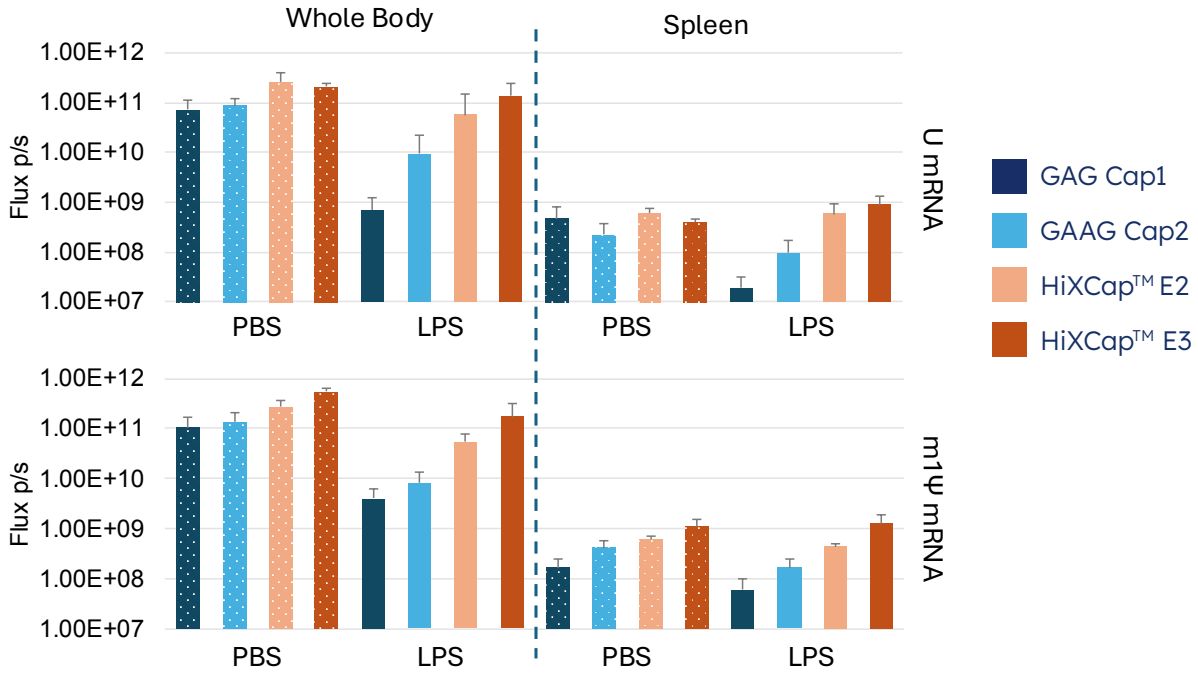

JAWSII cells pre-treated with INFα were transfected with 5′ capped eGFP mRNA using lipofectamine™; After 24 h, cells were analyzed by flow cytometry.

To assess translation under systemic immune activation, we evaluated HiXCap™ analogs in a murine LPS model using LNP-formulated Fluc mRNAs incorporating either uridine (U) or N1-methylpseudouridine (m1Ψ). Capping variants tested included GAG Cap1, GAAG Cap2, HiXCap™ E2, and HiXCap™ E3.

For **U-containing mRNAs**, GAG Cap1 showed strong suppression of translation in the LPS model, while GAAG Cap2 showed partial recovery. Both HiXCap™ analogs preserved high expression, with HiXCap™ E3 demonstrating the strongest performance in both whole-body and spleen imaging, which is consistent with superior evasion of IFIT1 resulting in enhanced translation.

For **m1Ψ-modified mRNAs**, translation was generally more stable in the LPS-treated mice; however, HiXCap™ analogs still outperformed conventional caps in both whole-body and spleen measurements, with HiXCap™ E3 again showing the highest expression. Notably, a similar performance trend was observed in the PBS control group (no LPS), suggesting that HiXCap™ analogs may also confer translational benefits under non-inflammatory conditions (Figure 9).

**Figure 9. HiXCap™ enhances mRNA translation in an LPS-induced in vivo inflammation model (whole body and spleen)**




C57BL/6 mice (n = 5) were dosed with PBS or LPS (3 mg/kg) by ip. After 4 h, 10 µg Fluc mRNA in LNP was dosed by iv. After 10 h, the mice were sacrificed and dissected for imaging and to collect spleen imaging data (Parhiz et al, JCR 2022).

These findings highlight the potential of HiXCap™ analogs to maintain mRNA translation under immune stress, which is an essential feature for clinical applications involving U- or m1Ψ-containing mRNAs, particularly in inflamed tissues or with repeated dosing regimens.

# Therapeutic Case Studies: Tumor Immunotherapy and Prophylactic Vaccination

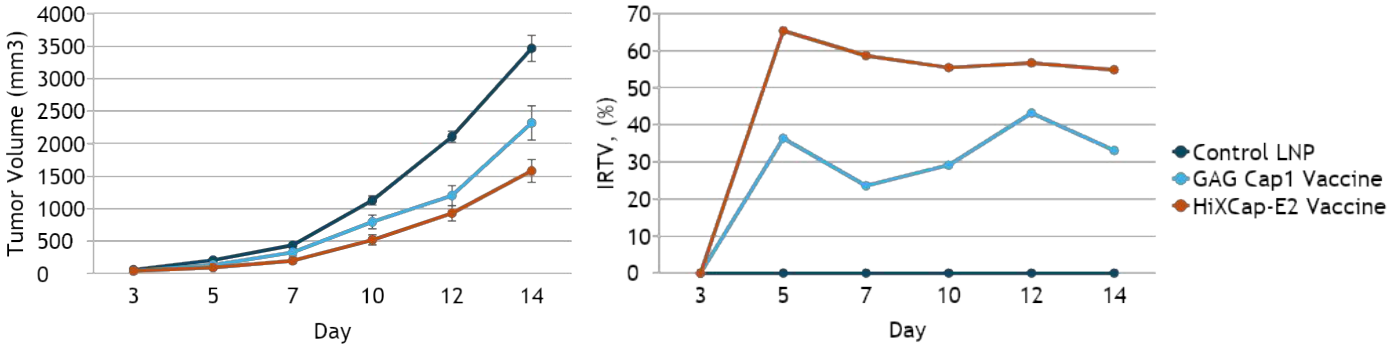
## Case study: Advancing personalized cancer vaccines with HiXCap™

Personalized cancer vaccines (PCVs) are emerging as a transformative class of mRNA drugs, offering advantages including individualized design and potent antigen expression. Critically, their chemical properties and therapeutic potential depends on the 5′ cap chemical structure[12]. Conventional cap analogs such as GAG Cap1 provide moderate activity, but chemistry optimization is required to achieve maximal efficacy and tumor growth inhibition (TGI).

This study evaluated the therapeutic potential of the HiXCap™ E2 cap analog in the context of an mRNA-based personalized cancer vaccine, using a melanoma tumor inhibition model in mice.

In a well-established B16F10 tumor model[13], mice received LNP-formulated mRNAs encoding tumor antigens and capped with either GAG Cap1 or HiXCap™ E2. Over a 14-day period, HiXCap™ E2-capped mRNA induced significantly greater TGI, achieving ~60% inhibition compared to ~30% for the GAG Cap1 comparator, relative to the empty LNP control. These results are consistent with reduced IFIT1-mediated suppression under inflammatory conditions driving enhanced protein translation because of the HiXCap™ E2 design (Figure 10).

## Figure 10. Tumor growth inhibition by HiXCap™ E2 in a cancer vaccine model compared to GAG Cap1




500,000 B16F10 melanoma cells were subcutaneously injected into transgenic mice to establish tumors. Beginning two days post-tumor inoculation, mice received intramuscular injections of 10 μg mRNA vaccine formulated in lipid nanoparticles (LNP); dosing was repeated three times at five-day intervals. Tumor growth was monitored over a 14-day period, and inhibition rates were calculated relative to an empty LNP control group

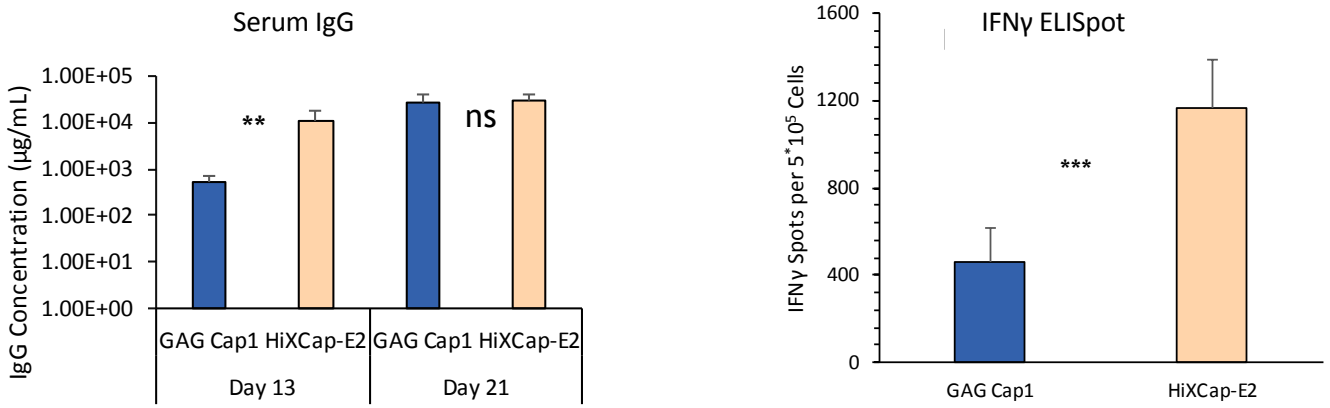
This case study underscores the critical role of cap analog chemistry in TGI and has implications that extend well beyond oncology to the broader field of mRNA therapeutics.

**HiXCap™ E2 capped mRNA personalized cancer vaccines (PCVs) have progressed to IIT clinical evaluation** at Shanghai Ruijin Hospital, affiliated with Shanghai Jiao Tong University School of Medicine. These studies are focused on patients with end-stage solid tumors, acute myeloid leukemia, and post surgical pancreatic cancer (NCT05916248; NCT06496373; NCT06141369; NCT06980155).

## Case study: Enhancing VZV mRNA Vaccine Immunogenicity With HiXCap™

The efficacy of mRNA vaccines against infectious diseases depends not only on antigen sequence design but also on optimized structural elements - most notably the 5′ cap. This study assessed the impact of cap structure on immunogenicity in a mouse model by comparing mRNA encoding varicella-zoster virus (VZV) glycoprotein E (wtgE) capped with either the conventional GAG Cap1 or the HiXCap™ E2 analog (Figure 11).

## Figure 11. Immunogenicity of HiXCap™ E2 in a VZV vaccine model compared to GAG Cap1




C57BL/6 mice (n=6 per group) received two intramuscular doses (14 days apart) of mRNA vaccines containing m1Ψ modification and encoding VZV glycoprotein E (wtgE), formulated with ALC-0315 lipid nanoparticles. The vaccines were capped with either the standard GAG Cap1 or the HiXCap™ E2 cap analog. Serum titers of anti-VZV IgG were measured on days 13 and 21 by ELISA and IFNγ-secreting splenocytes were quantified by ELISpot assay on day 21.

### Humoral Response:

On day 13, the HiXCap™ E2 group exhibited significantly higher serum anti-VZV IgG titers than the GAG Cap1 group, indicating a more rapid humoral response. By day 21, titers in both groups were comparable, suggesting the HiXCap™ E2 structure conferred faster onset of immunity.

### Cellular Response:

Splenocyte analysis on day 21 showed significantly increased IFNγ secretion in the HiXCap™ E2 cohort, reflecting enhanced Th1-type cellular immune activation, which is a key feature for viral clearance and long-term protection.

In this study, HiXCap™ E2 was observed to enhance both the speed and magnitude of the adaptive immune response, as evidenced by accelerated IgG production and elevated IFNγ-secreting T cells. These features are essential for effective virus neutralization and vaccine efficacy. The findings underscore the important role of 5′ cap chemistry in infectious disease mRNA vaccine design and support the incorporation of HiXCap™ designs into next-generation prophylactic vaccine platforms.

# Conclusions

HiXCap™ analogs demonstrate strong potential to enhance the performance of mRNA therapeutics by overcoming immune-mediated translational suppression and improving expression durability. Their rational, modular design supports broad utility across therapeutic areas including vaccines, oncology, and protein replacement.

Future development will focus on expanding structure activity relationships (SAR) to further optimize translation efficiency, stability, and duration of effect. HiXCap™ offers a flexible platform for next-generation mRNA therapeutics requiring improved potency, durability, and immune compatibility.

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Hongene Biotech Corporation

29520 Kouhotek Way,
Union City,
CA 94587

T: +1-919-371-4711
F: +1-919-371-0702
E: product.info@hongene.com
E: info@hongene.com

Hongene Biotech
Japan Co., Ltd

403, Kanda Iwamotocho
Plaza Building,
2-4-1 Iwamotocho,Cho,
Chiyoda-Ku, Tokyo,
101-0032

T: +81-3-5829-9770
F: +81-3-5829-9795
E: info@hongene.jp

Shanghai Hongene
Biotech Corporation

9F, Building 315-1,
No.333 Guiping Road,
Shanghai, China, 200233

T: +86-21-6475-7213
F: +86-21-6470-0613
E: info@hongene.com

Hongene Biotech
Germany GmbH

GroBmoorbogen 25, 21079
Hamburg, Germany

T: +49 40 8830 6130
F: +49 40 8830 6131
E: info-eu@hongene.com

www.hongene.com +1-510-931-4711 product.info@hongene.com Hongene Biotech