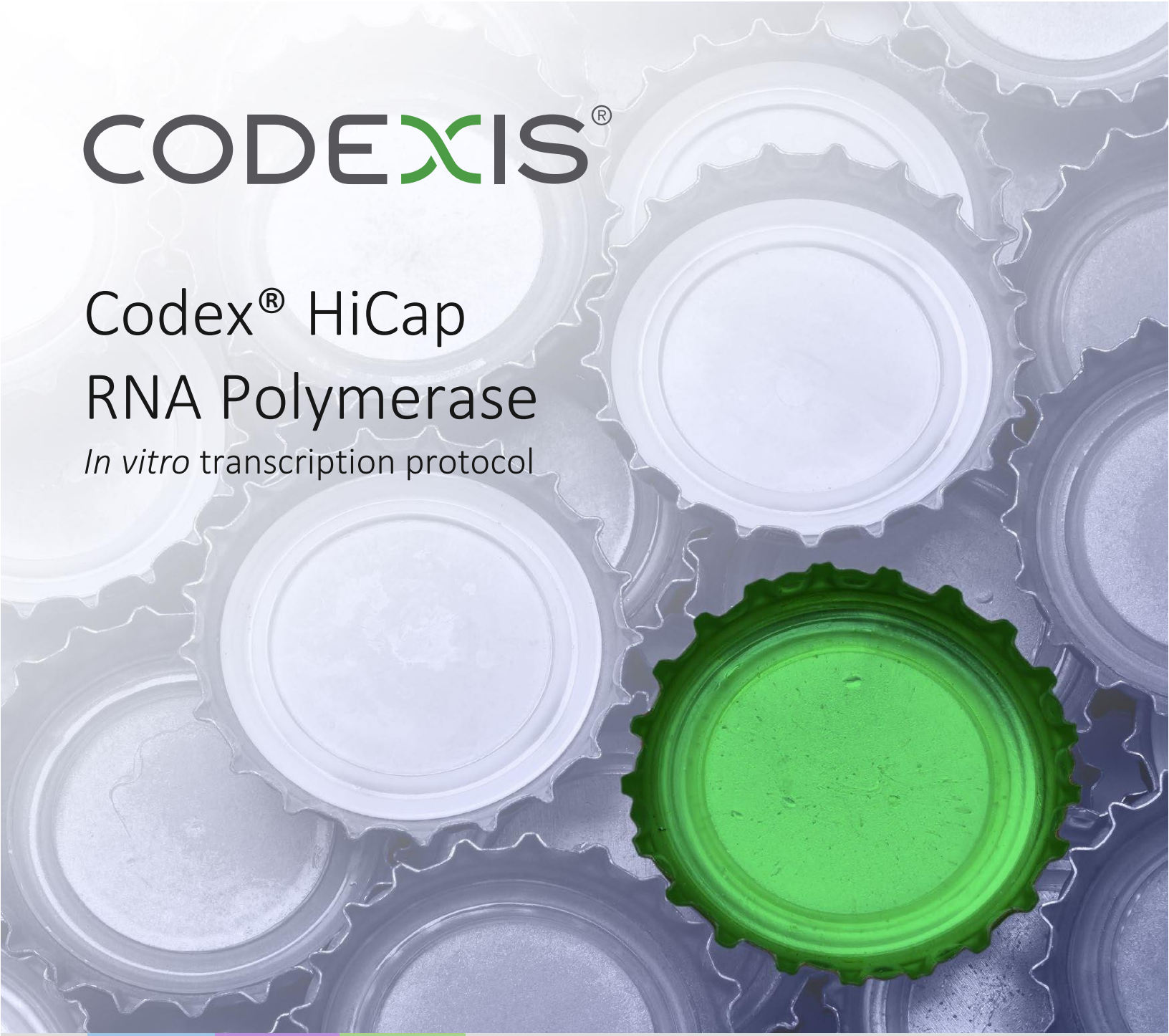
CODEXIS®

Codex® HiCap RNA Polymerase

In vitro transcription protocol



Enhanced co-transcriptional capping performance and low dsRNA generation

Purpose

The Codex® HiCap RNA Polymerase is an engineered T7 RNA polymerase designed to address the challenges of efficiently producing therapeutic-grade mRNA at scale. Compared to wild type T7, Codex® HiCap RNA Polymerase offers higher affinity for cap analogs leading to equivalent or better yields as well as higher capping efficiencies while also producing fewer dsRNA byproducts. While engineered enzymes offer more attractive activity profiles, they also exhibit unique characteristics compared to their wild type counterparts necessitating modified reaction conditions to achieve optimal performance. This document outlines a magnesium acetate-based buffer system that has been developed to maximize performance of the Codex® HiCap RNA Polymerase as well as a set of recommended protocols to guide customers in developing their own custom workflows.

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Buffer formulation

Buffer composition is crucial to the performance of the Codex® HiCap RNA Polymerase. When preparing the reaction buffer, it is important to observe proper precautions for maintaining RNase-free working conditions. The HiCap Buffer PDHC3 (10X) outlined below is formulated to be used with 20 mM NTPs (5 mM each A/U/G/C). If higher NTP concentrations are required, additional magnesium acetate should be added to the IVT reaction according to the following formula:

$$[Additional\ magnesium\ acetate]\ mM = [ATP]\ mM + [UTP]\ mM + [CTP]\ mM + [GTP]\ mM - 20\ mM$$

For example: A reaction utilizing 8 mM of each NTP would require an additional 12 mM magnesium acetate be added to the reaction.

$$8\ mM\ ATP + 8\ mM\ UTP + 8\ mM\ CTP + 8\ mM\ GTP - 20\ mM = 12\ mM\ Addtional\ magnesium\ acetate$$

User-provided materials

User-provided materials
Nuclease-free water (Invitrogen, P# AM9932)
1 M Tris, pH 8.0 (Invitrogen, P# AM9855G)
1 M Magnesium acetate (Sigma, P# 63052)
DTT (Sigma, P# 43815)

If desired 1 M Tris can be prepared by titrating Trizma® base (Sigma, P# T1503) with HCl (BDH, P# BDH7204-1). Ensure the resulting Tris stock is pH 8.0 ± 0.01 @ 25 °C. Do not adjust pH with base.

Buffer preparation

10X HiCap Buffer PDHC3†		
Component	**Volume**	**Final concentration (10X)**
Nuclease-free water	4.3 mL	
1 M Tris, pH 8.0	3 mL	300 mM
1 M Magnesium acetate	2.7 mL	270 mM
Total volume	***10 mL***	

†Note: The buffer should be prepared without DTT. DTT is known to be unstable in solution. 3 mM fresh DTT is required to be added to IVTs at run-time. Failure to add DTT to IVT reactions will result in lower-than-expected yields.

To prepare buffer, combine aqueous reagents in a suitable container. Do not adjust pH after formulation. Mix thoroughly and store at -20 ℃. Avoid excessive freeze thaw cycles.

The "High titer" and "Ultra-high titer" protocols described in this document require additional magnesium acetate over what is provided in the 10X HiCap Buffer PDHC3.

200 mM Magnesium acetate stock		
Component	**Volume**	**Final concentration**
Nuclease-free water	800 µL	
1 M Magnesium acetate	200 µL	200 mM
Total volume	***1 mL***	

To prepare magnesium acetate stock, combine aqueous reagents in a suitable container. Mix thoroughly and store at -20 ℃. Avoid excessive freeze thaw cycles.

Preparation and handling of DTT

DTT is required to maintain disulfide bonds present in the Codex® HiCap RNA Polymerase in a reduced state. DTT is known to be unstable in solution and failure to maintain sufficient reducing potential in IVTs will result in lower-than-expected yields. We recommend preparation and storage of DTT as a 1 M stock. 1 M DTT can be diluted 1:10 in nuclease-free water at runtime to prepare a 100 mM working stock.

1 M DTT Stock		
Component	**Volume**	**Final concentration**
Nuclease-free water	To final volume	
DTT (powdered)	154.25 mg	1 M
Total volume	***1 mL***	

To prepare 1 M DTT stock, resuspend powdered DTT in nuclease-free water. Store at -20 °C in single-use aliquots.

Co-transcriptional capping IVTs

In co-transcriptional capping workflows, a di- or tri-nucleotide cap analog is incorporated at the 5' end of the nascent transcript by the T7 RNA polymerase generating capped mRNA in a single reaction. Codexis has validated CleanCap® Reagent AG, Anti Reverse Cap Analog (ARCA), and m7Gpppm7G (sCap) for use with Codex® HiCap RNA Polymerase. Other cap analogs may be used but are not currently supported.

The initiator sequence used in the development of the Codex® HiCap RNA Polymerase is "AGGAAATA." This sequence includes the "AGG" nucleotides recommended for use with CleanCap® AG with wild-type phage polymerases in the +1 through +3 positions (underlined). In addition, the nucleotides in the +4 through +8 positions can significantly influence mRNA yield for wild type phage polymerases. For a general study of the effects of initiator sequences on mRNA yield for T7 RNA polymerase, see this publication by Conrad, T., Plumbom, I., Alcobendas, M. *et al*. .

NTP concentrations and mRNA titers

The concentration of NTPs in the IVT reaction will determine the concentration of mRNA produced with higher NTP concentrations producing higher concentrations of mRNA. To simplify the formulation of IVTs at various NTP concentrations Codexis provides the following three protocols:

- **Routine** – IVTs w/ 5 mM each NTP. Optimized for exploratory work and small-scale mRNA production.
- **High titer** – IVTs w/ 8 mM each NTP. Our default protocols. Optimized for high mRNA titers while maintaining compatibility with sodium salt NTPs.
- **Ultra-high titer w/ Tris-NTPs** – IVTs w/ 10 mM each NTP. Optimized for maximum mRNA titers, not compatible with sodium salt NTPs.

If you are just starting out, Codexis recommends starting with the **High titer** protocol for your desired cap analog.

Co-transcriptional capping IVTs with CleanCap® Reagent AG

CleanCap® Reagent AG is a trinucleotide cap analog used for the production mRNA with a 5' Cap 1 structure. When used in combination with Codex® HiCap RNA Polymerase, CleanCap® Reagent AG is expected to yield >95% capped material.

The Codex® HiCap RNA Polymerase was evolved to have a higher affinity for cap analogs. For CleanCap® Reagent AG, Codexis recommends a cap concentration of 0.3X the concentration of GTP used in the reaction.

User-provided materials

User-provided materials
Ribonucleotide Solution Set, Sodium Salts (NEB P# N0450L)
Ribonucleotide Solution Set, Tris Buffered (Thermo Fisher P# R1481)[1]
CleanCap® Reagent AG (TriLink P# N-7113)
RNase Inhibitor, Murine (NEB P# M0314L)
Pyrophosphatase, Inorganic (yeast) (NEB P# M2403L)
EDTA (Invitrogen P# AM9260G)
Linear double-stranded DNA template
HiCap Buffer PDHC3 (10X)
Magnesium acetate (Sigma, P# 63052)
DTT (Sigma, P# 43815)

[1] Required only for the Ultra-high titer w/ Tris-NTPs - CleanCap® IVT protocol (10 mM Each NTP)

When utilizing CleanCap® Reagent AG the linear double stranded DNA template must contain the T7 promoter sequence immediately followed by the "AGG" initiator.

Sense 5'- TAA TAC GAC TCA CTA TA ***AGG*** -3'
Antisense 3'- ATT ATG CTG AGT GAT AT ***TCC*** -5'
T7 Promoter Initiator

Routine – CleanCap® IVT protocol (5 mM each NTP)

1. Mix the following components **in order** and **at room temperature:**

Component	Volume	Final concentration
Nuclease-free water	Variable	
HiCap Buffer PDHC3 (10X)	10 µL	1X
DTT (100 mM)	3 µL	3 mM
Vortex gently		
ATP (100 mM)	5 µL	5 mM
CTP (100 mM)	5 µL	5 mM
GTP (100 mM)	5 µL	5 mM
UTP [1] (100 mM)	5 µL	5 mM
CleanCap® Reagent AG (100 mM)	1.5 µL	1.5 mM
RNase Inhibitor, Murine (40 U/µL)	2.5 µL	1 U/µL
Pyrophosphatase, Inorganic (yeast) (0.1U/µL)	2 µL	0.002 U/µL
Template (Variable concentration)	Variable	0.05 µg/µL
Vortex gently		
Codex® HiCap RNA Polymerase (40X)	2.5 µL	1X
Total reaction volume	***100 µL***	

[1] Pseudo-UTP (TriLink P# N-1019) or N1-Methylpseudouridine-5'-Triphosphate (TriLink P# N-1081) can be substituted for UTP as desired.

2. Ensure reaction components are mixed thoroughly and quickly spin down tube(s). Incubate at 37 °C for 2 hours.
3. The reaction may be stopped, and DNA template removed by the addition of RNase-free DNAse I according to the manufacturer's suggested protocol. Alternatively, the reaction may be stopped by adding 12 µL of 0.5 M EDTA if DNA removal is not required.

High titer - CleanCap® IVT protocol (8 mM each NTP)

1. Mix the following components **in order** and **at room temperature:**

Component	Volume	Final concentration
Nuclease-free water	Variable	
HiCap Buffer PDHC3 (10X)	10 µL	1X
Magnesium acetate (200mM)[2]	6 µL	12 mM
DTT (100 mM)	3 µL	3 mM
Vortex gently		
ATP (100 mM)	8 µL	8 mM
CTP (100 mM)	8 µL	8 mM
GTP (100 mM)	8 µL	8 mM
UTP [1] (100 mM)	8 µL	8 mM
CleanCap® Reagent AG (100 mM)	2.4 µL	2.4 mM
RNase Inhibitor, Murine (40U/µL)	2.5 µL	1 U/µL
Pyrophosphatase, Inorganic (yeast) (0.1U/µL)	2 µL	0.002 U/µL
Template (Variable concentration)	Variable	0.1 µg/µL
Vortex gently		
Codex® HiCap RNA Polymerase (40X)	5 µL	2X
Total reaction volume	***100 µL***	

[1] Pseudo-UTP (TriLink P# N-1019) or N1-Methylpseudouridine-5'-Triphosphate (TriLink P# N-1081) can be substituted for UTP as desired.

[2] HiCap Buffer PDHC3 is formulated for use with 20 mM NTPs. To support the 32 mM NTPs used in this protocol additional magnesium acetate is required to avoid lower than expected yields.

2. Ensure reaction components are mixed thoroughly and quickly spin down tube(s). Incubate at 37 °C for 2 hours.
3. The reaction may be stopped, and DNA template removed by the addition of RNase-free DNAse I according to the manufacturer's suggested protocol. Alternatively, the reaction may be stopped by adding 12 µL of 0.5 M EDTA if DNA removal is not required.

Ultra-high titer w/ Tris-NTPs - CleanCap® IVT protocol (10 mM each NTP)

1. Mix the following components **in order** and **at room temperature:**

Component	Volume	Final concentration
Nuclease-free water	Variable	
HiCap Buffer PDHC3 (10X)	10 µL	1X
Magnesium acetate (200 mM)[2]	10 µL	20 mM
DTT (100 mM)	3 µL	3 mM
Vortex gently		
Tris Buffered ATP (100 mM)[3]	10 µL	10 mM
Tris Buffered CTP (100 mM) [3]	10 µL	10 mM
Tris Buffered GTP (100 mM) [3]	10 µL	10 mM
Tris Buffered UTP [1] (100 mM) [3]	10 µL	10 mM
CleanCap® Reagent AG (100 mM)	3 µL	3 mM
RNase Inhibitor, Murine (40 U/µL)	2.5 µL	1 U/µL
Pyrophosphatase, Inorganic (yeast) (0.1 U/µL)	2 µL	0.002 U/µL
Template (Variable concentration)	Variable	0.1 µg/µL
Vortex gently		
Codex® HiCap RNA Polymerase (40X)	5 µL	2X
Total reaction volume	***100 µL***	

[1] Pseudo-UTP (TriLink P# N-1019) or N1-Methylpseudouridine-5'-Triphosphate (TriLink P# N-1081) can be substituted for UTP as desired.

[2] HiCap Buffer PDHC3 is formulated for use with 20 mM NTPs. To support the 40 mM NTPs used in this protocol additional magnesium acetate is required to avoid lower than expected yields.

[3] This protocol requires the use of Tris buffered NTPs (Thermo Fisher P# R1481). Use of sodium salt NTPs in this protocol will result in lower-than-expected yields.

2. Ensure reaction components are mixed thoroughly and quickly spin down tube(s). Incubate at 37 °C for 2 hours.
3. The reaction may be stopped, and DNA template removed by the addition of RNase-free DNAse I according to the manufacturer's suggested protocol. Alternatively, the reaction may be stopped by adding 12 µL of 0.5 M EDTA if DNA removal is not required.

Co-transcriptional capping IVTs with ARCA

The Anti Reverse Cap Analog (ARCA) is a dinucleotide cap analog used for the production of mRNA with a 5' Cap 0 structure. While wild type T7 RNA polymerases require GTP restriction to achieve high capping efficiency with ARCA and other dinucleotide caps, this strategy is not required when used in combination with Codex® HiCap RNA Polymerase. The protocol below is expected to generate >95% capped material with yields similar to those obtained with CleanCap® Reagent AG.

The Codex® HiCap RNA Polymerase was evolved to have a higher affinity for cap analogs. For dinucleotide cap analogs, Codexis recommends a cap concentration of 0.8X the concentration of GTP used in the reaction.

User-provided materials

User-provided materials
Ribonucleotide Solution Set, Sodium Salts (NEB P# N0450L)
Ribonucleotide Solution Set, Tris Buffered (Thermo Fisher P# R1481)[1]
ARCA (TriLink P# N-7003) or sCap
RNase Inhibitor, Murine (NEB P# M0314L)
Pyrophosphatase, Inorganic (yeast) (NEB P# M2403L)
EDTA (Invitrogen P# AM9260G)
Linear double-stranded DNA template
HiCap Buffer PDHC3 (10X)
Magnesium acetate (Sigma, P# 63052)
DTT (Sigma, P# 43815)

[1] Required only for the Ultra-high titer w/ Tris-NTPs - ARCA IVT protocol (10 mM Each NTP)

When utilizing ARCA the linear double-stranded DNA template must contain the T7 promoter sequence immediately followed by the "GGG" initiator.

Sense 5'- TAA TAC GAC TCA CTA TA ***GGG*** -3'
Antisense 3'- ATT ATG CTG AGT GAT AT ***CCC*** -5'
T7 Promoter Initiator

Routine – ARCA IVT protocol (5 mM each NTP)

1. Mix the following components **in order** and **at room temperature:**

Component	Volume	Final concentration
Nuclease-free water	Variable	
HiCap Buffer PDHC3 (10X)	10 µL	1X
DTT (100 mM)	3 µL	3 mM
Vortex gently		
ATP (100 mM)	5 µL	5 mM
CTP (100 mM)	5 µL	5 mM
GTP (100 mM)	5 µL	5 mM
UTP [1] (100 mM)	5 µL	5 mM
ARCA (100 mM)	4µL	4 mM
RNase Inhibitor, Murine (40 U/µL)	2.5 µL	1 U/µL
Pyrophosphatase, Inorganic (yeast) (0.1 U/µL)	2 µL	0.002 U/µL
Template (Variable concentration)	Variable	0.05 µg/µL
Vortex gently		
Codex® HiCap RNA Polymerase (40X)	2.5 µL	1X
Total reaction volume	***100 µL***	

[1] Pseudo-UTP (TriLink P# N-1019) or N1-Methylpseudouridine-5'-Triphosphate (TriLink P# N-1081) can be substituted for UTP as desired.

2. Ensure reaction components are mixed thoroughly and quickly spin down tube(s). Incubate at 37 °C for 2 hours.
3. The reaction may be stopped, and DNA template removed by the addition of RNase-free DNAse I according to the manufacturer's suggested protocol. Alternatively, the reaction may be stopped by adding 12 µL of 0.5 M EDTA if DNA removal is not required.

High titer - ARCA IVT protocol (8 mM each NTP)

1. Mix the following components **in order** and **at room temperature:**

Component	Volume	Final concentration
Nuclease-free water	Variable	
HiCap Buffer PDHC3 (10X)	10 µL	1X
Magnesium acetate (200 mM)[2]	6 µL	12 mM
DTT (100 mM)	3 µL	3 mM
Vortex gently		
ATP (100 mM)	8 µL	8 mM
CTP (100 mM)	8 µL	8 mM
GTP (100 mM)	8 µL	8 mM
UTP [1] (100 mM)	8 µL	8 mM
ARCA (100 mM)	6.4 µL	6.4 mM
RNase Inhibitor, Murine (40 U/µL)	2.5 µL	1 U/µL
Pyrophosphatase, Inorganic (yeast) (0.1 U/µL)	2 µL	0.002 U/µL
Template (Variable concentration)	Variable	0.1 µg/µL
Vortex gently		
Codex® HiCap RNA Polymerase (40X)	5 µL	2X
Total reaction volume	***100 µL***	

[1] Pseudo-UTP (TriLink P# N-1019) or N1-Methylpseudouridine-5'-Triphosphate (TriLink P# N-1081) can be substituted for UTP as desired.

[2] HiCap Buffer PDHC3 is formulated for use with 20 mM NTPs. To support the 32 mM NTPs used in this protocol additional magnesium acetate is required to avoid lower than expected yields.

2. Ensure reaction components are mixed thoroughly and quickly spin down tube(s). Incubate at 37 °C for 2 hours.
3. The reaction may be stopped, and DNA template removed by the addition of RNase-free DNAse I according to the manufacturer's suggested protocol. Alternatively, the reaction may be stopped by adding 12 µL of 0.5 M EDTA if DNA removal is not required.

Ultra-High Titer w/ Tris-NTPs - ARCA IVT Protocol (10 mM each NTP)

1. Mix the following components **in order** and **at room temperature:**

Component	Volume	Final concentration
Nuclease-free water	Variable	
HiCap Buffer PDHC3 (10X)	10 µL	1X
Magnesium acetate (200 mM)[2]	10 µL	20 mM
DTT (100 mM)	3 µL	3 mM
Vortex gently		
Tris Buffered ATP (100 mM)[3]	10 µL	10 mM
Tris Buffered CTP (100 mM) [3]	10 µL	10 mM
Tris Buffered GTP (100 mM) [3]	10 µL	10 mM
Tris Buffered UTP [1] (100 mM) [3]	10 µL	10 mM
ARCA (100 mM)	8µL	8 mM
RNase Inhibitor, Murine (40 U/µL)	2.5 µL	1 U/µL
Pyrophosphatase, Inorganic (yeast) (0.1 U/µL)	2 µL	0.002 U/µL
Template (Variable concentration)	Variable	0.1 µg/µL
Vortex gently		
Codex® HiCap RNA Polymerase (40X)	5 µL	2X
Total reaction volume	***100 µL***	

[1] Pseudo-UTP (TriLink P# N-1019) or N1-Methylpseudouridine-5'-Triphosphate (TriLink P# N-1081) can be substituted for UTP as desired.

[2] HiCap Buffer PDHC3 is formulated for use with 20 mM NTPs. To support the 40 mM NTPs used in this protocol additional magnesium acetate is required to avoid lower than expected yields.

[3] This protocol requires the use of Tris buffered NTPs (Thermo Fisher P# R1481). Use of sodium salt NTPs in this protocol will result in lower-than-expected yields.

2. Ensure reaction components are mixed thoroughly and quickly spin down tube(s). Incubate at 37 °C for 2 hours.
3. The reaction may be stopped, and DNA template removed by the addition of RNase-free DNAse I according to the manufacturer's suggested protocol. Alternatively, the reaction may be stopped by adding 12 µL of 0.5 M EDTA if DNA removal is not required.

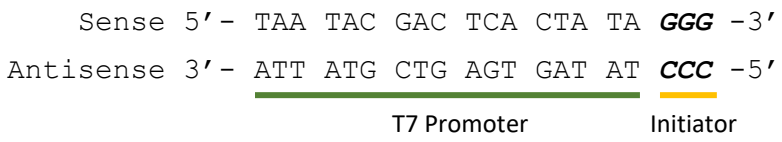
Uncapped IVTs

Many workflows call for the production of uncapped mRNA. Robust yields can be achieved with the Codex® HiCap RNA Polymerase.

User-provided materials

User-provided materials
Ribonucleotide Solution Set, Sodium Salts (NEB P# N0450L)
RNase Inhibitor, Murine (NEB P# M0314L)
Pyrophosphatase, Inorganic (yeast) (NEB P# M2403L)
EDTA (Invitrogen P# AM9260G)
Linear Double Stranded DNA Template
HiCap Buffer PDHC3 (10X)
Magnesium acetate (Sigma, P# 63052)
DTT (Sigma, P# 43815)

For uncapped IVTs the linear double stranded DNA template must contain the T7 promoter sequence immediately followed by the "GGG" initiator. In addition, the nucleotides in the +4 through +8 positions can significantly influence mRNA yield for wild type phage polymerases. For a general study of the effects of initiator sequences on mRNA yield for T7 RNA polymerase, see this publication by Conrad, T., Plumbom, I., Alcobendas, M. *et al*.



Routine - IVT protocol (5 mM each NTP)

1. Mix the following components **in order** and **at room temperature:**

Component	Volume	Final concentration
Nuclease-free water	Variable	
HiCap Buffer PDHC3 (10X)	10 µL	1X
DTT (100 mM)	3 µL	3 mM
Vortex gently		
ATP (100 mM)	5 µL	5 mM
CTP (100 mM)	5 µL	5 mM
GTP (100 mM)	5 µL	5 mM
UTP [1] (100 mM)	5 µL	5 mM
RNase Inhibitor, Murine (40 U/µL)	2.5 µL	1 U/µL
Pyrophosphatase, Inorganic (yeast) (0.1 U/µL)	2 µL	0.002 U/µL
Template (Variable concentration)	Variable	0.05 µg/µL
Vortex gently		
Codex® HiCap RNA Polymerase (40X)	2.5 µL	1X
Total reaction volume	***100 µL***	

[1] Pseudo-UTP (TriLink P# N-1019) or N1-Methylpseudouridine-5'-Triphosphate (TriLink P# N-1081) can be substituted for UTP as desired.

2. Ensure reaction components are mixed thoroughly and quickly spin down tube(s). Incubate at 37 °C for 2 hours.
3. The reaction may be stopped, and DNA template removed by the addition of RNase-free DNAse I according to the manufacturer's suggested protocol. Alternatively, the reaction may be stopped by adding 12 µL of 0.5 M EDTA if DNA removal is not required.

High titer - IVT protocol (8 mM each NTP)

1. Mix the following components **in order** and **at room temperature:**

Component	Volume	Final concentration
Nuclease-free water	Variable	
HiCap Buffer PDHC3 (10X)	10 µL	1X
Magnesium acetate (200 mM)[2]	6 µL	12 mM
DTT (100 mM)	3 µL	3 mM
Vortex gently		
ATP (100 mM)	8 µL	8 mM
CTP (100 mM)	8 µL	8 mM
GTP (100 mM)	8 µL	8 mM
UTP [1] (100 mM)	8 µL	8 mM
RNase Inhibitor, Murine (40 U/µL)	2.5 µL	1 U/µL
Pyrophosphatase, Inorganic (yeast) (0.1 U/µL)	2 µL	0.002 U/µL
Template (Variable concentration)	Variable	0.1 µg/µL
Vortex gently		
Codex® HiCap RNA Polymerase (40X)	5 µL	2X
Total reaction volume	***100 µL***	

[1] Pseudo-UTP (TriLink P# N-1019) or N1-Methylpseudouridine-5'-Triphosphate (TriLink P# N-1081) can be substituted for UTP as desired.

[2] HiCap Buffer PDHC3 is formulated for use with 20 mM NTPs. To support the 32 mM NTPs used in this protocol additional magnesium acetate is required to avoid lower than expected yields.

2. Ensure reaction components are mixed thoroughly and quickly spin down tube(s). Incubate at 37 °C for 2 hours.
3. The reaction may be stopped, and DNA template removed by the addition of RNase-free DNAse I according to the manufacturer's suggested protocol. Alternatively, the reaction may be stopped by adding 1 of 0.5 M EDTA if DNA removal is not required.

Ultra-high titer w/ Tris-NTPs - IVT protocol (10 mM each NTP)

1. Mix the following components **in order** and **at room temperature:**

Component	Volume	Final concentration
Nuclease-free water	Variable	
HiCap Buffer PDHC3 (10X)	10 µL	1X
Magnesium acetate (200mM)[2]	10 µL	20 mM
DTT (100 mM)	3 µL	3 mM
Vortex gently		
Tris Buffered ATP (100 mM)[3]	10 µL	10 mM
Tris Buffered CTP (100 mM) [3]	10 µL	10 mM
Tris Buffered GTP (100 mM) [3]	10 µL	10 mM
Tris Buffered UTP [1] (100 mM) [3]	10 µL	10 mM
RNase Inhibitor, Murine (40 U/µL)	2.5 µL	1 U/µL
Pyrophosphatase, Inorganic (yeast) (0.1U/µL)	2 µL	0.002 U/µL
Template (Variable concentration)	Variable	0.1 µg/µL
Vortex gently		
Codex® HiCap RNA Polymerase (40X)	5 µL	2X
Total reaction volume	***100 µL***	

[1] Pseudo-UTP (TriLink P# N-1019) or N1-Methylpseudouridine-5'-Triphosphate (TriLink P# N-1081) can be substituted for UTP as desired.

[2] HiCap Buffer PDHC3 is formulated for use with 20 mM NTPs. To support the 40 mM NTPs used in this protocol additional magnesium acetate is required to avoid lower than expected yields.

[3] This protocol requires the use of Tris buffered NTPs (Thermo Fisher P# R1481). Use of sodium salt NTPs in this protocol will result in lower-than-expected yields.

2. Ensure reaction components are mixed thoroughly and quickly spin down tube(s). Incubate at 37 °C for 2 hours.
3. The reaction may be stopped, and DNA template removed by the addition of RNase-free DNAse I according to the manufacturer's suggested protocol. Alternatively, the reaction may be stopped by adding 12 µL of 0.5 M EDTA if DNA removal is not required.

mRNA evaluation

The Codex® HiCap RNA Polymerase is engineered to address the challenges of efficiently producing therapeutic-grade mRNA at scale. Codex® HiCap has been shown to produce robust mRNA titers with high capping efficiency and low dsRNA contamination. To assist users in evaluating the performance of Codex® HiCap Codexis also offers the following companion protocols:

- *Evaluating Capping Efficiency with Long Transcripts*
- *dsRNA Evaluation by Qualitative Dot Blot*

Troubleshooting

Issue	Troubleshooting steps
Yields are lower than expected	DTT is known to be unstable in solution. Prepare fresh DTT and ensure 3 mM DTT is added to the reaction.
	The Codex® HiCap RNA Polymerase requires a magnesium concentration 7 mM greater than the total NTP concentration to achieve maximum performance. Ensure sufficient magnesium has been added to the reaction based on the NTP concentrations used.
	Shorter templates, PCR templates, and templates with significant secondary structure may require higher input concentrations for optimal yields. If necessary, perform a template titration experiment to determine the optimal input concentration for your template.
	The optimal pH for the polymerase is 8.0. Ensure that buffer stocks are of the proper pH.
	NTP concentrations above 20 mM (5 mM each NTP) require additional polymerase and template to achieve maximum yields. Ensure reactions are formulated with 2x HiCap RNA Polymerase and 0.1 µg/µL template.
	If using a buffering system other than the one described in this document, prepare fresh reaction buffer according to the buffer formulation section above.
Capping efficiency is lower than expected	DTT is known to be unstable in solution. Prepare fresh DTT and ensure 3 mM DTT is added to the reaction.
	The Codex® HiCap RNA Polymerase requires a magnesium concentration 7 mM greater than the total NTP concentration to achieve maximum performance. Ensure sufficient magnesium has been added to the reaction based on the NTP concentrations used.
	The optimal pH for the polymerase is 8.0. Ensure that buffer stocks are of the proper pH.
	NTP concentrations above 20 mM (5 mM each NTP) require additional polymerase and template to achieve maximum yields. Ensure reactions are formulated with 2x HiCap RNA Polymerase and 0.1 µg/µL template.
	If using a buffering system other than the one described in this document, prepare fresh reaction buffer according to the buffer formulation section above.

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