

Ternaria
Biosciences

ZEBRASCRIBE SP6 TRANSCRIPTION KIT

USER GUIDE

ZebraScribe SP6 Transcription Kit

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Description

The ZebraScribe SP6 Transcription Kit is designed to produce high yields of ARCA-capped mRNA via *in vitro* transcription reaction using the SP6 RNA polymerase.

The kit includes an anti-reverse cap analog (ARCA, m7,3'-OGpppG), which presents a 3'-O-methylation of the m7 guanine moiety and, unlike the traditional cap analog (m7GpppG), prevents it from being incorporated into RNA in the wrong orientation.

The ARCA-capped mRNA produced with the ZebraScribe SP6 kit has a significantly higher translatability than m7GpppG-capped mRNA and can be used in downstream applications such as microinjections, transfection and *in vitro* translation experiments.

The high amount of RNA produced in each reaction (~20-25 µg with the provided control DNA template) is achieved by using high nucleotide concentrations (ATP, CTP, UTP) with an optimized ratio of GTP to ARCA to ensure maximum yields and capping efficiency.

The nucleotides and the ARCA can be provided in a convenient 5X mix, or in individual vials to allow the incorporation of modified nucleotides into the *in vitro* transcription reactions.

Components included in the kit

The ZebraScribe SP6 Transcription Kit is available in 25 or 50 reactions and includes the following components:

- **With NTP/ARCA mix**

Component	Concentration	25 reactions	50 reactions
SP6 Enzyme Mix	NA	62.5 µL	125 µL
SP6 IVT Transcription Buffer	10X	50 µL	100 µL
5X NTP/ARCA mix	25 mM ATP 25 mM CTP 25 mM UTP 5 mM GTP 20 mM ARCA	100 µL	200 µL
GTP solution	20 mM	100 µL	200 µL
Control template	100 ng/µL	10 µL	10 µL
DNase I	2 U/µL	25 µL	50 µL
DNase I Buffer	10X	50 µL	100 µL
DEPC-treated water	NA	500 µL	1 mL
EDTA	50 mM	50 µl	100 µl

Table 1. Components included in ZebraScribe SP6 Transcription Kits with NTP/ARCA mix.

- **With individual NTPs and ARCA**

Component	Concentration	25 reactions	50 reactions
SP6 Enzyme Mix	NA	62.5 µL	125 µL
SP6 IVT Transcription Buffer	10X	50 µL	100 µL
ATP	100 mM	25 µL	50 µL
CTP	100 mM	25 µL	50 µL
GTP	20 mM	125 µL	250 µL
UTP	100 mM	25 µL	50 µL
ARCA	100 mM	20 µL	40 µL
Control template	100 ng/µL	10 µL	10 µL
DNase I	2 U/µL	25 µL	50 µL
DNase I Buffer	10X	50 µL	100 µL
DEPC-treated water	NA	500 µL	1 mL
EDTA	50 mM	50 µl	100 µl

Table 2. Components included in ZebraScribe SP6 Transcription Kits with individual NTPs and ARCA.

Materials not included in the kit

- Products for RNA purification:
  - Isopropanol
  - Lithium chloride
  - Ammonium acetate
  - Phenol:chloroform or phenol:chloroform:isoamylalcohol
  - Spin columns
  - Ethanol
  - RNase and DNase-free reaction tubes

In vitro transcription procedure

Important notes: The enzyme mix included in the ZebraScribe SP6 Kit contains an RNase inhibitor to prevent contamination during *in vitro* transcription. However, the user should ensure that the work environment and the solutions used are RNAse-free. The following steps are recommended prior to starting any RNA procedure:

- Clean surfaces and pipettes with an RNase decontamination solution.
- Use RNAse-free, sterile materials, such as tubes and pipette tips.
- Wear clean gloves when handling samples and reagents.
- Make sure that the reagents are closed properly during storage and before and after the preparation of the reaction procedure.

○ Preparation of DNA template

The DNA template must contain an SP6 promoter sequence (5'- ATTTAGGTGACACTATAG-3') upstream the region of interest to be transcribed. The linearized template can be produced by enzymatic digestion, with a restriction enzyme site downstream of the insert of interest, or by PCR amplification.

For linearization using restriction enzyme digestion, it is important to ensure that the restriction enzyme used generates 5' overhangs or 3' blunt ends. Complete cleavage of the plasmid DNA can be verified by running the digested template on a gel. Due to the processivity of SP6 RNA polymerase, small amounts of circular plasmid can result in generation of very long transcripts.

PCR-amplified products that contain an SP6 promoter upstream the region of interest can be used as DNA templates for *in vitro* transcription directly after amplification. However, digestion of the methylated parental DNA template with DpnI is recommended.

The PCR product should be visualized on a gel to confirm the expected size of the DNA template.

○ Purification of DNA templates

Linearized DNA templates should be clean to ensure high yields and quality of the RNA products. Spin columns for DNA purification, following the instructions provided by the manufacturer, or the protocol below can be used prior to *in vitro* transcription to generate high quality DNA templates.

- After linearization, add the following reagents to the restriction digestion reaction:
    - 1/20th volume of 0.5 M EDTA

- 1/10th volume of 3 M sodium acetate or 5 M ammonium acetate
- 2 volumes of ethanol

- Mix thoroughly and incubate the reaction at -20°C for at least 30 minutes.
- Centrifuge the reaction at top speed for 30 minutes at 4°C. Carefully remove the supernatant.
- Spin the tube for a few seconds and remove the residual liquid with a pipette tip.
- Resuspend the pelleted DNA with nuclease-free water or TE buffer and measure the concentration.

Although PCR-amplified DNA templates can be used directly after the PCR amplification protocol, spin columns or the DNA purification protocol describe above can be performed to ensure DNA products free of contaminants or inhibitors that might interfere with the reaction.

- **Assembly of capped IVT reaction**

The volumes and concentrations detailed in this section are for single 20 µl reactions. The volumes can be scaled up or down accordingly.

- Thaw the reagents listed in Table 3 or Table 4, depending on the format of the kit, by putting them on ice.
- Assemble the *in vitro* transcription reaction at room temperature by mixing the indicated volumes of each reagent.

  - **With NTP/ARCA mix**

Component	Amount
Linear DNA template*	0.1 – 1 µg
10X SP6 IVT Transcription Buffer	2 µL
5X NTP/CAP mix	4 µL
SP6 Enzyme Mix	2.5 µL
DEPC-treated water	Up to 20 µL

*The recommended amount is 0.1 – 0.2 µg of PCR-product template or 1 µg of linearized plasmid template.

Table 3. Assembly of 20 µl of capped in vitro transcription reactions with NTP/ARCA mix.

- **With individual NTPs and ARCA**

Component	Amount
Linear DNA template*	0.1 – 1 µg
10X SP6 IVT Transcription Buffer	2 µL
ATP 100 mM	1 µL
CTP 100 mM	1 µL
GTP 20 mM	1 µL
UTP 100 mM	1 µL
ARCA 100 mM	0.8 µL
SP6 Enzyme Mix	2.5 µL
DEPC-treated water	Up to 20 µL

*The recommended amount is 0.1 – 0.2 µg of PCR-product template or 1 µg of linearized plasmid template.

Table 4. Assembly of 20 µl of capped in vitro transcription reactions with individual NTPs and ARCA.

- Mix the reaction thoroughly by gently flicking the tube 3-5 times and briefly spinning down by centrifugation.
- Incubate 1-2 hours at 37°C.

  Attention: Longer incubation times (2 hours) can increase mRNA yields.

  - **Removal of DNA template (optional)**

- Add 1 µl of DNase I and 2 µl of 10X DNAse I buffer to the reaction.
- Mix thoroughly by flicking and briefly spinning down the tube.
- Incubate for 15-30 minutes at 37°C.
- Inactivate the DNase I by adding 2 µl of EDTA to the reaction, to a final concentration of 5 mM.
- Mix thoroughly and incubate for 10 minutes at 70°C.

  - **Purification of mRNA**

The method and degree of purification required for the generated mRNA will depend on the downstream applications. Three different purification methods and their applications are described in Table 5.

Method	Properties	Observations
Precipitation with lithium chloride (not included)	Efficiently removes unincorporated nucleotides and most proteins, while precipitating RNA molecules of at least 100 nucleotides.	Samples with low concentrations of RNA (< 400 µg/ml) might not be as efficiently precipitated.
Spin columns for RNA purification (not included)	Kits including spin columns and purification buffers offer a simple and reliable method for the rapid preparation and recovery of high-quality total RNA.	Purification kits can limit the yield of recovered RNA.
Precipitation with phenol:chloroform and isopropanol (not included)	The most rigorous method for RNA purification. Efficiently removes proteins and free nucleotides while providing high recovery yields.	Due to the multiple steps involved in the purification process, this method can result in significant contamination of the RNA sample.

Table 5. RNA purification methods after in vitro transcription.

- **Precipitation with lithium chloride**

- Dilute the RNA sample to a starting volume of 50 µl by adding nuclease-free water.
  Attention: if the RNA concentration in the reaction is low, skip this step. See Table 5, Precipitation with lithium chloride, Observations.
- Add 30 µl of lithium chloride solution (7.5 M) and mix thoroughly.
- Incubate it at -20°C for at least 30 minutes.
- Centrifuge at 4°C and maximum speed for 15-30 minutes.
- Remove the supernatant and wash the pellet by adding 500 µl of ethanol 70%.
- Centrifuge the tube for a few seconds and discard the supernatant. Make sure to remove all the remaining ethanol with a pipette tip.
- Air-dry the pellet for 5-10 minutes to allow residual ethanol to evaporate.
  Attention: Longer drying times might cause the pellet to become difficult to resuspend.

- Resuspend the pellet in nuclease-free water, Tris-EDTA buffer or a buffer appropriate for your downstream application.

    - **Spin columns for RNA purification**

- Follow the manufacturer's instructions.

    - **Precipitation with phenol:chloroform and isopropanol**

- Adjust the volume of the initial sample by adding 115 µl of nuclease-free water.
- Add 15 µl of ammonium acetate solution (5 M) and mix thoroughly.
- Add 1 volume of phenol:chloroform and mix thoroughly by vortexing for a few seconds.
- Centrifuge at top speed for 5 minutes and transfer the aqueous phase to a new tube.
- Repeat the previous two steps once.
- Add 1 volume of isopropanol and mix thoroughly by vortexing.
- Incubate the mixture at -20°C for at least 30 minutes.
- Centrifuge at 4°C and maximum speed for 15-30 minutes.
- Remove the supernatant and wash the pellet by adding 500 µl of 70% ethanol.
- Centrifuge the tube for a few seconds and discard the supernatant. Make sure to remove all the remaining ethanol with a pipette tip.
- Air-dry the pellet for 5-10 minutes to allow residual ethanol to evaporate.
  Attention: Longer drying times might cause the pellet to become difficult to resuspend.
- Resuspend the pellet in nuclease-free water, Tris-EDTA buffer or a buffer appropriate for your downstream application.

Quantification and analysis of *in vitro* transcription products

There are several methods to quantify and analyze the products generated after in vitro transcription reactions.

- **Quantification using UV-Vis spectrophotometry**

Quantification of RNA samples using UV light absorbance at 260 nm is the easiest way to determine the yield of a reaction. Unincorporated nucleotides and residual DNA template will interfere with the absorbance; therefore, it is better to use UV-Vis spectrophotometry after RNA purification. Spectrophotometers like Nanodrop include a setting to directly quantify RNA and provide the total concentration of the sample. Alternatively, 1 unit at a 260 nm reading corresponds to 40 µg/ml of RNA, which can be used to calculate the yield as:

$$\frac{\mu g}{ml} RNA = A260 * dilution\ factor * 40\ \mu g/ml$$

- **Analysis on agarose gel**

Visualization of transcription products can be performed on agarose or polyacrylamide gels stained with fluorescent dyes that intercalate with RNA, such as ethidium bromide or SYBR™ Green II RNA Gel Stain.

Agarose gels are suitable for large transcripts, while polyacrilamide gels work better for shorter transcription products.

RNA products tend to fold into secondary structures that affect their size and interfere with their migration on gels. Denaturing polyacrylamide gels with urea, or agarose gels with formaldehyde or glyoxal, will keep the RNA fragments in a denatured state during the electrophoresis and allow a more accurate separation based on their size compared to non-denaturing gels.

Additional information and troubleshooting

- **Increasing product yield with higher GTP concentration**

- The volume and concentration of each reagent in the *in vitro* transcription protocol have been optimized to ensure high yields of mRNA with maximum capping efficiency. Additional GTP is provided with the kit. Increasing the concentration of GTP in the reaction can lead to higher yields of mRNA, however, it might compromise capping efficiency.

- **Low yield, multiple RNA products or wrong transcript size**

- Any issues regarding the yield or quality of the products obtained from the *in vitro* transcription reaction should be first troubleshooted by running a reaction under standard conditions (see Assembly of capped IVT reaction) using the control template provided with the kit.

Contact

For additional information or questions, contact us at ternariabio@ternariabio.com.