Promega

Technical Bulletin

HeLaScribe® Nuclear Extract in vitro Transcription System

INSTRUCTIONS FOR USE OF PRODUCTS E3110, E3091 AND E3092.

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HeLaScribe® Nuclear Extract in vitro Transcription System

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1. Description

The most well characterized cell-free system for in vitro transcription of eukaryotic genes is derived from HeLa cell nuclei (1). HeLa nuclear extracts can support accurate transcription initiation by RNA polymerase II and exhibit both basal and regulated patterns of RNA polymerase transcription (2–4). These nuclear extracts also are a source for a variety of transcription factors, DNA binding proteins and the enzymatic machinery involved in RNA processing (5–8).

The HeLaScribe® Nuclear Extract included in this system is prepared by a modification of the method of Dignam *et al.* (1). The system includes all of the necessary components for in vitro transcription as well as a positive control template that provides run-off transcripts from a CMV immediate early promoter.

This system is designed for runoff transcription. Alternatively, transcription products can be analyzed by primer extension (9).

2. Product Components and Storage Conditions

Product	Size	Cat.#
HeLaScribe® Nuclear Extract in vitro Transcription System	40 reactions	E3110

Each system contains sufficient reagents for 40 reactions. Includes:

- 320u HeLaScribe® Nuclear Extract, in vitro Transcription Grade
- 400µl HeLa Nuclear Extract 1X Transcription Buffer
- 1 × 15ml HeLa Extract Stop Solution
- 500µl $MgCl_2$, 50mM
- 15µl rATP, 100mM
- 15µl rCTP, 100mM
- 15µl rGTP, 100mM
- 15µl rUTP, 100mM
- 300ng HeLa Nuclear Extract Positive Control DNA (CMV)
- 1ml Loading Dye
- 1.25ml Nuclease-Free Water

Product	Size	Cat.#
HeLaScribe® Nuclear Extract, in vitro Transcription Grade	40 reactions	E3091
	160 reactions	E3092

Storage Conditions: Store HeLaScribe® Nuclear Extract at –70°C; avoid repeated freeze-thaw cycles. Divide the HeLa Extract Stop Solution into single-use aliquots in RNase-free tubes after the first thaw and store at –70°C. Store rATP, rCTP, rGTP and rUTP at –20°C. Store all other components at –70°C.

Divide the HeLa Extract Stop Solution into single-use aliquots in RNase-free tubes. Store at –70°C.

Composition: The extract is dialyzed into a storage buffer containing 40mM Tris (pH 7.8 at 25°C), 100mM KCl, 0.2mM EDTA, 0.5mM PMSF, 0.5mM DTT and 25% glycerol.

Unit Definition: One unit is defined as the amount of extract required for the incorporation of 50fmol of nucleotides into a 363-nucleotide runoff transcript generated from the CMV immediate early promoter fragment per hour at 30°C under standard conditions.

3. HeLaScribe® Nuclear Extract in vitro Transcription System Protocol

3.A. Transcription Reaction

The standard control reaction contains 8 units of Nuclear Extract and 100ng of linearized HeLa Nuclear Extract Positive Control DNA (referred to as CMV Positive Control DNA) in a total volume of 25µl. When using different promoters, the volume of the extract, template, magnesium chloride concentration, ribonucleotide concentration, temperature and time of incubation may need to be optimized; adjust the volume of water accordingly.

The $MgCl_2$ concentration is important for specific promoter function, and different promoters work optimally at slightly different $MgCl_2$ concentrations. We recommend a final concentration of 3mM $MgCl_2$ for the CMV Positive Control DNA. For the Adenovirus Major Late Promoter, we recommend a final concentration of 4mM $MgCl_2$ (2,10). Table 1 provides volumes of 50mM $MgCl_2$ needed to achieve the required final $MgCl_2$ concentration in a 25µl reaction.

Table 1. Volumes of 50mM $MgCl_2$ Needed to Achieve the Required $MgCl_2$ Concentration in a 25µl Reaction.

Final $MgCl_2$ Concentration	Volume of 50mM $MgCl_2$
3mM	1.5µl
4mM	2.0µl
5mM	2.5µl
6mM	3.0µl
7mM	3.5µl
8mM	4.0µl

Materials to Be Supplied by the User
(Solution compositions are provided in Section 4.)

- phenol:chloroform:isoamyl alcohol (25:24:1) OR TE-saturated phenol and chloroform:isoamyl alcohol (24:1)
- ethanol (100%)
- [α-^{32}P]rGTP (3,000Ci/mmol, 10mCi/ml)

1. Prepare an appropriate ribonucleotide mix using the 100mM rNTP stock solutions and Nuclease-Free Water. For example, when using the CMV Positive Control DNA and [α-^{32}P]rGTP (3,000Ci/mmol, 10mCi/ml), we recommend preparing a 25X stock containing 10mM rATP, 10mM rCTP, 10mM rUTP and 0.4mM rGTP. This ribonucleotide mix will be referred to as the "25X rNTP Mix." If using a radiolabeled ribonucleotide other than rGTP, decrease the concentration of that corresponding ribonucleotide in the mix and maintain a 10mM concentration of the other 3 ribonucleotides.

A final concentration of unlabeled nucleotides in a typical transcription reaction would be 0.4mM rATP, 0.4mM rCTP, 0.4mM rUTP and 16µM rGTP.

3.A. Transcription Reaction (continued)

2. Determine the volume of HeLaScribe® Nuclear Extract that provides 8 units of activity (or the number of units required for your experiment, see Notes 1 and 2, below). In most cases, 5–11µl of Nuclear Extract will provide 8 units of activity. Calculate the volume of HeLa Nuclear Extract 1X Transcription Buffer needed to bring the total volume of extract plus buffer to 11µl. This volume of HeLa Nuclear Extract 1X Transcription Buffer is designated "(11 – x)µl," where x is the volume of Nuclear Extract used.

   Step 2 Notes:

   1. The activity for each lot of HeLaScribe® Nuclear Extract is based upon transcriptional activity of the CMV immediate early promoter, and lot-to-lot variation may be observed for the activities of other promoters. Also, the volume of HeLaScribe® Nuclear Extract that provides 8 units of CMV activity may vary from lot to lot; therefore, the volume of Nuclear Extract to be added to the in vitro transcription reaction needs to be calculated with each lot of Nuclear Extract. The volume of extract that provides 8 units of activity (CMV promoter) is indicated on the vial label for each lot.
   2. When using different promoters, the volume of the extract, template, magnesium chloride concentration, ribonucleotide concentration, temperature and time of incubation may need to be optimized.

3. Thaw a tube of extract on ice just before use (approximately 5 minutes). Gently mix the extract by pipetting, stirring with a pipette tip or flicking the tube before taking a sample. Assemble the following components on ice in a 1.5ml sterile siliconized microcentrifuge tube:

HeLa Nuclear Extract 1X Transcription Buffer (where x is the volume of Nuclear Extract used)	(11 – x)µl
$MgCl_2$, 50mM	1.5µl
25X rNTP Mix	1µl
CMV Positive Control DNA (100ng)	4µl
[α-^{32}P]rGTP (3,000Ci/mmol, 10mCi/ml)	1µl

   Add Nuclease-Free Water such that the final volume after addition of the Nuclear Extract will be 25µl.

   Add 8 units (xµl) of HeLaScribe® Nuclear Extract to initiate the reaction. Mix briefly and gently.

   Step 3 Notes:

   1. Higher yields have been obtained by using siliconized tubes for Steps 3 and 6 and transferring the ethanol mixture to nonsiliconized tubes for the centrifugation (Step 9).
   2. For some applications, preincubation of Nuclear Extract with template DNA is preferred, and the reaction is initiated by the addition of ribonucleotides (9).

4. Incubate at 30°C for 60 minutes.

5. To decrease variability, the HeLa Extract Stop Solution should be warmed at 25°C for one hour and vortexed well to mix. Terminate the reaction by adding 175µl of the Stop Solution.

6. Add 200µl TE-saturated phenol, vortex 60 seconds and centrifuge at 14,000 × *g* for 5 minutes. Transfer 150µl of the upper, aqueous phase to a clean tube. Re-extract the phenol phase by adding 200µl of Stop Solution. Vortex and centrifuge as described above. Combine the aqueous layers and extract with 300µl chloroform:isoamyl alcohol (24:1). Vortex and spin as above. Transfer aqueous phase to a clean siliconized tube.

   Alternatively, extract with 200µl phenol:chloroform:isoamyl alcohol (25:24:1), vortex 60 seconds and centrifuge at 14,000 × *g* for 5 minutes. Transfer the upper, aqueous phase to a fresh tube.

7. Add 700µl of 100% ethanol, mix by vortexing and place at –70°C for at least 15 minutes. For best results, incubate overnight.

8. Transfer ethanol mixture to a sterile, 1.5ml microcentrifuge tube (nonsiliconized).

9. Position the tube in the microcentrifuge so that the pellet will form in a known orientation. Centrifuge at 14,000 × *g* for 10 minutes at 4°C. Carefully remove the supernatant and do not disturb the pellet. The pellet may not be visible. Dry the pellet in a vacuum desiccator.

10. Suspend the pellet carefully in 10–20µl of Nuclease-Free Water, the amount depending upon the volume and percentage of sample that will be loaded onto the gel.

11. Add an equal volume of Loading Dye to the RNA. Heat the samples at 90°C for 10 minutes just prior to loading onto a gel. If only a portion of the sample is loaded onto the gel, store the remainder at –20°C.

3.B. Gel Analysis

We typically analyze runoff transcription products on a 16 × 18cm denaturing polyacrylamide gel containing 6% acrylamide, 7M urea and TBE 0.5X buffer (see Figure 1). Size markers are prepared by kinasing dephosphorylated, digested ϕX174 HinfI DNA (Cat.# E3511) with [γ-^{32}P]ATP. For more information on preparing 5´ end-labeled DNA molecules, refer to the *DNA 5´ End-Labeling System Technical Bulletin* #TB096. This document may be requested from Promega Corporation or is available at: **www.promega.com**. Prerun the gel at 250V for 10 minutes. Flush the wells with TBE 0.5X buffer and load the samples and kinased size markers. Run the gel at 250V in TBE 0.5X buffer until the bromophenol blue dye just runs off the gel.

3.B. Gel Analysis (continued)

Wrap the gel in plastic wrap and expose to X-ray film overnight at –70°C with an intensifying screen. Orientation of the gel with the film is necessary if the runoff products are to be excised from the gel and counted in scintillation fluid.

Alternatively, the gel can be exposed to a phosphorimaging screen. These systems may provide greater sensitivity, speed and the ability to quantitate the radioactive bands.

Specific transcription from the CMV Positive Control DNA results in a runoff transcript 363 nucleotides in length. Larger products may be visible on the gel and may represent end-to-end transcription of the 1.2kb promoter-containing restriction fragment used as the template DNA. If 50% of the transcription mixture is loaded on the gel, a prominent band at 363 nucleotides should be visible after an overnight exposure of the gel to X-ray film with an intensifying screen at –70°C.

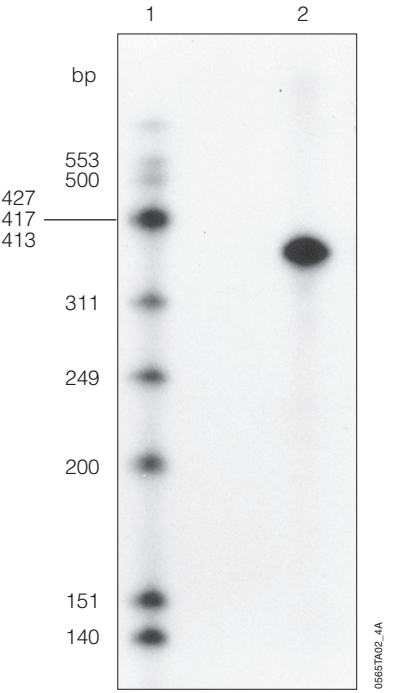

Figure 1. Runoff transcription product generated from in vitro transcription of the HeLa Nuclear Extract Positive Control DNA (CMV). HeLaScribe® Nuclear Extract was incubated with a fragment of DNA containing the immediate early gene of CMV as described. Lane 1, ϕX174 HinfI size markers; lane 2, CMV runoff product. The product was subjected to electrophoresis on a 6% denaturing polyacrylamide gel and exposed to film for 16 hours at –70°C with an intensifying screen.

4. Composition of Buffers and Solutions

HeLa Nuclear Extract 1X Transcription Buffer

20mM	HEPES (pH 7.9 at 25°C)
100mM	KCl
0.2mM	EDTA
0.5mM	DTT
20%	glycerol

HeLa Extract Stop Solution

0.3M	Tris-HCl (pH 7.4 at 25°C)
0.3M	sodium acetate
0.5%	SDS
2mM	EDTA
3µg/ml	tRNA

Loading Dye

98%	formamide
10mM	EDTA
0.1%	xylene cyanol
0.1%	bromophenol blue

TBE 1X buffer

89mM	Tris base
110mM	boric acid
2mM	EDTA

TE buffer

10mM	Tris-HCl (pH 8.0 at 25°C)
1mM	EDTA

TE-saturated phenol

Mix equal parts of TE buffer and phenol and allow the phases to separate. Repeat until the aqueous phase reaches a pH of approximately 8.0.

TE-saturated phenol:chloroform: isoamyl alcohol (25:24:1)

Mix equal parts of TE buffer and phenol and allow the phases to separate. Then mix 1 part of the lower, phenol phase with 1 part of chloroform:isoamyl alcohol (24:1).

5. Related Products

Product	Size	Cat.#
rATP, 100mM	400µl	E6011
rCTP, 100mM	400µl	E6041
rGTP, 100mM	400µl	E6031
rUTP, 100mM	400µl	E6021
rCTP, rATP, rUTP, rGTP, 100mM each	4 × 400µl	E6000

For Laboratory Use

Product	Size	Cat.#
HeLaScribe® Nuclear Extract Positive Control DNA	300ng	E3621
Primer Extension System—AMV Reverse Transcriptase	40 reactions	E3030
Gel Shift Assay Core System	100 reactions	E3050
Gel Shift Assay System	100 reactions	E3300
Gel Shift Binding 5X Buffer	5 × 200µl	E3581
SP1, Human, Recombinant	15µg	E6391

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