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Kool™ NC-45™ RNAP Activity & Inhibitor Screening Kit

Kool™ NC-45™ Universal RNA Polymerase Template

Cat. No. KNK49025 and KN411100

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1. Introduction

The Kool™ NC-45™ RNAP Activity & Inhibitor Screening Kit+ is based on the patented Rolling Circle Transcription (RCT) technology,* developed in the laboratory of Dr. Eric Kool.[1-4] RCT technology takes advantage of the observations that certain small, circular, single-stranded DNAs (DNA nanocircles) are efficiently transcribed by RNA Polymerases without the requirement for promoter sequences or primers.

The Kool™ NC-45™ Universal RNA Polymerase Template+, supplied separately and in the kit, is a 45-base single-stranded circular DNA that functions as template for *in vitro* transcription by bacterial and phage RNA Polymerases. The Kool NC-45 Template has been demonstrated to function in RCT assays using purified RNA Polymerases from *E. coli* (core and holoenzyme), *S. aureus*, *T. thermophilus* and phages T7 and N4.

RCT can be followed in real-time with fluorescence monitoring or the synthesized RNA can be detected by a variety of methods without removal of the template DNA.

Applications

- Screening for bacterial DNA-dependent RNA Polymerase activity.
- Screening compounds for RNA Polymerase inhibitor activity.

2. Product Specifications

Storage: Store only at –20°C in a freezer without a defrost cycle. Do not store at –70°C.

Storage Buffer: *E. coli* Core RNA Polymerase, Core Enzyme is supplied in a 50% glycerol solution containing 50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 0.1 mM EDTA, and 1 mM DTT.

***E. coli* RNA Polymerase 5X Reaction Buffer:** 200 mM Tris-HCl (pH 7.5), 250 mM KCl, 50 mM $MgCl_2$, and 0.05% Triton® X-100.

3. Kit Contents

Desc.	Concentration	Quantity
The 25-reaction size kit contains:		
E. coli RNA Polymerase, Core Enzyme	(15 Units @ 1 U/µl)	15 µl
E. coli RNA Polymerase		
5X Reaction Buffer		125 µl
RiboGuard™ RNase Inhibitor	(800 Units @ 40 U/µl)	20 µl
Kool™ NC-45™ Template	(700 ng @ 14 ng/µl = 1 pmol/µl)	50 µl
NTP Solution Contains 5 mM each ATP, CTP, GTP, and UTP		65 µl
100 mM Dithiothreitol (DTT)		50 µl
RNase-Free Water		1 ml

Note: *A detection dye is not included in this kit. One must be purchased separately for real time fluorescent detection.*

Kool™ NC-45™ Universal RNA Polymerase Template	1 pmol/µl	100 µl

4. Related Products

The following products are also available:

- *E. coli* RNA Polymerase (Core and Holoenzymes)
- CircLigase™ ssDNA Ligase
- CircLigase™ II ssDNA Ligase
- T7 Phage RNA Polymerase
- NTP Solutions

Unit Definition: One Unit of *E. coli* Core RNA Polymerase, Core Enzyme catalyzes the incorporation of one nanomole of ribonucleoside triphosphates (NTPs) into RNA in 10 minutes at 37°C.

Contaminating Activity Assays: All of the components of the Kool NC-45 RNAP Activity & Inhibitor Screening Kit are free of detectable exo- and endonuclease and RNase activities.

Notes on Using the Kool™ NC-45™ RNAP Activity & Inhibitor Screening Kit

1. **Pre-incubation of RNA Polymerase with Inhibitor:** Rifampicin is a known inhibitor of *E. coli* RNA Polymerase, Core Enzyme (EcRNAP) provided in the kit. Effective inhibition of EcRNAP by rifampicin requires pre-incubation of the EcRNAP with the inhibitor prior to the addition of the Kool NC-45 DNA Template and NTPs. The pre-incubation step is written into this protocol. The user must determine experimentally if pre-incubation of EcRNAP (or other bacterial or phage RNA Polymerases) with the inhibitor of interest is necessary, and if so, determine the optimal pre-incubation conditions.
2. **Screening Alternative Bacterial RNA Polymerases:** The Kool NC-45 RNAP Activity & Inhibitor Screening Kit can be used to screen the activity of, or inhibitors of other bacterial or phage RNA Polymerases. In addition to the *E. coli* RNA Polymerase, Core Enzyme, the Kool NC-45 Template efficiently serves as transcription template for *E. coli* RNA Polymerase, Holoenzyme, *S. aureus*, and *T. thermophilus* RNA Polymerases. Presumably other bacterial RNA Polymerases will also be able to utilize the Kool NC-45 Template. If assaying the activity of a bacterial RNA Polymerase, substitute the desired RNA Polymerase for the *E. coli* RNA Polymerase, Core Enzyme in the reaction procedure presented on pages 4 or 5.
   - It may be necessary to first test serial dilutions of the RNA Polymerase of interest to determine the optimum amount to use in the assay.
   - It may be necessary to increase the amount of Kool NC-45 Template in the reactions.
   - It may be necessary to adjust the concentration of the fluorescent dye in the reaction.

3. **Detection Methods:**

   Note: *A detection dye is not included in this kit. One must be purchased separately for real time fluorescent detection.*

   Real time fluorescent detection: RNA synthesis can be detected in a real-time format using a fluorometer or real-time PCR instrument capable of 490-nm excitation and 530-nm emission detection. The following fluorescent dyes exhibiting fluorescence enhancement upon binding to RNA have been tested: SYBR® Green, SYBR Gold, and RiboGreen® dyes.

   Note: *The dilution of fluorescent dyes was optimized for EcRNAP using the BioRad iCycler iQ® Real-Time Detection System. When using different enzymes or instruments, the concentration of the fluorescent dye may need to be adjusted.*

   If using SYBR Green, prepare a 1:1,500 dilution in 0.1X TE Buffer (1 mM Tris-HCl [pH 7.5], 0.1 mM EDTA) and add 2.5 µl in the procedure described below.

   If using SYBR Gold, prepare a 1:2,000 dilution in 0.1X TE Buffer (1 mM Tris-HCl [pH 7.5], 0.1 mM EDTA) and add 2.5 µl in the procedure described below.

   If using RiboGreen, prepare a 1:100 dilution in 0.1X TE Buffer (1 mM Tris-HCl [pH 7.5], 0.1 mM EDTA) and add 2.5 µl in the procedure described below.

   Note: *Molecular beacons provide another alternative for real-time fluorescent detection of RCT*[5].

End-point detection: If performing end-point detection, do not add any fluorescent dye to the reaction: *instead*, add 2.5 µl of RNase-Free Water or 0.1X TE Buffer in place of the dye in the procedure described on page 5. In the absence of fluorescent dyes, the standard reaction produces up to 600 ng of RNA in 60 minutes using *E. coli* RNA Polymerase Core enzyme provided in the kit. The yield of RNA is significantly lower when the reaction is performed in the presence of a fluorescent dye.

A procedure for fluorescent end-point quantitation using RiboGreen dye is presented in Procedure B. End-Point detection using RiboGreen on page 6.

Reaction products can be visualized by 1% denaturing agarose gel electrophoresis followed by staining with SYBR Gold or ethidium bromide. After a 60 minute reaction (in the absence of dye), load 8-10 µl of the standard 25 µl reaction (200-240 ng of RNA) onto the gel. Run the gel and stain with SYBR Gold.

Radioactive detection: can be performed by adding a small amount of a radiolabeled-NTP to the reaction. The radiolabeled RNA produced in the reaction can be visualized by gel electrophoresis followed by autoradiography or counted in a scintillation counter after removal of the unincorporated NTPs.

5. Procedures

Additionally Required Reagents and Equipment
Water bath
Fluorometer or real-time thermocycler capable of 490-nm excitation and 530-nm emission detection.
RNase-Free Water
Optional: Fluorescent dye for detection or quantification
Optional: E. coli RNA Polymerase Inhibitor
(e.g., rifampicin)

Two procedures are presented. Real-time detection is the standard procedure on which the kit was developed. End-point detection using RiboGreen requires the user to provide the RiboGreen dye. The user should consult the **Notes on Using the Kool NC-45 RNAP Activity & Inhibitor Screening Kit** for guidelines to modify the procedures for using alternative *E. coli* RNA Polymerase, Core Enzyme inhibitors, alternative bacterial or phage RNA Polymerases, or alternative real-time or end-point detection methods.

A. Real-Time detection

The procedure described below is for identification of *E. coli* RNA Polymerase, Core Enzyme inhibitors based on real-time detection of RNA synthesis. The recommended EcRNAP control inhibitor, rifampicin (not provided in the kit), requires pre-incubation with the EcRNAP prior to initiating the RCT reaction. The pre-incubation step is written into this protocol. The user must determine experimentally if a pre-incubation of EcRNAP with their inhibitor of interest is necessary and, if so, determine the optimal pre-incubation conditions.

1. Combine the following on ice:

8.8	µl	RNase-Free Water
5	µl	*E. coli* RNA Polymerase 5X Reaction Buffer
2	µl	100 mM DTT
0.7	µl	RNase Inhibitor (40 U/µl)
1	µl	0.25 mM Rifampicin (substitute RNase-Free Water for no-inhibitor reactions)
0.5	µl	*E. coli* RNA Polymerase, Core Enzyme (1 U/µl)
18	µl	Total reaction volume

2. Pre-incubate the reaction for 10 minute at 37°C in a water bath or thermocycler.

   Pre-incubation ensures complete inhibition of the EcRNAP in the samples containing rifampicin.

3. At room temperature, combine the following:

18	µl	Pre-incubated reaction from Part A, Step 2
2	µl	Kool NC-45 Template (14 ng/µl; 1 pmol/µl)
2.5	µl	Fluorescent Dye (user provided)
22.5	µl	Total reaction volume

4. Transfer the tubes/microtiter plate to 37°C.
5. Initiate the reaction by adding:

2.5	µl	NTP Solution (substitute RNase-Free Water for the control reaction)
25	µl	Total reaction volume

6. Immediately begin recording fluorescence using 490-nm excitation and 530-nm emission wavelengths. RNA synthesis continues even after the fluorescence signal reaches a plateau.

B. End-Point detection using RiboGreen

RNA produced by the Rolling Circle Transcription (RCT) reaction can be quantified in an end-point assay using the fluorescent dye RiboGreen (excitation max. = 500 nm, emission max. = 525 nm; provided by the user). The end-point assay can be performed without removing the Kool NC-45 Template or other reaction components.

The procedure described below is for end-point RiboGreen detection of *E. coli* RNA Polymerase, Core Enzyme inhibitors. The recommended EcRNAP control inhibitor, rifampicin (provided by the user), requires pre-incubation with the EcRNAP prior to initiating the RCT reaction. The pre-incubation step is written into this protocol. The user must determine experimentally if a pre-incubation of EcRNAP with their inhibitor of interest is necessary and, if so, determine the optimal pre-incubation conditions.

1. Combine the following on ice:

8.8	µl	RNase-Free Water
5	µl	*E. coli* RNA Polymerase 5X Reaction Buffer
2	µl	100 mM DTT
0.7	µl	RNase Inhibitor (40 U/µl)
1	µl	0.25 mM Rifampicin (substitute RNase-Free Water for no-inhibitor reactions)
0.5	µl	*E. coli* RNA Polymerase, Core Enzyme (1 U/µl)
18	µl	Total reaction volume

2. Pre-incubate the reaction for 10 minute at 37°C in a water bath or thermocycler.

   Pre-incubation ensures complete inhibition of the EcRNAP in the samples containing rifampicin.
3. At room temperature, combine the following:

18	µl	Pre-incubated reaction from Part B, Step 2
2.5	µl	RNase-Free Water or 0.1X TE Buffer
2	µl	Kool NC-45 Template (14 ng/µl; 1 pmol/µl)
22.5	µl	Total reaction volume

4. Transfer the tubes/microtiter plate to 37°C.
5. Initiate the reaction by adding:

2.5	µl	NTP Solution (substitute RNase-Free Water for the control reaction)
25	µl	Total reaction volume

6. Incubate the reactions at 37°C for 60 minutes.
7. While the reactions incubate, prepare an RNA standard curve using RNA of known concentrations according to one of the following procedures:

i If measuring fluorescence in a 200-µl volume, dilute the RiboGreen 1:200 in TE Buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA). Then, prepare RNA standards by combining:

100 µl of TE Buffer + 100 µl diluted RiboGreen
100 µl of 0.04 ng/µl RNA + 100 µl diluted RiboGreen
100 µl of 0.2 ng/µl RNA + 100 µl diluted RiboGreen
100 µl of 1.0 ng/µl RNA + 100 µl diluted RiboGreen
100 µl of 2.0 ng/µl RNA + 100 µl diluted RiboGreen

Incubate for 2-5 minutes at room temperature. Then, read the fluorescence of the standards. Construct a standard curve by plotting the fluorescence of each sample against its RNA concentration. Store the remaining 1:200 diluted RiboGreen at room temperature, in the dark for use in Part B, Step 8.

Note: *Do not store the diluted RiboGreen for more than 2 hours.*

ii If measuring fluorescence in a 2-ml volume, dilute the RiboGreen 1:2,000 in TE Buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA). Then, prepare RNA standards by combining:

1 ml of TE Buffer + 1 ml diluted RiboGreen
1 ml of 2 ng/µl RNA + 1 ml diluted RiboGreen
1 ml of 10 ng/µl RNA + 1 ml diluted RiboGreen
1 ml of 50 ng/µl RNA + 1 ml diluted RiboGreen
1 ml of 100 ng/µl RNA + 1 ml diluted RiboGreen

Incubate for 2-5 minutes at room temperature. Then, read the fluorescence of the standards. Construct a standard curve by plotting the fluorescence of each sample against its RNA concentration. Store the remaining 1:2,000 diluted RiboGreen at room temperature, in the dark for use in Step B8.

Note: *Do not store the diluted RiboGreen for more than 2 hours.*

8. After the 60-minute RCT reaction is complete, transfer tube/microtiter plate from Part B, Step 6 to ice. Then, measure the quantity of RNA produced by one of the following procedures:
    - i If measuring fluorescence in a 200-µl volume, add 2.5 µl of each RCT reaction to 95 µl of TE Buffer. Then, add 100 µl of the 1:200 diluted RiboGreen to the diluted sample.
    - ii If measuring fluorescence in a 2-ml volume, add 2.5 µl of each RCT reaction to 1 ml of TE Buffer. Then, add 1 ml of the 1:2,000 diluted RiboGreen to the diluted sample.
9. Incubate each for 5 minutes at room temperature.
10. Read the fluorescence of the RCT reaction samples. Subtract the fluorescence of the control reaction from that of the RCT samples and determine the RNA concentration using the RNA standard curve produced in Part B, Step 7.

6. References

1. Daubendiek, S.L. *et al.*, (1995) *J. Am. Chem. Soc.* **117**, 7818.
2. Diegelman, A.M. *et al.*, (1998) *Nucl. Acid Res.* **26**, 3235.
3. Ohmichi, T. *et al.*, (2002) *Proc. Natl. Acad. Sci.* **USA** 99 (1), 54.
4. Daubendiek, S.L. *et al.*, (1997) *Nat. Biotechnol.* **15**, 273.
5. Marras, S.A. *et al.*, (2004) *Nucl. Acids Res.* **32**, e72.

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