Preparation of Custom Synthesized RNA Transcript Standard

Version 3

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Abstract

This protocol is from:

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Please see the full chapter for additional details.

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Guidelines

Required materials

• Equipment: 4 degrees Celsius microcentrifuge, 10-, 20-, 200-, and 1000-uL pipettes, water bath, 37 degrees Celsius shaking incubator, thermocycler, gel electrophoresis equipment and reagents, microfluidic electrophoresis instrument or fluorometry-based instrument for measuring nucleic acid concentration.

• Media: LB agar, LB agarþampicillin (100 ug/mL final concentration), LB medium, LB mediumþampicillin (100 ug/mL final concentration), SOC medium (2% tryptone, 0.5% yeast extract, 10 mM sodium chloride, 10 mM magnesium sulfate, 20 mM glucose).

• Bacterial cell line: One Shot® Top10 Chemically Competent Escherichia coli (Life Technologies, Grand Island, NY).

• TemplateDNA: Custom synthesizedDNAtemplate (T7 RNA polymerase promoter, internal standard sequence, unique restriction site) inserted into a plasmid.

• Restriction digest and end repair: Restriction enzyme matching unique restriction site and corresponding buffers, mung bean nuclease for end repair on digests that do not produce blunt ends.

• Commercially available kits: Ambion MEGAscript® T7 Kit (Life Technologies), Quant-iT™ RiboGreen® RNA Assay Kit (Life Technologies), miniPrep plasmid extraction kit.

• Other reagents: phenol:chloroform:isoamyl alcohol (24:24:1, pH 7), citrate-saturated phenol:chloroform:isoamyl alcohol (24:24:1, pH 4.7), sterile 2-propanol, ice cold 70% ethanol, nuclease-free 3M sodium acetate, nuclease-free TE buffer, sterilized 100% glycerol; 1% agarose gel,

nuclease-free water, RNaseZap® (Life Technologies).

• Disposables: nuclease-free 10-, 20-, 200-, and 1000-uL filter tips, nuclease-free PCR tubes, nuclease-free microcentrifuge tubes, gloves.

Plasmid amplification and stock preparation

Resuspension of plasmid DNA

If beginning with lyophilized plasmid DNA, spin briefly to ensure the contents are at the bottom of the tube. Resuspend the plasmid DNA in a volume of TE buffer to produce a stock concentration of 0.1 ug/uL. To prepare a working solution, add 1 uL of the stock solution to 99 uL of nuclease-free water to produce a final concentration of 1 ng/uL. The resuspended plasmid DNA can be stored at -20 degrees Celsius.

Chemical transformation of plasmid into Top10 E. coli cells

Prior to beginning the transformation, ensure that all required media are prepared and sterilized. Place frozen competent cells and a prelabeled tube on ice. Prewarm a hot water bath to 42 degrees Celsius. To a tube on ice, add 2 uLof (~2 ng) the plasmid working solution to 100 uL of thawed competent cells and flick the tube gently to mix. Incubate the mixture for 30 min on ice, then heat shock in the 42 degrees Celsius hot water bath for 45 s. Immediately place the tube on ice for 2 min and then add 500 uL of SOC or LB liquid medium to the tube and incubate at 37 degrees Celsius for 1 h with shaking (~225 rpm). During this time prewarm LB-Amp agar plates in a 37 degrees Celsius incubator. From the tube, pipet and spread 10, 100, and 200 uL on three separate LB-Amp agar plates. Place the plates upside down in a 37 degrees Celsius incubator for 12â□□24 h. Following incubation, inoculate a single, well-isolated colony from one of the plates and place into 5-mL LB-Amp media. Grow the liquid culture at 37 degrees Celsius for ~8h with vigorous shaking (~300 rpm). Remove 850 uL of the starter culture and place into a 2-mL freezer vial with 150 uL of sterilized 100% glycerol, mix thoroughly, and store at -80 degrees Celsius. To work from the frozen stocks, place a loopful of stock into 10 mL of LB-Amp liquid medium and grow at 37 degrees Celsius with vigorous shaking (~300 rpm) for 12â□□16 h. Harvest cells by centrifugation at 6000 x g for 15 min at 4 degrees Celsius. Discard the supernatant and recover the plasmid DNA using a commercially available plasmid mini-prep kit.

Plasmid linearization and in vitro transcription

Linearization of plasmid template

Digest 2 ug of plasmid with restriction enzyme targeting the site at the end of the template sequence according to the restriction enzyme protocol. Sticky ends created by nonblunt-end cutting enzymes should be removed using mung bean nuclease. After digestion and end repair, bring the reaction to 100 uL by adding TE buffer, add 100 uL of phenol:chloroform:isoamyl alcohol (25:24:1, pH 7), and mix by vortexing. Spin the mixture for 5 min at 12,000 x g. Transfer the aqueous phase to a new tube and add 0.1 volumes (10 uL) of 3M sodium acetate and 0.7 volumes (70 uL) of isopropanol to the tube.Mix thoroughly and incubate for 10 min at room temperature, and centrifuge for 30 min at 12,000 x g at 4 degrees Celsius. Discard supernatant and wash pellet with 200 uL of ice cold 70% ethanol. Centrifuge for 5 min and discard the supernatant, being careful not to disturb the pellet. Air-dry the pellet to remove residual ethanol before resuspending the pellet in 5 uL of nuclease-free water. Transfer 2 uL of linearized plasmid into a newtube and add 2 uL of nuclease-free water. Use 1 uL of the diluted sample to check the concentration and analyze the remaining 3 uL on a 1% agarose gel to check for

complete digestion and the presence of a single-sized product. Retain the 3 uL of undiluted DNA template for subsequent steps.

Synthesis and purification of mRNA internal standard
Synthesis of the internal standards from a template containing a T7 promoter is completed through the use of an in vitro transcription reaction using the Ambion MEGAscriptÂ® High Yield T7 Kit. In a 0.2-mL tube at room temperature, combine 2 uL of ATP solution, 2 uL of CTP solution, 2 uLof GTP solution, 2 uL of UTP solution, 2 uL of 10x reaction buffer, 1 ug of linearized template DNA (up to 8 ul), and 2 uL of enzyme mix, and bring the total reaction volume to 20 uL with nuclease-free water. Mix thoroughly by flicking and incubate themixture at 37 degrees Celsius in a thermocycler with a heated lid for 16 h. Degrade the plasmid DNA by adding 1 uL of Turbo DNAse to the reaction tube and incubating for 15 min at 37 degrees Celsius. Add 20 uL of citrate-saturated (pH 4.7) phenol:chloroform:isoamyl alcohol (25:24:1) to the tube. Vortex the mixture for 1 min and centrifuge for 2 min at 12,000 x g to separate the phases. Transfer the upper aqueous phase to a fresh tube and add 1 volume of chloroform:isoamyl alcohol (24:1). Vortex the mixture for 1 min and centrifuge for 2 min at 12,000 x g. Transfer the upper aqueous phase to a fresh tube and add 0.1 volumes of 3M sodium acetate and 0.7 volumes of isopropanol. Mix by vortexing and incubate for 10 min at roomtemperature and then centrifuge for 30 min at 4 degrees Celsius. Carefully discard the supernatant and wash the pelletwith 200 uL of ice cold 70% ethanol. Centrifuge for 5 min and carefully remove the supernatant without disturbing the pellet. Air-dry the pellet until no residual ethanol remains and resuspend the dried pellet in 50 uL of nuclease-free water. Quantify the RNA fluorometrically using a QuantiTâ□¢ RiboGreenÂ® RNA Assay Kit and check the transcript size using a microfluidic electrophoresis instrument (e.g., Experion Automated Electrophoresis System, Agilent 2100 Bioanalyzer, or Agilent 2200 TapeStation). Store the mRNA internal standard stock at -80 degrees Celsius.

Materials

One Shot® TOP10 Chemically Competent E. coli C4040-10 by Thermo Scientific

MEGAscript® T7 Transcription Kit AM1334 by Thermo Scientific

Quant-iT™ RiboGreen® RNA Assay Kit R11490 by Thermo Scientific

RNaseZap® AM9780 by Thermo Scientific

Protocol

Resuspension of lyophilized plasmid DNA

Step 1.
Briefly spin the tube to ensure the contents are on the bottom

Resuspension of lyophilized plasmid DNA

Step 2.
Resuspend the sample in a volume of TE Buffer resulting in a stock concentration of 0.1 ug/uL

Resuspension of lyophilized plasmid DNA

Step 3.
To prepare a working solution add 1 uL of the stock solution to 99 uL of water to reach a concentration of 1.0 ng/uL

Resuspension of lyophilized plasmid DNA

Step 4.

Store resuspended DNA at -20 °C

Chemical transformation of plasmid into Top10 E. coli cells

Step 5.

Place frozen competent cells and pre-labeled tubes on ice

NOTES

Christa Smith 29 Jul 2016

Prior to beginning the transformation, ensure that all required media are prepared and sterilized.

Chemical transformation of plasmid into Top10 E. coli cells

Step 6.

Pre-warm a hot water bath to 42 °C

Chemical transformation of plasmid into Top10 E. coli cells

Step 7.

In a tube on ice add 2 uL (2 ng) of plasmid working solution to 100 uL of thawed competent cells, flick the tubes to gently mix

Chemical transformation of plasmid into Top10 E. coli cells

Step 8.

Incubate the mixture on ice for 30 minutes

DURATION

00:30:00

Chemical transformation of plasmid into Top10 E. coli cells

Step 9.

Heat shock the tubes for 45 seconds in the 42 °C bath

DURATION

00:00:45

Chemical transformation of plasmid into Top10 E. coli cells

Step 10.

Immediately place the tubes on ice for 2 minutes

DURATION

00:02:00

Chemical transformation of plasmid into Top10 E. coli cells

Step 11.

Add 500 uL of SOC or LB to each tube and incubate the tubes at 37°C for 1 hour with shaking (225 rpm)

DURATION

01:00:00

Chemical transformation of plasmid into Top10 E. coli cells

Step 12.

Pre-warm agar plates to 37°C in incubator

Chemical transformation of plasmid into Top10 E. coli cells

Step 13.

Take 10 uL, 100 uL, and 200 uL and spread on LB-Amp agar plates

Chemical transformation of plasmid into Top10 E. coli cells

Step 14.

Place the plates upside down in 37°C incubator for 12-24 hours

DURATION

12:00:00

Chemical transformation of plasmid into Top10 E. coli cells

Step 15.

Inoculate a 5 uL liquid LB-Amp culture by picking a single well-isolated colony from the LB-Amp plate grown overnight

Chemical transformation of plasmid into Top10 E. coli cells

Step 16.

Grow liquid culture at 37°C for 8 hours with vigorous shaking (300 rpm)

DURATION

08:00:00

Chemical transformation of plasmid into Top10 E. coli cells

Step 17.

Remove 850 uL of starter culture and place into a 2 mL freezer vial with 150 uL of sterilized 100% glycerol, mix thoroughly, and store at -80 °C

Chemical transformation of plasmid into Top10 E. coli cells

Step 18.

Dilute the starter culture 1/500 to 1/1000 into a larger volume of LB-Amp

Chemical transformation of plasmid into Top10 E. coli cells

Step 19.

Grow the culture at 37°C with vigorous shaking (300 rpm) for 12-16 hours

DURATION

12:00:00

Chemical transformation of plasmid into Top10 E. coli cells

Step 20.

Harvest the culture by centrifugation at 6000 x g for 15 min at 4°C

DURATION

00:15:00

Chemical transformation of plasmid into Top10 E. coli cells

Step 21.

Discard supernatant

Chemical transformation of plasmid into Top10 E. coli cells

Step 22.

Recover plasmid DNA using a commercial plasmid mini-prep and the corresponding protocol

Linearization of plasmid (2 ug)

Step 23.

In a PCR tube combine in the order listed:

12.8 uL Nuclease-free water
2.00 uL NEBuffer 4 (10X)
0.20 uL BSA (100X)
4.00 uL Plasmid DNA (0.5 ug/uL)

Linearization of plasmid (2 ug)

Step 24.

In a thermocycler incubate the mixture for 1 hour at 37 °C

DURATION

01:00:00

Linearization of plasmid (2 ug)

Step 25.

Inactivate the enzyme by incubating at 65 °C for 20 minutes

DURATION

00:20:00

Extract the linearized plasmid DNA

Step 26.

Bring the reaction to 100 uL by adding TE buffer

Extract the linearized plasmid DNA

Step 27.

Add 100 uL of Phenol:Chloroform:Isoamyl alcohol (25:24:1)

Extract the linearized plasmid DNA

Step 28.

Vortex Centrifuge for 5 minutes at 11,000 rpm

DURATION

00:05:00

Extract the linearized plasmid DNA

Step 29.

Transfer the aqueous phase to a new tube

Extract the linearized plasmid DNA

Step 30.

Add 0.1 volumes (10 uL) of 3M Sodium Acetate and 0.7 volumes (70 uL) of Isopropanol to the aqueous phase

Extract the linearized plasmid DNA

Step 31.

Mix and incubate for 10 minutes at room temperature

DURATION

00:10:00

Extract the linearized plasmid DNA

Step 32.

Spin for 30 minutes at 11,000 rpm at 4 degrees Celsius

DURATION

00:30:00

Extract the linearized plasmid DNA

Step 33.

Discard the supernatant

Extract the linearized plasmid DNA

Step 34.

Wash the pellet with 200 uL of ice cold 70% ethanol

Extract the linearized plasmid DNA

Step 35.

Centrifuge for 5 minutes

DURATION

00:05:00

Extract the linearized plasmid DNA

Step 36.
Air dry the pellet

Extract the linearized plasmid DNA

Step 37.
Resuspend in 5 uL of nuclease-free water

Extract the linearized plasmid DNA

Step 38.
Transfer 2 uL of linearized plasmid prep into a new tube

Extract the linearized plasmid DNA

Step 39.
Add 2 uL sterile water to the 2uL sample

Extract the linearized plasmid DNA

Step 40.
Use 1 uL of the diluted sample to check the concentration of the plasmid prep

Extract the linearized plasmid DNA

Step 41.
Run remaining 3 uL of diluted plasmid prep on a 1% agarose gel to check for complete digestion

In vitro transcription

Step 42.
In a tube combine the following at room temperature (Ambion MEGAscript T7 kit):

PROTOCOL

. In vitro transcription mixture, Moran Lab

CONTACT: Christa Smith

Step 42.1.
2.00 uL ATP Solution

Step 42.2.
2.00 uL CTP Solution

Step 42.3.
2.00 uL GTP Solution

Step 42.4.
2.00 uL UTP Solution

Step 42.5.
2.00 uL 10X Reaction Buffer

Step 42.6.
0.1 - 1.00 ug Linearized Vector

Step 42.7.
2.00 uL Enzyme mix

Step 42.8.
X.X uL Nuclease-free water to 20 uL Total Volume

In vitro transcription

Step 43.
Mix Thoroughly by flicking

In vitro transcription

Step 44.
Incubate mixture at 37 degrees Celsius for 16 hours

DURATION
16:00:00

DNase treatment to remove the DNA template

Step 45.
Add 1 uL Turbo DNase to a concentration

DNase treatment to remove the DNA template

Step 46.
Incubate for 15 minutes at 37° Celsius

DURATION
00:15:00

DNase treatment to remove the DNA template

Step 47.
Extract with 1 volume of citrate-saturated (pH 4.7) phenol:chloroform:isoamyl alcohol (25:24:1)

DNase treatment to remove the DNA template

Step 48.
Vortex for 1 minute

DURATION
00:01:00

DNase treatment to remove the DNA template

Step 49.
Centrifuge for 2 minutes at 12,000 x g

DURATION
00:02:00

DNase treatment to remove the DNA template

Step 50.
Transfer the upper, aqueous phase to a fresh tube

DNase treatment to remove the DNA template

Step 51.
Add 1 volume chloroform:isoamyl alcohol (24:1)

DNase treatment to remove the DNA template

Step 52.
Vortex for 1 minute

DURATION
00:01:00

DNase treatment to remove the DNA template

Step 53.
Centrifuge for 2 minutes at 12,000 x g

DURATION
00:02:00

DNase treatment to remove the DNA template

Step 54.
Transfer the upper aqueous phase to a fresh tube

DNase treatment to remove the DNA template

Step 55.

Add 0.1 volumes of 3.0 M sodium acetate and 0.7 volumes of isopropanol

DNase treatment to remove the DNA template

Step 56.

Mix and incubate at room temperature for 10 minutes

DURATION

00:10:00

DNase treatment to remove the DNA template

Step 57.

Centrifuge for 30 minutes at 4 °C

DURATION

00:30:00

DNase treatment to remove the DNA template

Step 58.

Carefully discard supernatant

DNase treatment to remove the DNA template

Step 59.

Wash pellet with 200 uL of 70% ethanol

DNase treatment to remove the DNA template

Step 60.

Dry the pellet under vacuum

DNase treatment to remove the DNA template

Step 61.

Resuspend the RNA pellet in 50 uL of water

DNase treatment to remove the DNA template

Step 62.

Store at -70° Celsius

DNase treatment to remove the DNA template

Step 63.

Quantify RNA using nanodrop

DNase treatment to remove the DNA template

Step 64.

Check transcript size using Experion/Bioanalyzer