Protocol to add 5’ cap structures to exogenous synthetic RNA references (spike-ins)

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Contact information

Sílvia Carbonell Sala
Bioinformatics & Genomics Program
Center for Genomic Regulation, PRBB
Dr. Aiguader, 88 - 08003 Barcelona - Catalonia, Spain
Email: silvia.carbonell@crg.eu

Julien Lagarde
Bioinformatics & Genomics Program
Center for Genomic Regulation, PRBB
Dr. Aiguader, 88 - 08003 Barcelona - Catalonia, Spain
Email: julien.lagarde@crg.eu

Roderic Guigó Serra
Bioinformatics & Genomics Program
Center for Genomic Regulation, PRBB
Dr. Aiguader, 88 - 08003 Barcelona - Catalonia, Spain
Email: roderic.guigo@crg.eu

Overview

Exogenous synthetic RNA references (spike-ins) are widely used to calibrate measurements in RNA assays, such as gene expression microarray experiments, RT-qPCR, and RNA-Seq[1,2]. Synthetic spike-ins such as SIRV (Spike-In RNA Variants) and ERCC (External RNA Controls Consortium) mixes lack the **7-Methylguanosine** (**m^7G**) cap structure that most natural eukaryotic RNA transcripts bear at their 5’ end. As a result, in their native form, commercial spike-in mixes are unsuitable for library preparation protocols involving 5’ cap enrichment steps. This protocol describes a method to add 5’ m^7G caps to RNA spike-ins to address this issue. The preparation can then be used as external reference when producing full-length cDNA libraries that rely on the presence of m^7G cap structures.

Briefly, the pp5'N structure present at the 5' end of spike-in sequence is used by the vaccinia capping enzyme to add 7-methylguanylate cap structures[3,4]. After the capping reaction, Agencourt RNAClean XP magnetic beads are used to effectively remove the protein components of the capping reaction (enzyme) from the spike-ins before their use.

General considerations

The protocol aims to obtain spike-ins that will be used in RNA experiments, making it crucial to obtain a final product free of RNAses. Usual recommendations for handling RNA should be followed (*e.g.* wear latex or vinyl gloves while handling reagents). Gloves must be changed frequently and the tubes should be closed whenever possible. All buffers and solutions need to be prepared using nuclease-free water (UltraPure DNase/RNase-Free Distilled Water) and molecular grade reagents. All reaction mixes are prepared using 1.5 - 2 ml RNAse free tubes.

The SIRV-Set 4 (Iso Mix E0 / ERCC / Long SIRVs LOT; numbers: 013760 and 013761) kit was used as a template for the capping reaction. More information regarding SIRV kit sets at: https://www.lexogen.com/sirvs/sirv-sets/.

Description of the concentrations for the SIRV set 4 and considerations:

- The kit contains 1, or 3, tube(s) (Cat. No 141.01 and 141.03 respectively) with SIRV isoform mix E0 combined with ERCC mix and the long SIRVs.
- Each separate tube of SIRVs has a volume of 10 µl and contains 53.5 ngs.
- SIRV isoform mix E0 is present at a uniform concentration of 60 amoles/µl for each of the separate SIRV transcripts.
- long SIRV set is also present at 60 amoles/µl for each long SIRV transcript
- ERCC set of transcripts is present in its usual range of concentrations from 1500 amoles/uL to 1.4 X 10-3 amoles/µl.

The Vaccinia Capping System from New England BioLabs (M2080S) was used to add m7G cap structures to the 5′ end of SIRV-Set 4 (Iso Mix E0 / ERCC / Long SIRVs) spike-ins from Lexogen. The manufacturer's recommendations were followed to set up the reaction. The New England BioLabs protocol is designed to cap up to 10 µg of RNA (100 nt or larger) in a 20 µl reaction. Reaction size can be scaled up as needed.

The protocol uses harmful chemicals. Pay special attention to always wear a suitable lab coat, disposable gloves, and protective goggles, and follow appropriate regulations when handling chemicals and disposing of waste.

Protocol

Capping reaction

A total of ten vials of SIRV-Set 4 were used to perform the capping reaction. The mass of all 10 SIRV sets is 53.5 X 14 = 535 ng. This easily fits within the mass range for the NEB Vaccinia capping protocol (max mass = 10 µg).

Take all SIRV-Set 4 vials and mix them together to generate a 100 µl pool to make things more homogeneous as possible.

Denature the spike-ins by combining them with 3.5 µl of RNAse inhibitor and 1.5 µl of UltraPure DNase/RNase-Free Distilled Water in a 1.5 ml microfuge tube to a final volume of 105 µl. Use a thermal block to incubate the mixture at 65°C for 5 minutes. Immediately place the tube on ice for 5 minutes.

Prepare the capping reaction by adding the following components in the order specified:

	µl 1x
Denatured Spike-ins (from above)	105
10X Capping Buffer	14
GTP (10 mM)	7
SAM (2 mM, dilute 32 mM stock to 2 mM)	7
Vaccinia Capping Enzyme	7
Total	140

Incubate at 37°C for 2 hours.

Capping reaction purification

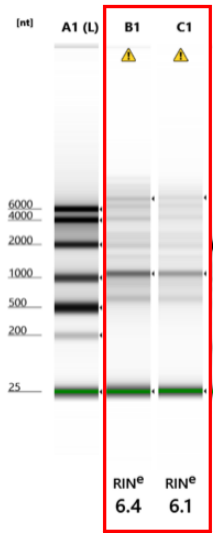
After the capping incubation step proceed immediately to purify the sample by using 1.8x AMPure RNA Clean XP beads, incubate the sample for 5 min at room temperature (RT) using the huLa mixer, quick spin, place the sample on the magnet and wait for the solution to clear, remove the supernatant, wash twice with freshly prepared 70% ethanol solution without disturbing the beads, air dry for 1 min, resuspend the sample in 102 µl of nuclease-free water, incubate at 37ºC for 5 minutes in a thermo block with gentle rotation, place the sample on the magnet and wait for the solution to clear, recover 100 µl of supernatant and move into a clean tube.

The final sample is quantified with qBIT and quality checked with TapeStation:

1. Check the ratio of SIRV-Set 4 capped vs uncapped using a Qubit quantitation platform. – Use 1 µl of the capped purified sample and 1 µl of uncapped stock solution to measure the SIRV-Set 4 concentration using a Qubit fluorometer and Quanti-it RNA assay kit according to the manufacturer's recommendations.

   NOTE from Lexogen technical support: SIRV-Sets contain a stabilizing agent which may interfere with the Nanodrop measurement and produce a slightly different concentration when it is not also used in the blank. (We cannot disclose what it is or provide a blank). Therefore, Lexogen does not advise to remeasure the concentration by Nanodrop.

2. Use 1 µl of capped and 1 µl of uncapped sample for SIRV-Set 4 sizing QC in Tapestation with High Sensitivity RNA kit.
   - Lane B1 uncapped SIRV-Set 4
   - Lane C1 capped SIRV-Set 4



Capped synthetic spike-in stocks are now ready for downstream applications. Prepare single-use Capped SIRV-Set 4 aliquots and store them at -80ºC until use.

Reagents, manufacturer and catalog number:

Name	Manufacturer	Catalog number
Agencourt RNAClean XP	Beckman Coulter Life Sciences	A63987
SIRV-Set 4 (Iso Mix E0 / ERCC / Long SIRVs)	Lexogen	141.0
Vaccinia Capping System (10,000 units/ml)	New England BioLabs	M2080S
RNasin Plus RNase Inhibitor	Promega	N2611
RNaseZap RNase Decontamination Solution	Sigma	R2020-250ML
UltraPure DNase/RNase-Free Distilled Water	Invitrogen	10977-035
Ethanol Absolute - Molecular biology grade	PanReac - AppliChem	A8075,1000PE
High Sensitivity RNA ScreenTape (Tape Station)	Agilent Technologies	5067-5579
High Sensitivity RNA ScreenTape Ladder (Tape Station)	Agilent Technologies	5067-5581
High Sensitivity RNA sample buffer (Tape Station)	Agilent Technologies	5067-5580
Quanti-it RNA assay kit	Life Technologies	Q32852
Tube 0.2 ml PCR DNAse/RNAse free	Any brand	-
Tube 1.5 ml LoBind DNAse/RNAse free	Any brand	-
Tube 1.5 safe lock DNAse/RNAse free	Any brand	-
Tube 2 ml DNAse/RNAse free	Any brand	-
10 µL (0.1 - 10 µL) LongReach Barrier DNAse/RNAse	Any brand	-
1000 µL (100-1000 µL) Barrier DNAse/RNAse	Any brand	-

20 µL (1-20 µL) Barrier DNAse/RNAse	Any brand	-
200 µL (1-200 µL) Barrier DNAse/RNAse	Any brand	-

Equipment:

These pieces of equipment are used in our laboratory. You can use any equivalent you have.

Name	**Manufacturer**
Agilent 4200 TapeStation	Agilent Technologies
Qubit 4 Fluorometer	Thermo Fisher Scientific
MJ Research PTC-100 Thermal Cycler	MJ Research
MiniSpin (Centrifuge)	Labnet
MiniSpin (Centrifuge for 8strip tube strips)	Labnet
IKA MS3 Basic Vortex	IKA
HuLa mixer	Invitrogen
DynaMag-2 (Magnetic Particle Concentrator)	Invitrogen
Thermomixer Compact	Eppendorf
PIPETMAN Classic, P10	Gilson
PIPETMAN Classic, P1000	Gilson
PIPETMAN Classic, P2	Gilson
PIPETMAN Classic, P20	Gilson
PIPETMAN Classic, P200	Gilson

References

1. Yang, I. V. Use of external controls in microarray experiments. *Methods Enzymol.* **411**, 50–63 (2006).
2. Jiang, L. *et al.* Synthetic spike-in standards for RNA-seq experiments. *Genome Res.* 21, 1543–51 (2011).
3. Guo, P. X. & Moss, B. Interaction and mutual stabilization of the two subunits of vaccinia virus mRNA capping enzyme coexpressed in Escherichia coli. *Proc. Natl. Acad. Sci. U. S. A.* 87, 4023–4027 (1990).
4. Mao, X. & Shuman, S. Intrinsic RNA (guanine-7) methyltransferase activity of the vaccinia virus capping enzyme D1 subunit is stimulated by the D12 subunit. Identification of amino acid residues in the D1 protein required for subunit association and methyl group transfer. *J. Biol. Chem.* 269, 24472–24479 (1994).