Quick-Start Protocol March 2015

REPLI-g® Single Cell RNA Library Kit

The REPLI-g Single Cell RNA Library Kit (cat. no. 150073) should be stored immediately upon receipt at –30°C to –15°C. If stored under these conditions, the kit is stable until the date indicated on the QC label inside the kit lid.

Further information

- *REPLI-g Single Cell RNA Library Handbook*: **www.qiagen.com/handbooks**
- Safety Data Sheets: **www.qiagen.com/safety**
- Technical assistance: toll-free 00800-22-44-6000, or **www.qiagen.com/contact**

Notes before starting

- This protocol is for generating and amplifying cDNA from single cells and constructing sequencing libraries for Illumina® NGS platforms. Refer to the kit handbook for additional protocols, required materials and kits.
- High-molecular-weight cDNA generated by random extension of primers in no-template controls does not contain genetic information that affects downstream applications.
- Thaw all enzyme mixes and gDNA Wipeout Buffer on ice. All other components can be thawed at room temperature (15–25°C). Maintain an RNase-free working environment.
- Enzymatically or mechanically shear 5 µg amplified cDNA and start library prep with 1 µg sheared cDNA. Refer to the kit handbook for protocols and cleanup steps.
- Median fragment sizes depend on the applications and read length.
- Library enrichment is not required.

Procedure: Amplification of total RNA or 3′-enriched mRNA from single cells

1. Place 7 µl of cell material (suspended in PBS) into a microcentrifuge tube.

   Note: If using <7 µl of cell material, add H20 sc to bring the volume up to 7 µl.

2. Add 4 µl Lysis Buffer. Mix carefully by flicking the tube and centrifuge briefly. Avoid getting cell material stuck to the wall of the tube and prevent any contact with pipet tips.
3. Incubate at 24°C for 5 min, followed by 95°C for 3 min. Cool to 4°C.
4. Add 2 µl gDNA Wipeout Buffer. Mix by vortexing and centrifuge briefly.
5. Incubate at 42°C for 10 min.
6. Prepare Quantiscript RT mix according to Table 1 and mix. Add 6 µl freshly prepared Quantiscript RT mix to the lysed cell sample. Mix by vortexing and centrifuge briefly.

Table 1. Preparing Quantiscript RT mix*

Component	Volume/reaction*
RT/Polymerase Buffer	4 µl
Oligo dT Primer	1 µl
Quantiscript RT Enzyme Mix	1 µl
Total volume	**6 µl**

* Scale up accordingly if performing several reactions simultaneously.

7. Incubate at 42°C for 60 min. Incubate 95°C for 3 min to stop the reaction, then cool on ice.
8. Prepare the ligation mix by adding the components as shown in Table 2. Add 10 µl of freshly prepared ligation mix to the Quantiscript RT reaction mix reaction from step 7. Mix by vortexing and centrifuge briefly.

Table 2. Preparing ligation mix*

Component	Volume/reaction*
Ligase Buffer	8 µl
Ligase Mix	2 µl
Total volume	**10 µl**

* Scale up accordingly if performing several reactions simultaneously.

9. Incubate at 24°C for 30 min. Stop the reaction by incubating at 95°C for 5 min.
10. Prepare the REPLI-g SensiPhi amplification mix (Table 3). Add 30 µl of freshly prepared REPLI-g SensiPhi amplification mix to the ligation reaction from step 9. Mix by vortexing

and centrifuge briefly.

Table 3. Preparing REPLI-g SensiPhi amplification mix*

Component	Volume/reaction*
REPLI-g sc Reaction Buffer	29 µl
REPLI-g SensiPhi DNA Polymerase	1 µl
Total volume	**30 µl**

* Scale up accordingly if performing several reactions simultaneously.

11. Incubate at 30°C for 2 h. Stop the reaction by incubating at 65°C for 5 min. If not being used directly, store the amplified cDNA (>100 ng/µl) at –30°C to –15°C.

12. Amplified cDNA behaves like purified genomic DNA and has an approximate length of 2000 bp up to 70,000 bp. See the kit handbook for QC of the WTA reaction.

Procedure: Library preparation

Note: High-quality cDNA is essential for obtaining good sequencing results. Residual traces of contaminants (salts or proteins) will degrade the DNA or decrease the efficiency of the enzyme activities necessary for optimal library preparation.

End repair

1. Prepare a reaction mix for end-repair on ice according to Table 4, dispensing the reagents into a PCR tube or the well of a PCR plate, and mix thoroughly.

Table 4. Reaction mix for end-repair

Component	Volume/reaction
DNA	1 µg
RNase-free water	Variable
End-Repair Buffer, 10x	2.5 µl
End-Repair Enzyme Mix	2 µl
Total reaction volume	**25 µl**

2. Program a thermocycler to incubate for 30 min at 25°C, followed by 20 min at 75°C to inactivate the enzyme.

A-addition

3. Prepare a reaction mix for A-addition according to Table 5, adding the components to the PCR tube containing the end-repaired DNA and mix.

Table 5. Reaction mix for A-addition

Component	Volume/reaction
End-repaired DNA (from step 2)	25 µl
A-Addition Buffer, 10x	3 µl
Klenow Fragment (3'→5' exo–)	3 µl
Total reaction volume	**31 µl**

4. Program a thermocycler to incubate for 30 min at 37°C, followed by 10 min at 75°C to inactivate the enzyme.

Adapter ligation

5. Prepare a reaction mix for adapter ligation according to Table 6, adding the components to the PCR tube containing DNA that has undergone end-repair and A-addition (step 4) and mix. Avoid any cross-contamination of adapters.

Table 6. Reaction setup for adapter ligation

Component	Volume/reaction
DNA from step 4 (has undergone end-repair and A-addition)	31 µl
Ligation Buffer, 2x	45 µl
GeneRead™ Adapter (ready to use)	2.5 µl*
T4 DNA Ligase	4 µl
RNase-free water	Variable
Total reaction volume	**90 µl**

* Alternatively, add the correct amount of adapter according to the supplier's directions.

6. Program a thermocycler without a heated lid to incubate for 10 min at 25°C.
7. Proceed with size selection of the library for adapter removal and QC of the library as described in the kit handbook.