Quick-Start Protocol June 2016

QIAseq FX Single Cell RNA Library Kit

The REPLI-g Single Cell RNA Library Kit (cat. no. 180733, 180735) should be stored immediately upon receipt at –15°C to –30°C. If stored under these conditions, kits are stable up to the date indicated on the QC label inside the kit lid.

Further information:

- *QIAseq FX Single Cell RNA Library Handbook*: **www.qiagen.com/HB-2139**
- Safety Data Sheets: **www.qiagen.com/safety**
- Technical assistance: **www.qiagen.com/support**

Notes before starting:

- This protocol is for the generation of cDNA from single cells and production of NGS libraries for RNA-Seq experiments. Refer to the kit handbook for additional information.
- Maintain RNAse-free environment, and thaw all enzyme mixes and gDNA Wipeout Buffer on ice. All other components can be thawed at room temperature (15–25°C).
- This protocol is optimized for eukaryotic single cells from species that lack a cell wall and for microdissected cells from frozen sections. It cannot be used for cells that are treated with formalin or other cross-linking agents.
- Library amplification is not required; this entire process is PCR-free.
- This kit is for use with Illumina® instruments.

Procedure: Amplification of 3′-enriched mRNA from Single Cells

1. Place 7 µl of cell material (1-1000 cells suspended in PBS) into a microcentrifuge tube.
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.
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, centrifuge and incubate at 42°C for 10 min.
5. Prepare Quantiscript RT mix according to Table 1 and mix well.

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.

6. Add 6 µl of freshly prepared Quantiscript RT to the lysed cell sample. Mix, centrifuge and incubate at 42°C for 60 min.
7. Stop the reaction by incubating at 95°C for 3 min, then cool on ice.
8. Prepare the ligation mix by adding the components as shown in Table 2.

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. Add 10 µl of freshly prepared ligation mix to the Quantiscript RT reaction from step 7. Mix, centrifuge briefly and incubate at 24°C for 30 min.
10. Stop the reaction by incubating at 95°C for 5 min.
11. Prepare the REPLI-g SensiPhi amplification mix (Table 3).

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.

12. Add 30 µl of freshly prepared REPLI-g SensiPhi amplification mix to the ligation reaction from step 10. Mix, centrifuge briefly and incubate at 30°C for 2 h.
13. Stop the reaction by incubating at 65°C for 5 min.

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

Procedure: PCR-Free Library Construction from Amplified cDNA

FX Single-Tube Fragmentation, End Repair and A-addition

1. Thaw all kit components on ice, program a thermocycler according to Table 4 and start the program. When the thermocycler block reaches 4°C, pause the program.

Table 4. Reaction mix for end-repair FX reaction cycling conditions

Step	Temperature	Incubation time
1	4°C	1 min
2	32°C	15 min *
3	65°C	30 min
4	4°C	Hold

* The insert size of the completed libraries is determined by the duration of step 2. Using 200–1000 ng input amplified cDNA, 15 min fragmentation time produces a fragment distribution of around 350 bp. For detailed recommendations, please refer to the QIAseq FX Single Cell RNA Library Handbook. Use a thermocycler with heated lid.

2. Prepare a WTA DNA dilution 200–1000 ng in 10 µl H_2O sc.
3. Prepare the FX Reaction Mix on ice according to Table 5 and mix by pipetting.

Table 5. FX Reaction Setup for >200 ng input WTA-DNA

Component	Volume/reaction*
FX Buffer, 10x	5 µl
H2O sc	20 µl
FX Enhancer	5 µl
FX Enzyme Mix	10 µl
Total reaction volume	**40 µl**

Note: Mix by pipetting and keep on ice.

4. Add 40 µl FX Reaction Mix to each WTA cDNA sample on ice and gently mix.
5. Briefly spin down the PCR plate/tubes and immediately transfer to the pre-chilled thermocycler (4°C) and resume the program. Once the fragmentation program is complete, transfer samples to ice. Proceed with the next step.

Adapter Ligation

6. Mix and spin down the adapter plate. Remove the protective adapter plate lid, pierce the foil seal and transfer 5 µl from one DNA adapter well to each sample. Track the barcodes used. Freeze unused adapters.
7. Prepare the Ligation master mix on ice according to Table 6. Mix by gently mixing.

Table 6. Ligation master mix (per sample)

Component	Volume/reaction*
DNA Ligase Buffer, 5x	20 µl
H_2O sc	15 µl
DNA Ligase	10 µl
Total reaction volume	**45 µl**

8. Add 45 µl master mix to each sample. Mix well and incubate at 20°C for 15 min.
9. Proceed immediately to adapter ligation cleanup (steps 10–18).
10. Add 80 µl resuspended Agencourt AMPure XP beads to each sample and mix.
11. Incubate for 5 min at room temperature.
12. Pellet the beads on a magnetic stand and carefully discard the supernatant.
13. Add 200 µl fresh 80% ethanol to each pellet on the magnetic stand and carefully discard the supernatant. Repeat this step once for a total of 2 ethanol washes.
14. Incubate on the magnetic stand for 5–10 min or until the beads are dry. Avoid over-drying since this may result in lower DNA recovery. Remove from the magnetic stand.
15. Elute by resuspending in 52.5 µl Buffer EB. Pellet beads on the magnetic stand. Carefully transfer 50 µl supernatant to a new PCR plate.
16. Add 50 µl of resuspended Agencourt AMPure XP beads to each sample and mix.
17. Follow steps 11–14 and continue to step 18.
18. Elute by resuspending in 26 µl Buffer EB. Pellet the beads on the magnetic stand. Carefully transfer 23.5 µl of supernatant into a new PCR plate. Store purified libraries at –20°C until ready for sequencing.

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