QIAGEN Supplementary Protocol

Whole Genome Amplification in 96-Wells from Single Cells using the REPLI-g® Single Cell Kit

This protocol describes whole genome amplification of single cell material in a 96-well format with the REPLI-g Single Cell Kit (96) using a fast, 3 hour, incubation time. It can be used with all species of, for example, vertebrates, bacteria (gram positive and gram negative), plants (without the cell wall), sorted cells, tissue culture cells, and cells. It results in up to 10 µg of amplified DNA per reaction.

Since the amplification success is dependent on the well diameter and volume-to-surface ratio, we strongly recommend using 96-well plates with cone-shaped wells and following the described reaction volumes.

IMPORTANT: Please read the handbooks supplied with the REPLI-g Single Cell Kit, paying careful attention to the “Safety Information” and “Important Notes” sections, before beginning this procedure. The REPLI-g Single Cell Kit is intended for molecular biology applications. This product is not intended for the diagnosis, prevention, or treatment of a disease.

Equipment and reagents

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

- REPLI-g Single Cell Kit (cat. no. 150345 for 96 reactions)
- Vortexer
- Centrifuge suitable for 96-well plates
- 96-well plates
- Pipets and pipet tips
- Heating block or water bath capable of reaching 56ºC
- Ice

Important points before starting

- This protocol is optimized for 96-well format, whole genome amplification using single cell material from all species of, for example, vertebrates, bacteria (gram positive and gram negative), plants (without the cell wall), sorted cells, tissue culture cells, and cells. The protocol cannot be used with fixated cells that are treated with formalin or other cross-linking agents (e.g., single cell samples obtained by laser microdissection from formalin-fixed, paraffin-embedded tissues).
- Samples of 1–1000 intact cells (e.g., human or bacterial cells) are optimal for 2 X 96 whole genome amplification reactions using the REPLI-g Single Cell Kit (96).
- Avoid DNA contamination of reagents by using separate laboratory equipment (e.g., pipets, filter pipet tips, reaction vials, etc.). Set up the REPLI-g Single Cell Kit reaction in a location free of DNA.
- REPLI-g sc DNA Polymerase should be thawed on ice (see step 7). All other components can be thawed at room temperature (15–25°C).
- Buffer D2 (denaturation buffer) should not be stored longer than 3 months.
- Small amounts of DNA (up to 5 µg) will be present in negative (no-template) controls because DNA is generated during the REPLI-g Single Cell Kit reaction by random extension of primer-dimers, generating high-molecular-weight product. This DNA will not affect the quality of the actual samples and will not give a positive result in downstream assays.

Things to do before starting

- Prepare Buffer DLB by adding 500 µl H_2O sc to the tube provided. Mix thoroughly and centrifuge briefly to dissolve.

  Note: Reconstituted Buffer DLB can be stored for 6 months at –15 to –30°C. Buffer DLB is pH-labile.
- All buffers and reagents should be vortexed before use to ensure thorough mixing.

Procedure

1. **Prepare sufficient Buffer D2 (denaturation buffer) for the total number of whole genome amplification reactions (Table 1).**

   Note: The total volume of Buffer D2 given in Table 1 is sufficient for 96 reactions. If performing fewer reactions, store residual Buffer D2 at –15 to –30°C. Buffer D2 should not be stored for longer than 3 months.

Table 1. Preparation of Buffer D2

Component	Volume*
DTT, 1M	13 µl
Reconstituted Buffer DLB†	143 µl
Total volume	156 µl

*Volumes given are sufficient for 96 reactions.

†Reconstitution of Buffer DLB is described in "Things to do before starting".

2. **Place 2 µl cell material (supplied with PBS) into each well of the 96-well plate. If using less than 2 µl of cell material, add PBS sc to bring the volume up to 2 µl.**

   Note: The amount of PBS sc supplied with the REPLI-g Single Cell Kit is insufficient to prepare serial dilutions of cell material.

3. **Add 1.5 µl Buffer D2 into each well and then seal the plate using a tape sheet. Centrifuge the plate briefly at 1000 rpm to ensure that the cell material and Buffer D2 are collected at the bottom of the wells.**

4. **Mix by a short vortexing step, then briefly centrifuge again at 1000 rpm.**

   Ensure that after centrifugation the cell material and Buffer D2 are collected at the bottom of the wells.

5. **Incubate at 65°C for 10 min. Cool down to 4°C.**

   Note: If a thermal cycler is used, the temperature of the heating lid should be set at 70°C to avoid evaporation. Alternatively, incubation can be performed in a water bath.

6. **Briefly centrifuge at 1000 rpm to ensure that cell material and Buffer D2 are collected at the bottom of the well.**

7. **Remove the tape sheet and add 1.5 µl Stop Solution. Seal the plate, centrifuge briefly, and store on ice.**

   Ensure that after centrifugation the lysed cell material and Stop Solution are collected at the bottom of the wells.

8. **Thaw REPLI-g sc DNA Polymerase on ice. Thaw all other components at room temperature, vortex, and then centrifuge briefly.**

   The REPLI-g sc Reaction Buffer may form a precipitate after thawing. The precipitate will dissolve by vortexing for 10 s.

9. **Prepare a master mix according Table 2. Mix and centrifuge briefly.**

   IMPORTANT: Add the master mix components in the order listed in Table 2. After the addition of water and REPLI-g sc Reaction Buffer, briefly vortex and centrifuge the mixture before adding REPLI-g sc DNA Polymerase. The master mix should be kept on ice and used immediately upon addition of REPLI-g sc DNA Polymerase.

10. For each reaction, add 20 µl master mix to 5 µl denatured DNA (from step 6). Seal the plate with a tape sheet and centrifuge briefly at 1000 rpm.

Table 2. Preparation of master mix

Component	Volume*
H_2O sc	450 µl
REPLI-g sc Reaction Buffer	1450 µl
REPLI-g sc DNA Polymerase	100 µl
Total volume	2000 µl

* To prepare a master mix for multiple reactions, scale up accordingly for the number of reactions and add 10%.

11. Mix by a short vortexing step and centrifuge again briefly.

Ensure that after centrifugation the lysed cell material and REPLI-g sc master mix are collected at the bottom of the wells.

12. Incubate at 30°C for 3 h.

After incubation at 30°C, heat the water bath or heating block up to 65°C if the same water bath or heating block will be used in step 12.

Note: If a thermal cycler is used with a heated lid, the temperature of the lid should be set to 70°C.

13. Inactivate REPLI-g sc DNA Polymerase at 65°C for 3 min.

14. If not using immediately, store amplified DNA at 4–8°C for short-term storage. For long- term storage, store at –15 to –30°C.

DNA amplified using the REPLI-g Single Cell Kit should be treated as genomic DNA with minimal freeze-thaw cycles. We therefore recommend storage of nucleic acids at a concentration of at least 100 ng/µl.

15. For further use in a variety of downstream applications, use the correct amount of REPLI-g single cell amplified DNA diluted in water or TE according to the manufacturer's instructions. If performing PCR analysis, dilute an aliquot of amplified DNA 1:25 and use 2 µl of diluted DNA for each PCR reaction.

16. **Amplified DNA behaves like purified genomic DNA and has an approximate length of 2000–7000 bp. It is highly suited for use in a variety of downstream applications, including next-generation sequencing, array CGH, and quantitative PCR.**

Note: Typical DNA yields are approximately 10 µg per 25 µl reaction and need to be diluted appropriately. Optical density (OD) measurements overestimate REPLI-g amplified DNA. Refer to Appendix A, page 21, of the *REPLI-g Single Cell Handbook* for an accurate method of quantifying REPLI-g amplified DNA without further purification. Alternatively, purify the reaction with the QIAamp® DNA Mini Kit using the protocol "*Purification of REPLI-g amplified DNA using the QIAamp DNA Mini Kit* "or purify the reaction with EtOH using the protocol "*Purification of DNA amplified using REPLI-g Kits*". Following purification, determine the amount of DNA by a standard OD measurement.

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