FACS sorting and genome amplification of single cells

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Abstract

This protocol describes fluorescence-activated single cell sorting using a MoFlo Astrios EQ cell sorter (Beckman Coulter) and whole genome amplification of single sorted cells in plate formats using the REPLI-g Single Cell kit (Qiagen).

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Guidelines

General cleanroom rules

FACS sorting and whole genome amplification is performed in a cleanroom. Protective clothing such as lab coats, shoe covers, are required for entering the room and additional protection with gloves, hair nets and mouth covers are needed when the plates are sorted into and further processed. Lab coats should be dedicated to a specific task/analysis i.e. sorting or whole genome amplification. The screening of the amplified material is done outside the cleanroom in a PCR hood dedicated to DNA work. To keep a clean environment, take off your gloves when using the computer or touch screen. For a more detailed description of the different steps and critical issues see the paper by Rinke et al 2014 (1).

Whole genome amplification

Whole genome amplification is performed as described for the “amplification of genomic DNA from single cells” in the REPLI-g Single Cell Handbook (2), with the exception that volumes are scaled down with a quarter of the original volume, hence 12.5 µl in total.

Lysis and neutralization reagents from the REPLI-g Single Cell kit are first added to each well of the sorted plate to facilitate genome amplification. The MDA master mix is then added, the plate is sealed with an optical sealing and the reaction is monitored every 15 minutes in real-time at 30 °C for 8 h using a DNA-binding dye (SYTO13) and an instrument suitable for fluorescent readings e.g. a PCR machine or a plate reader. Note that the REPLI-g Single Cell kit mastermix should not be UV-decontaminated as it is already treated to minimize contamination. Tubes, water, lysis and stop solution can however be UVed for 2 Joules before use.

Protocol

I. Fluorescence-activated cell sorting for single cell genomics

Step 1.

General

FACS sorting and whole genome amplification is performed in a cleanroom. Protective clothing such as lab coats, shoe covers, are required for entering the room and additional protection with gloves, hair nets and mouth covers are needed when the plates are sorted into and further processed. Lab coats should be dedicated to a specific task/analysis i.e. sorting or whole genome amplification. The screening of the amplified material is done outside the cleanroom in a PCR hood dedicated to DNA work. To keep a clean environment, take off your gloves when using the computer or touch screen. For a more detailed description of the different steps and critical issues see the paper by Rinke et al 2014 (1).

Sort plate preparation

Step 2.

- 1 µl of sterile filtered 1x PBS is pipetted to each well of a 96-well plate. The plate is covered by a clear sealing and then UV-treated for 2 Joule.
- Prepared plates are kept at 4°C until sorting the same day.

Staining of sample

Step 3.

- Thaw the sample on ice.
- Run the sample through a 70 µm cell strainer to get rid of cell aggregates.
- Run the unstained sample for sample analysis.
- Depending on the event rate (eps = events per second), the sample is diluted accordingly.
- Add SYBR Green I to a final concentration of 1x to the sample.
- Stain the sample in the dark and on ice for at least 20 min.
- During analysis of the stained samples the region of interest for sorting are chosen.
- Define the sort layout in the CyClone plate layout. Always include a few empty wells as negative controls and a few wells with more than 1 cell (e.g 10 cells) as positive controls.
- Clean the sort chamber and the work space with a decontamination solution, e.g. DNA AWAY®.

Running a clean single cell sort

Step 4.

- Take one plate, carefully remove the sealing.
- Place the plate into the sort chamber, pay attention that the plate is positioned correctly.
- Start the sample, then start the sort.
- Once the sort is finished, carefully take out the plate and immediately cover it with a sealing. Spin down, and transfer the plate to the freezer.
- Continue with the next plate.
- Sorted plates are kept at -20°C for short-term, or -80°C for long-term storage.

II. Cell lysis and whole genome amplification using MDA

Step 5.

Whole genome amplification is performed as described for the “amplification of genomic DNA from single cells” in the REPLI-g Single Cell Handbook (2), with the exception that volumes are scaled down with a quarter of the original volume, hence 12.5 µl in total.

Lysis and neutralization reagents from the REPLI-g Single Cell kit are first added to each well of the sorted plate to facilitate genome amplification. The MDA master mix is then added, the plate is sealed with an optical sealing and the reaction is monitored every 15 minutes in real-time at 30 °C for 8 h using a DNA-binding dye (SYTO13) and an instrument suitable for fluorescent readings e.g. a PCR machine or a plate reader. Note that the REPLI-g Single Cell kit mastermix should not be UV-decontaminated as it is already treated to minimize contamination. Tubes, water, lysis and stop solution can however be UVed for 2 Joules before use.

General cleanroom rules and cleaning procedure

Step 6.

- Always wear shoe covers when entering the cleanroom.
- Use a dedicated lab coat, and only wear it inside the cleanroom, use it at all times.
- Use hair net and mouth cover when you set up the MDA reaction.
- Use fresh gloves each time you re-enter the cleanroom, use gloves for every single step.
- Wipe all working areas and pipets with DNA AWAY®.
- Clean your gloves and everything that goes into the MDA hood with DNA AWAY®.

General considerations

Step 7.

To minimize contamination

- Change tips between every well (or rows/columns if you use a multichannel pipette).
- Mix and spin down all reagents before use to avoid possible drops in the lid coming in contact with your gloves.
- Open sorted plates only in the PCR hood.
- Include negative controls such as “no sort controls” and no template controls if possible, to keep track of potential contamination.

Reagent handling

- Keep all reagents on ice after thawing or preparation. The polymerase should be kept in a cooling block.
- After adding the nucleic binding dye SYTO13, protect the MDA mastermix from light using foil.

Preparation for MDA

Step 8.

- Clean the hood working area with DNA AWAY®.
- Wipe pipettes with DNA AWAY®.
- Wipe all equipment that you will need, including enough pipette tips, with DNA AWAY® and place them in the hood.
- Turn on the UV in the hood for 20 to 30 min.
- Fill an ice-box with ice and place a lid wrapped in foil on top of it, fill this lid with ice (will be used for the UV-linker).
- Fill an ice-box with ice for the reagents later on.

Prepare the lysis, stop solution and MDA master mix

Step 9.

- Thaw plates on ice in an ice box
- Add lysis buffer (Buffer D2) in an 8-well strip and close with an 8-cap lid. Do the same for the stop solution.
- Mix all reagents for the MDA, including the SYTO13 dye, and distribute it in an 8-well strip and close with an 8-cap lid. Protect the reaction from light.

Lysis and neutralization

Step 10.

- Add 0,75 µl lysis reagents on the rim of the wells in each row using a multichannel pipette.
- Close the plate with a sealing and spin down.
- Put the plate in a thermocycler at 65° C for 10 min.
- Add 0,75 µl stop solution on the rim of the in each row using a multichannel pipette.
- Close the plate with sealing and spin down.

MDA

Step 11.

- Add 10 µl of MDA-mix reagents on the rim of the wells in each row using a multichannel pipette.
- Close the plate with a sealing and spin down.
- Put the plate in a qPCR machine or a plate reader and incubate the plate at 30 °C for 8 h while reading the fluorescence every 15 minutes.
- The reaction is inactivated at 3 min 65 °C

Screening of amplified material

Step 12.

Screening of whole genome amplification (WGA) products is done using the ROCHE lightCycler 480 and the ROCHE kit LightCycler 480 SYBR Green I Master in 10 ul reactions. Dilute PCR products x 100 and add 2 ul of the DNA to the PCR-mix including the forward and reverse primer. Add a melting curve analysis after the PCR cycling for evaluation of positive reactions by comparing Tm-values of the samples and positive as well as negative controls.

Purification of PCR-products

Step 13.

Purified PCR-products or WGA can be quantified in a plate format using the Quant-IT PicoGreen® dsDNA Assay kit (Thermo Fisher Scientific) and a plate reader as described in the REPLI-g Single Cell handbook from Qiagen Appendix B (2).