



DROPLET DIGITAL PCR

Counting Genetically Modified Cells Using Whole Cell–Based Droplet Digital PCR (wc-ddPCR)

Protocol

This protocol is designed for counting cells by encapsulating whole cells and targeting the transgene and reference gene to amplify the DNA within. This application allows precise and accurate cell counts of an edited or modified cell population with minimal input cells. It is recommended that the end user validate the assay prior to use.

Materials

Note: Additional materials and equipment are needed to carry out the ddPCR portions of this protocol.

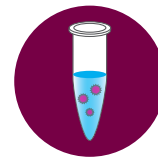
- ddPCR Enhancer Reagent A, 1,000 reactions (catalog #12013425)
- ddPCR Multiplex Supermix, 500 reactions (#12005910)
- RPP30 FAM Copy Number Assay, 1,000 reactions (#dHsaCP2500313)
- SOD1 HEX Copy Number Assay, 1,000 reactions (#dHsaCNS642774914)
- DTT, 2 ml (#12012171)
- Restriction enzymes HaeIII (3,000 units) or BsrI (1,000 units) (NEB catalog #R0108S or R0527S)

Equipment and Consumables

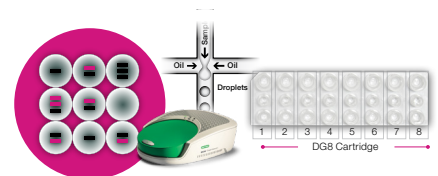
- QX200 Droplet Generator (#1864002)
- QX200 Droplet Digital PCR System, includes QX200 Droplet Generator (#1864001)
- PX1 PCR Plate Sealer (#1814000)
- C1000 Touch Thermal Cycler with 96–Deep Well Reaction Module (#1851197)
- ddPCR 96-Well Plates, 25 plates (#12001925)
- Droplet Generation Oil, 10 x 7 ml (#1863005)
- DG8 Cartridges for QX200 Droplet Generator, 5 pkg of 24 (#1864007)
- PCR Plate Heat Seal, pierceable foil, 100 seals (#1814040)
- ddPCR Droplet Reader Oil, 2 L (#1863004)
- TC20 Automated Cell Counter (#1450102)

General Workflow

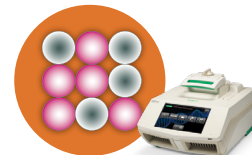
1 Add cells to 1x ddPCR reaction



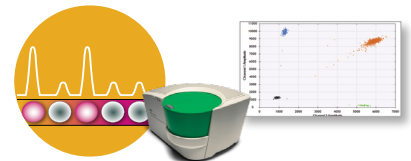
2 Encapsulate whole cells in droplets



3 Perform endpoint PCR



4 Read and analyze results



Key Workflow Considerations

- **Tip 1** — The order in which cells and master mix are prepared is important. Prepare cells first and place on ice, then prepare the 1x master mix. Use the master mix within 20 min. Load droplet generation cartridges and generate droplets within 5 min of adding cells to master mix
- **Tip 2** — An efficient workflow that makes droplets within 5 min of adding cells to the master mix is important for counting more cells and reducing free-floating DNA. Nevertheless, the linkage calculation will count only intact cells, even if some lysis occurs
- **Tip 3** — Thorough mixing/vortexing of each component is essential for workflow success. Vortex master mix and cells individually before combining and after combining. The supermix is concentrated and highly viscous and vortexing is critical to distribute the components evenly among all droplets
- **Tip 4** - Prepare samples in microcentrifuge tubes to make vortexing easier

Best Practices

Setting Up Your Assay

Two options can be used to support this protocol. The first is an all-in-one-well triplex assay and the second is a single-well duplex assay (Figure 1). For best results use RPP30 and/or SOD1 with your CAR-T-specific or transgene assay and HaeIII as the restriction enzyme. If needed, redesign the CAR-T-specific assay to ensure that the amplicon is not cut by HaeIII.

Triplex Assay:

- Recommended for best results: Use RPP30 and/or SOD1 with your CAR-T-specific or transgene assay and HaeIII as the restriction enzyme
- For the all-in-one triplex assay, the CAR-T-specific or transgene assay will need to be ordered as a FAM assay and again as a HEX assay. Mix the FAM and HEX assays together at a 1:1 ratio before adding to the well along with the RPP30 in FAM and SOD1 in HEX. This mixture will create a triplex assay with three positive clusters and the expected co-occupied droplet clusters (Figure 2). If clusters are not clearly separated, it may be necessary to reduce the concentration of one of the assays

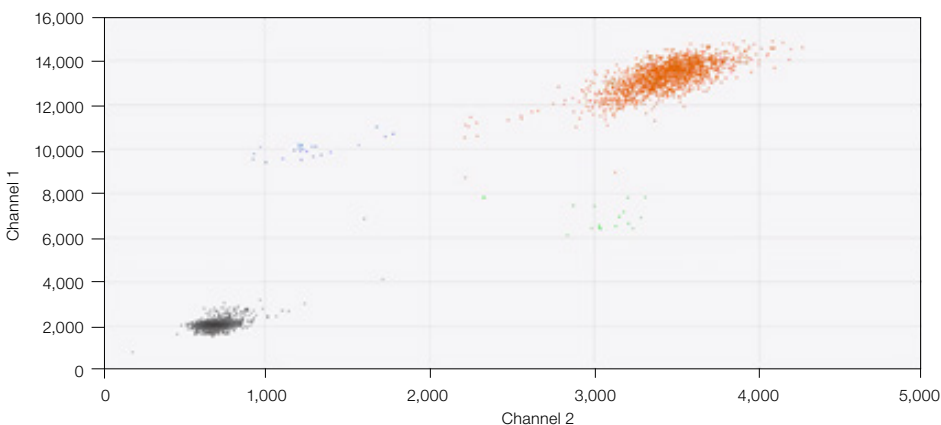


Fig. 2. Triplex assay. Orange cluster includes droplets positive for the three assays. Green and blue clusters are free-floating DNA. Black cluster is negative droplets.

Assay 1: Gene of interest (*AP3B1*), FAM
 Assay 2: Reference gene (*RPP30*), FAM/HEX mix
 Assay 3: Reference gene (*SOD1*), HEX

Duplex Assay:

- For the duplex strategy, choose either RPP30 in FAM or SOD1 in HEX and add the CAR-T-specific assay in the opposite fluorophore (FAM or HEX). With this strategy you will calculate the total cells from the RPP30 or SOD1 assay and they should be comparable to the TC20 Automated Cell Counter (Bio-Rad #1450102) results. The transgene will be calculated using the transgene copies/ μ l (cells/ μ l). Finally, linkage percentages will also be calculated using these two assays. If linkage percentage values are low, consider using the triplex method as an additional control to be sure the protocol is performed correctly. If needed, a second well with the RPP30/SOD1 duplex can be run with the RPP30/transgene or SOD1/transgene duplex

Controls and Test Run:

- To ensure quality data are generated, it is recommended that a simple control run be performed using RPP30 and SOD1 assays in duplex with K562 cell-line cells and the sample cells of interest. Since all human cells should contain both *RPP30* and *SOD1*, the data control run should produce equal *RPP30* and *SOD1* copies/ μ l (cells/ μ l) so that the ratio plot will show a ratio of 1 (Figure 1). Additionally, the total number of cells should be equal to that predicted by the TC20 Cell Counter cell counts. Finally, linkage percentage should be >90% (see analysis)

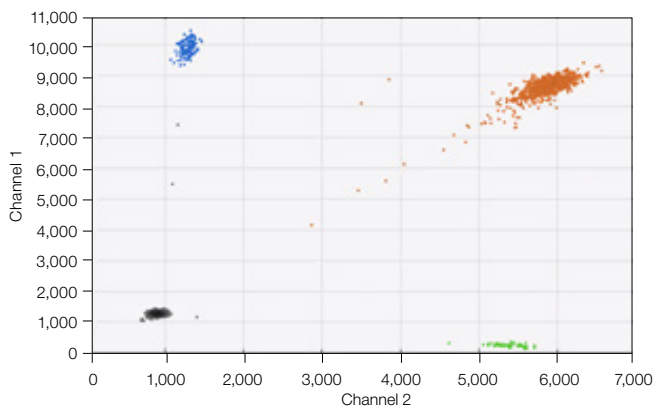


Fig. 1. Duplex assay. Orange cluster includes droplets positive for both assays. Green and blue clusters are free-floating DNA. Black cluster is negative droplets.

Assay 1: Reference gene (*RPP30*), FAM
 Assay 2: Reference gene (*SOD1*), HEX

Protocol

1 Preparation, counting, assessment of viability of cells
Prepare your cells in PBS + 0.1% BSA before setting up the ddPCR reaction mixture. Fresh or frozen cells and nucleosomal preparations may be used. It is recommended that 1–2 1x washes be performed before counting and adding cells to the ddPCR reaction. Keep cells stored on ice until added to the ddPCR reaction. Some cells and nucleosomal preparations may require a user-defined and -optimized cell-preparation protocol. For nucleosomal preparations see the OMNI-ATAC protocol and buffers ([SureCell® ATAC-Seq Library Preparation Kit User Guide, section 3](#)) to get started. Bio-Rad's TC20 Automated Cell Counter with replicates is recommended for an accurate cell count and to assess cell viability. A count of 4,000 cells/μl in 5 μl (20,000 cells total) is recommended for detection of an edit frequency down to 0.1%.*

* Up to 60,000 cells per well can be used.

2 Prepare 1x ddPCR reaction
After preparing the cells or nucleosomes and storing on ice, prepare the ddPCR reaction with all components according to Table 1. Make master mix + cells for technical replicate wells in one microcentrifuge tube to simplify and expedite the vortexing step.

It is important to wait to prepare the ddPCR reaction until the cells are on ice and ready to be added to the master mix. This ensures that all reaction components are fresh, which has an impact on workflow success.

Important: Mix thoroughly. Seal, vortex, and centrifuge plate or prepare in a tube and vortex.

3 Add cells to 1x ddPCR reaction
Vortex to resuspend cells, add to the 1x ddPCR reaction, and mix well as in step 2.

Important: For good results it is critical to thoroughly vortex the cells prior to making droplets.

Table 1. Preparation of 1x ddPCR reaction.

Component	Volume, μl
ddPCR Multiplex Supermix	6.25
Restriction enzyme** ≥10 U	1
20x assay 1	1.25
20x assay 2	1.25
DTT	0.34
ddPCR Enhancer Reagent A	0.5
Cells in PBS + 0.1% BSA***	Up to 5 μl (do not exceed 20% PBS)
Water	Variable
Total	25

** Non-amplicon-cutting restriction enzymes that are recommended and verified: *HaeIII* or *BsrI* + *SmaI*.

*** Cells up to 60,000 per well (3,000 copies/μl), as reported in QuantaSoft Software, can be directly converted from copies/μl to cells/μl.

4 Encapsulate cells in droplets
Make droplets using ddPCR protocol (20 μl of reaction and 70 μl of Droplet Generator Oil). Refer to QX200 Droplet Generator Instruction Manual (10031907) for full instructions.

5 Thermal cycle to endpoint
Seal plate and thermal cycle (according to Table 2). Proceed to reading of droplets in the QX200 Droplet Reader. Droplet reading is recommended within 24 hours of thermal cycling completion.

Note: This protocol normally generates droplet counts of ~10,000 droplets or fewer per well.

Table 2. Thermal cycling parameters.

Step	Temperature, °C	Time	Ramp, °C/sec	Number of Cycles
1	37	30 min	2	1
2	95	10 min	2	1
3	94	30 sec	2	60
4	55	2 min	2	60
5	98	10 min	2	1
6	4	Hold	—	Hold

6 Analysis
Analyze with QuantaSoft Software, version 1.7, QuantaSoft Analysis Pro Software, or QX Manager Software. QX Manager Software may be used for the triplexing method. After carefully labeling the wells, set the thresholds. The reported concentrations in copies/μl from the software are directly equivalent to cells/μl when performing the sc-ddPCR reaction. Copies/μl for total cells will come from the two reference assays RPP30 and SOD1. These two numbers should be equal to each other:

$$\text{RPP30 cells/}\mu\text{l} = \text{SOD1 cells/}\mu\text{l}.$$

The reported copies/μl number can be used as the cells/μl because cells are being counted.

Well	E	Experiment	T	Target	Status	Conc(copies/µL)	Superplating	Copies/20µL Well	Linkage	Accepted Droplets	Ratio	TotalRatioMax	TotalRatioMin	TotalRatioMax68	TotalRatioMin68	PoissonRatioMax
C02	A	ABS	U	RPP30_v1.1	Manual	154	ddPC...	3080	145	8491	1.02					1.1
C02	A	ABS	U	SOD1	Manual	151	ddPC...	3020	145	8491	1.02					1.1
D02	A	ABS	U	RPP30_v1.1	Manual	156	ddPC...	3120	147	10002	1.03					1.11
D02	A	ABS	U	SOD1	Manual	152	ddPC...	3040	147	10002	1.03					1.11
E02	A	ABS	U	RPP30_v1.1	Manual	144	ddPC...	2880	136	9624	1.01					1.09
E02	A	ABS	U	SOD1	Manual	143	ddPC...	2860	136	9624	1.01					1.09
F02	A	ABS	U	RPP30_v1.1	Manual	146	ddPC...	2920	141	10019	1.01					1.09
F02	A	ABS	U	SOD1	Manual	145	ddPC...	2900	141	10019	1.01					1.09

Fig. 3. Access linkage values in table view in QuantaSoft Software 1.7.

Analysis Tips

Note: It is normal to see a few free-floating segments of DNA coming from the cell solution. In order to account for free-floating DNA it can be useful to know the linkage percentage.

Use the RPP30_FAM+SOD1_HEX well to calculate linkage of two DNA targets inside a cell. The linkage percentage can be calculated using the Linkage copies/µl reported in the Table view of the software (Figure 3). This linkage value is the number of copies/µl of double positives above and beyond those expected to occur through random distribution. Use this linkage to calculate percentage linked by taking the (linkage concentration [copies/µl]/ concentration [copies/µl] x 100).

Cells run with two DNA copy number assays should have a concentration ratio of 1 so approximately the same concentration and a high percentage of the total positives should be linked. An optimized experiment can be expected to produce a linkage of 95–98%. If the linkage percentage is close to 100%, then using the nonlinkage concentrations for the reference gene and gene of interest (GOI) will be accurate. For more information on Poisson distribution and linkage, please reference the Droplet Digital PCR Applications Guide (bulletin 6407) and Guidelines for Phasing Variants Using Bio-Rad’s Droplet Digital PCR System (bulletin 6718).

To ensure that the protocol is working for your cells, it is important to have a control well of two copy number DNA assays, such as RPP30 and SOD1, duplexed. Alternatively, a triplex assay can be created that has the two DNA assays plus the GOI assay (see Best Practices: Setting Up your Assay section). If you choose to use a triplex assay, the linkage can be calculated between each of the pairwise combinations of assays using QX Manager or QuantaSoft Analysis Pro Software. When duplexing the two DNA reference assays, every cell should have both the genes and therefore should be FAM and HEX positive (double-positive).

Visit bio-rad.com/ddPCR-wholecell for more information on counting genetically modified cells using whole cell–based Droplet Digital PCR.

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