Protocol

Using Digital Polymerase Chain Reaction to Detect Single-Nucleotide Substitutions Induced by Genome Editing

Yuichiro Miyaoka,[1] Amanda H. Chan,[1] and Bruce R. Conklin[1,2,3]

[1]*Gladstone Institute of Cardiovascular Disease, San Francisco, California 94158;* [2]*Departments of Medicine, and Cellular and Molecular Pharmacology, University of California, San Francisco, California 94143*

This protocol is designed to detect single-nucleotide substitutions generated by genome editing in a highly sensitive and quantitative manner. It uses a combination of allele-specific hydrolysis probes and a new digital polymerase chain reaction (dPCR) technology called droplet digital PCR (ddPCR). ddPCR partitions a reaction into more than 10,000 nanoliter-scale water-in-oil droplets. As a result, each droplet contains only a few copies of the genome so that ddPCR is able to detect rare genome-editing events without missing them.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

Reagents

ddPCR Buffer Control Kit (Bio-Rad 1863052)
ddPCR Supermix for Probes (no dUTP) (Bio-Rad 1863024)
Droplet Generation Oil for Probes (Bio-Rad 1863005)
Genomic DNA isolated from cells treated with genome-editing tools to induce single-nucleotide substitutions, diluted to 100–150 ng/µL in distilled water or TE buffer (if original concentration is higher)
Positive-control plasmids with the original and changed allelic sequences

Alternatively, allele-specific DNA fragments may be used. These DNA fragments should be contain all the primer and probe binding sequences.

Equipment

DG8 Cartridge Holder (Bio-Rad 1863051)
DG8 Cartridges for Droplet Generator (Bio-Rad 1864008)
DG8 Gaskets for QX200 Droplet Generator (Bio-Rad 1863009)
PCR eight-tube strips (optional; see Step 17)
PCR plates (Eppendorf twin.tec; 96-wells; semiskirted) (Fisher 951020346)
Pierceable Foil Heat Seal (Bio-Rad 1814040)
Pipettes (eight-channel; 20- and 50-µL)
Primer Express Software 3.0 (Life Technologies 4363991)
PX1 PCR Plate Sealer (Bio-Rad 1814000)

[3]Correspondence: bconklin@gladstone.ucsf.edu

Cite this protocol as *Cold Spring Harb Protoc;* doi:10.1101/pdb.prot086801

QX100 or QX200 Droplet Digital PCR system (Bio-Rad 1864001)

The ddPCR system includes a droplet generator and droplet analyzer.

Thermocycler capable of holding 96-well plates

METHOD

Preparation of Hydrolysis Probes and Primers

Allele-specific hydrolysis probes (e.g., TaqMan) and primers (Fig. 1A) are required for this procedure. They may be obtained by following Steps 1–3 or by sending target sequences to a vendor (e.g., Integrated DNA Technologies).

1. Use Primer Express Software 3.0, following the manufacturer's instructions for "TaqMan MGB Allelic Discrimination." Design probes and primers by treating the single-nucleotide substitution as a single-nucleotide polymorphism (SNP) in the software.
2. Select and order a primer pair such that at least one of the two primers binds outside of the donor oligo-DNA sequence (Fig. 1A).

   Typically the amplicon size is <200 bp.
3. Order two allele-specific MGB probes conjugated with fluorescent dyes FAM and VIC/HEX, respectively, from Life Technologies.
4. Prepare two 100-µL hydrolysis probe and primer mixtures, each of which is specific for one allele.

Forward primer (allele-specific; 100 µM)	18 µL
Reverse primer (allele-specific; 100 µM)	18 µL
Probe (FAM or VIC/HEX; 100 µM)	5 µL
Distilled water	59 µL

Store the mixtures for up to 1 yr at −20°C.

Validation of Probes and Primers for the ddPCR System

This method is adapted from Hindson et al. (2011).

5. Dilute the positive control as appropriate for their size (e.g., to 0.5 pg/µL for a 3000-bp plasmid or to 0.05 pg/µL for a 300-bp DNA fragment) and combine them to make a 1:1 mixture.

   Handle positive-control mixtures carefully. If a reagent is contaminated with this mixture, it will completely disrupt the assay.
6. Assemble a master mix on ice by combining the following reagents (volumes are indicated for one 25-µL reaction).

Distilled water	9 µL
ddPCR Supermix for Probes (no dUTP)	12.5 µL
FAM probe and primer mixture (from Step 4)	1.25 µL
VIC/HEX probe and primer mixture (from Step 4)	1.25 µL
1:1 plasmid mixture (from Step 5)	1 µL

7. Carefully apply 20 µL of the mixture into each of the eight "sample" wells of a DG8 Cartridge for Droplet Generator.

   Bubbles floating on the surface of the sample do not affect droplet generation, but bubbles in the bottom of the well must be removed. Take note of orientation of the cartridge with respect to the sample order to ensure correct loading of the final Eppendorf twin.tec 96-well plate.
8. Apply 70 µL of Droplet Generation Oil for Probes into each of the eight "oil" wells of the DG8 Cartridge for Droplet Generator.

   Do not load the oil before the samples as this will reduce the droplet number.
9. Hook a DG8 Gasket for QX200 Droplet Generator onto the DG8 Cartridge Holder.

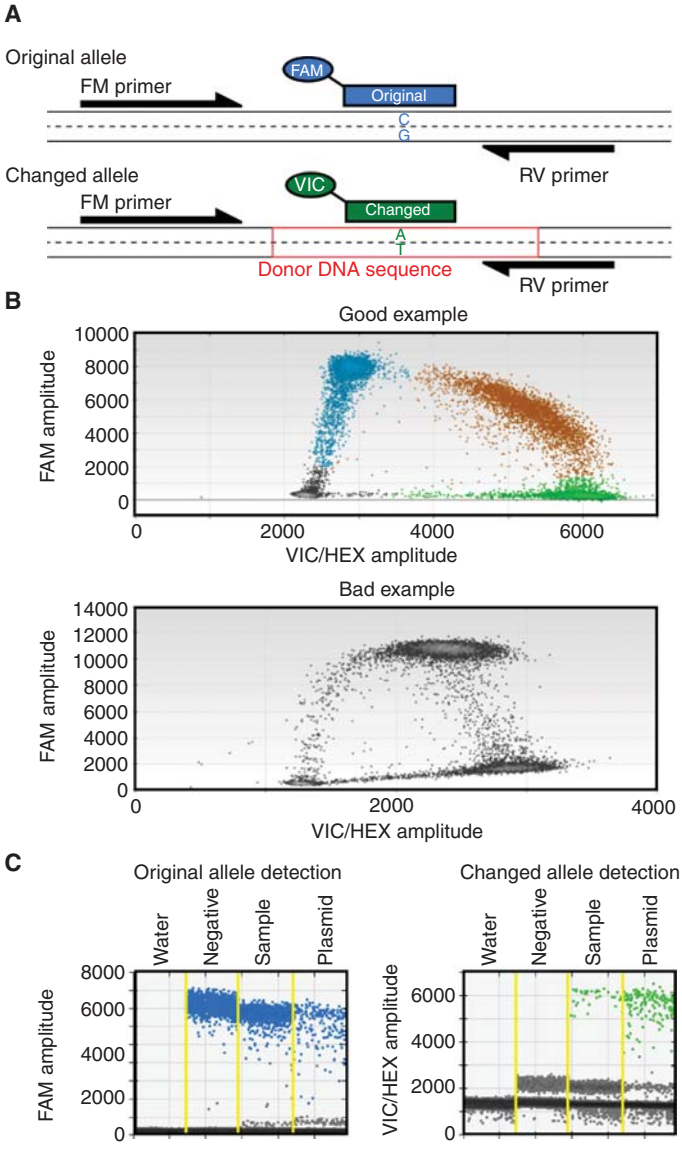

FIGURE 1. (*A*) PCR design with allele-specific hydrolysis probes. A C-to-A substitution is shown as an example. These two alleles are discriminated by different fluorophores; hydrolysis probes specific to the original and changed alleles are conjugated to FAM and VIC (or HEX; not shown), respectively. The primers, however, are identical. One of the two primers hybridizes to a region outside of the donor DNA sequence to amplify the correctly recombined allele. (*B*) Good and bad examples of primer and probe sets to detect single-nucleotide substitutions. In the good example, distinct negative (black), FAM-positive (blue), VIC/HEX-positive (green), and double-positive (brown) populations are clearly seen. However, in the bad example, the FAM-positive and double-positive populations are fused into one population. In such cases, the probes and/or primers must be redesigned. (*C*) Example of ddPCR analysis to detect a single-nucleotide substitution. The original (blue) and changed (green) alleles are detected by the FAM and VIC/HEX signals, respectively. The distilled water control indicates that the system was free from noise, and the negative control indicates that genome-editing tools that targeted an unrelated genomic region did not induce aberrant HDR at the target locus. The positive-plasmid control indicates the amplitudes of the two alleles. In this case, 1.45% of the sample had the changed allele.

10. Put the holder in the droplet generator to generate droplets in eight wells.
11. Transfer droplets into a semiskirted Eppendorf twin.tec 96-well plate using an eight-channel pipette set to 45 µL.

    Do not press the pipette tightly to the bottom of the cartridge or pipette too vigorously as this will shear the droplets. Cover the PCR plate with a foil sheet immediately after transfer to reduce the risk of contamination.

12. Seal the plate with a Pierceable Foil Heat Seal using the PX1 PCR Plate Sealer set to 180°C.
13. Perform thermal cycling using the following program.

1 cycle	95°C	10 min
40 cycles	94°C	30 sec
	50°C–60°C gradient	1 min
1 cycle	98°C	10 min
1 cycle	12°C	hold

14. Analyze the droplets using the ddPCR analyzer.
    i. Go to "Analyze" and then "2-D Amplitude."

    In a successful assay, distinct negative, FAM-positive, VIC/HEX-positive, and double-positive populations should be seen in the 2D plot (Fig. 1B). See Troubleshooting.

    ii. Determine the best temperature for annealing and extension by identifying the highest temperature at which the best separation of the four populations is achieved.
15. (Optional) Make a dilution series of the changed allele to determine the sensitivity of the ddPCR assay for the target.

    A typical dilution series is 0.01%, 0.1%, 1%, 10%, 50%, and 100% of the changed allele over the original allele. Repeat Steps 5–14 with a dilution series to determine the limit of detection.

Detection of Single-Nucleotide Substitutions in Genomic-DNA Samples

16. Assemble a master mix on ice by combining the following reagents. Prepare a total volume sufficient for every sample as well as a distilled water–only negative control and a 1:1 positive-control mixture.

Distilled water	9 µL
ddPCR Supermix for Probes (no dUTP)	12.5 µL
FAM probe and primer mixture (from Step 4)	1.25 µL
VIC/HEX probe and primer mixture (from Step 4)	1.25 µL

In Step 18, 1 µL of 100–150 ng/µL genomic DNA will be added to bring the final volume of each reaction to 25 µL. If using a different volume of genomic DNA in Step 18, adjust the volume of distilled water used here accordingly.

17. Aliquot 24 µL of master mix from Step 16 into PCR eight-tube strips or any 96-well plate.
18. Add 1 µL (100–150 ng) of genomic DNA per sample. Also, add controls to tubes or wells designated for the controls.
19. If the total number of samples to be analyzed is not a multiple of eight, fill the remaining empty tubes of the PCR strip with 25 µL of ddPCR Buffer Control Kit diluted to 1× with distilled water.
20. Briefly centrifuge the PCR tubes or plates.
21. Gently pipette up and down to mix the reactions using a 20-µL eight-channel pipette, and carefully apply the mixtures into "sample" wells.
22. Repeat Steps 8–11 until droplets are generated for all the samples.
23. Seal the plate with a Pierceable Foil Heat Seal using the PX1 PCR Plate Sealer set to 180°C.
24. Perform thermal cycling with the optimal temperature identified in Step 14.ii.

1 cycle	95°C	10 min
40 cycles	94°C	30 sec
	temperature from Step 14.ii	1 min
1 cycle	98°C	10 min
1 cycle	12°C	hold

25. Analyze the droplets using the ddPCR analyzer (Fig. 1C).
    i. Go to "Analyze" and then "2D Amplitude."

ii. Gate negative, FAM-positive, VIC/HEX-positive, and double-positive populations by comparing samples with controls.

iii. Go to "concentration" to obtain the allelic frequencies.

iv. Export data as a CSV file by "Export CSV."

TROUBLESHOOTING

Problem (Step 14.i): Four distinct populations cannot be detected (e.g., see Fig. 1B).

Solution: The 50°C–60°C gradient may not yield optimal separation of the four populations; performing the probe/primer validation again using an adjusted temperature gradient may solve the problem. Alternatively, the probes and/or primers may have to be redesigned.

RELATED INFORMATION

For background information on this protocol, see Miyaoka et al. (2014) and Introduction: **Detecting Single-Nucleotide Substitutions Induced by Genome Editing** (Miyaoka et al. 2016).

REFERENCES

Hindson BJ, Ness KD, Masquelier DA, Belgrader P, Heredia NJ, Makarewicz AJ, Bright IJ, Lucero MY, Hiddessen AL, Legler TC, et al. 2011. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. *Anal Chem* **83:** 8604–8610.

Miyaoka Y, Chan AH, Judge LM, Yoo J, Huang M, Nguyen TD, Lizarraga PP, So PL, Conklin BR. 2014. Isolation of single-base genome-edited human iPS cells without antibiotic selection. *Nat Methods* **11:** 291–293.

Miyaoka Y, Chan AH, Conklin BR. 2016. Detecting single-nucleotide substitutions induced by genome editing. *Cold Spring Harb Protoc* doi: 10.1101/pdb.top090845.

Using Digital Polymerase Chain Reaction to Detect Single-Nucleotide Substitutions Induced by Genome Editing

Yuichiro Miyaoka, Amanda H. Chan and Bruce R. Conklin

Cold Spring Harb Protoc; doi: 10.1101/pdb.prot086801; published online June 1, 2016