Promega

Product Application

Using droplet digital PCR to confirm CRISPR knock-in of the HiBiT tag

Use droplet digital PCR (ddPCR) on a BioRad ddPCR system to confirm and quantify HiBiT integration in cell pools or clones.

This protocol was developed by Promega Applications Scientists and is intended for research use only.

Users are responsible for determining suitability of the protocol for their application.

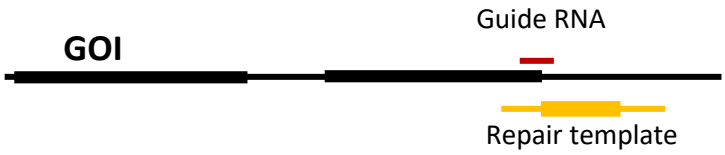
Sample Type(s): Purified DNA from cell cultures

Input: 10ng – 50ng

Materials Required:

- Probe-based assays (IDT) – Assay design is shown in Fig. 1. Ordering is described in Appendix A.
  - Primers spanning the knock-in region in the gene of interest
  - Probe for the gene of interest, upstream of the CRISPR cut site
  - Probe for the HiBiT tag (including the linker sequence)
    56-FAM/TAATCTTCT/ZEN/TGAACAGCCGCCAGCC/3IABkFQ *or*
    5-HEX/TAATCTTCT/ZEN/TGAACAGCCGCCAGCC/3IABkFQ
- ddPCR Supermix for Probes (no dUTP), BioRad, Cat. #1863024
- Droplet Generation Oil for Probes, BioRad Cat. #1863005
- DG8 Cartridges, BioRad Cat. #1864008
- Droplet Generator DG8 Gasket, BioRad Cat. #1863009
- Pierceable Foil Heat Seal, BioRad Cat. #1814040
- BioRad QX200 Droplet Generator with Cartridge Holder
- BioRad C1000 Touch Thermal Cycler
- BioRad PX1 Plate Sealer
- BioRad QX200 Droplet Reader

A. CRISPR knock-in strategy for HiBiT tag



B. Detection of HiBiT knock-ins by ddPCR

Figure 1. Targeted HiBiT tag insertion using CRISPR and detection of HiBiT knock-in. A) CRISPR design to target the HiBiT tag to the carboxy terminus of a gene of interest (GOI) using a repair template with 50bp homology arms (yellow) and guide RNA (red). B) Design of ddPCR assays. Primers (gray arrows) are designed outside the repair template and are used to amplify the integration site, with multiplexed probes for the HiBiT tag (green) and the GOI (blue) for normalization.

Protocol:

Note: For more detailed instructions regarding the setup of ddPCR assays, please refer to the BioRad Droplet Digital PCR Applications Guide (#6407)

1. Assemble the following master mix, minus the DNA, for ddPCR reactions. The following table assumes assembly of 23µl reactions (for each 20µl reaction required) to avoid transferring air bubbles into droplet generator cartridges, which can interfere with droplet generation. Volumes of primers and probes assume 20X stocks.

Reagent	Final concentration	Volume per reaction (µl)
Nuclease Free Water	NA	2.30
ddPCR Supermix for Probes (no dUTP)	1X	11.50
Primers (20X mix)	900nM	1.15
HiBiT Probe (20X)	250nM	1.15
Gene of Interest Probe (20X)	250nM	1.15
Sample DNA (2ng/µl – 10ng/µl)	10ng – 50ng	5.75

2. Add 17.25µl of the assembled master mix per well of an 8 well strip tube. All 8 wells must contain master mix for appropriate function of the droplet generation system.
3. Dilute DNA to 2ng/µl – 10ng/µl.
4. Add 5.75µl diluted DNA per reaction. The final DNA input will be 5µl (10ng – 50ng) per 20µl reaction. Higher concentrations of DNA may be used if restriction digest is incorporated. Refer to the BioRad Droplet Digital PCR Applications Guide (#6407) for more information.
5. Cap strip tubes securely and vortex to ensure homogenous reactions. Centrifuge briefly to collect liquid at the bottom of each well.
6. Place a new DG8 Cartridge in a Cartridge Holder and close.
7. Taking care to avoid transferring air bubbles, pipet 20µl of the complete reaction mixes into the 8 sample wells of the cartridge (middle row of the cartridge).
8. Add 70µl of Droplet Generation Oil for Probes into each of the 8 oil wells in the cartridge (bottom row of the cartridge).
9. Cover the cartridge with a gasket, hooking the gasket in place to secure.
10. Place the Cartridge Holder containing the assembled DG8 Cartridge and gasket in the QX100 Droplet Generator.
11. Close the QX100 Droplet Generator. Droplet generation will initiate automatically (~2 minutes).
12. Remove the Cartridge Holder from the QX100 Droplet Generator and dispose of the gasket.
13. Transfer droplets (from the top row of the cartridge) to a 96 well PCR plate. To avoid shearing the droplets, transfer them slowly and carefully; a multi-channel electronic pipet with a slow speed setting may be used to improve consistent transfer and reduce droplet shearing.
14. Repeat steps 6-13 as needed to complete all reactions.
15. Seal the PCR plate using Pierceable Foil Heat Seals and a BioRad PX1 Plate Sealer according to the directions.
16. Place the sealed PCR plate in a BioRad C1000 Touch Thermal Cycler and cycle as follows. The annealing temperature may require optimization, but temperatures of 58°C – 60°C are appropriate for most assays designed in primer design software with the default conditions.

Step	Time	Temperature (°C)	Cycles	Ramp
Activation	10 min	95	1	2.2 °C/s
Denature	30 sec	94	40	
Anneal/Extend	1 min	58-60		
Enzyme deactivation	10 min	98	1	
Hold	Hold	4	1	

17. After cycling, count droplets using a BioRad QX200 Droplet Reader and Absolute Quantification (ABS) method according to the instrument protocol. Select detection of both Target 1 and Target 2 with appropriate fluorophores for the multiplexed probes.
18. When analyzing the data, all droplets with HiBiT-positive droplets should also be positive for the gene of interest (GOI). The fraction of HiBiT-positive/GOI-positive indicates the fraction of successfully targeted loci. Note that this does not guarantee correct sequence, reading frame, or expression of the fusion.

Appendix A: Designing and Ordering Assays

Use of IDT PrimerQuest to design primers and probes is detailed in the instructions below. All primers were designed to be outside of the repair template homology arms so they could be used on cell pools following CRISPR integration, when residual template is still expected to be present. ddPCR primers are typically designed for amplification of a target ≤ 250bp for optimal amplification efficiency. We can meet that length suggestion here because the HiBiT sequence is 39bp with 50bp homology arms included on either side of HiBiT in the repair template. Longer homology arms or insert sequences will increase the required amplicon size for detection with ddPCR and may require further optimization. The probe for detection of the gene of interest (GOI) should be designed upstream of the CRISPR cut site and guide RNAs to minimize the chance of sequence alteration at the probe hybridization site.

1. Copy ~400bp of sequence spanning the entire repair template DNA plus at least 100 bases of the gene on either side. Include the homology arms, guide and VS-HiBiT sequences as it would appear in a correctly integrated sequence.
2. Identify the base pair locations of the guide RNAs and HiBiT on the copied sequence. One way to do this is to paste the sequence into Word, search for each element using the Find function, and color code each sequence. Then highlight the entire sequence up to the first or last base in the element and use Review > Word Count to identify the position. These positions will be used to specify regions for primer and probe design.
3. Open the online PrimerQuest tool from IDT (http://www.idtdna.com/PrimerQuest/Home/Index).
4. Paste your ~400bp sequence in the Sequence Entry box. Name the sequence if desired.
5. Under Choose Your Design, click “qPCR 2 primers + probe”.
6. This selection will automatically identify primers without any sequence or location restrictions. Do not use these assays. Instead, click “Customize Assay Design”.
7. Scroll down to Amplicon Criteria settings and change them to 75 (Min)/150 (Opt)/ 250 (Max).
8. In Custom Target Region > Included Region, indicate a ~300bp range centered on your HiBiT sequence (e.g. 50-350). This is the region the software will use for primer design, so we recommend keeping the amplicon size fairly small. If the initial primer design does not generate quality primers, you can increase the size of the included region and repeat.
9. In Custom Target Region > Excluded Region List, indicate the start and end of the repair template (in this case, 50bp homology arms upstream and downstream of the HiBiT insertion). Exclude this region to force primer design outside of the repair template.
10. In Custom Target Region > Excluded Region List – Probe, indicate the start of the guide RNA through the end of the Included Region. This will force the probe to be 5’ with respect to the guide RNAs.
11. Click Get Assays.
12. Order the gene-specific probe and HiBiT probes with opposite fluorophores and ZEN/IBlkHQ quenching. Order forward primer, reverse primer, and probe separately, not pre-mixed.