CRISPY™ Master Mix for qPCR-based Gene Editing Quantitation

Cat # CRISPY100A-1

User Manual

Storage: Store at -20˚ upon receipt

Version 5
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Product Description

Better than a mismatch detection assay in every way

Accelerate your CRISPR/Cas9 workflows with CRISPY Master Mix, a reagent that enables fast, sensitive, and accurate quantitation of genome editing success using qPCR. Leveraging a "snapback" primer and a DNA polymerase that lacks both 5′-to-3′ exonuclease and strand displacement activity, CRISPY assays can be used to measure the percentage of successful genome edits at the target site from purified genomic DNA or cells.

The CRISPY assay offers a wealth of advantages over widely-used gel-based mismatch detection assays, including:

- Faster workflows that are complete in less than 1hour with only 15 minutes of hands-on time
- Simpler workflows that involve as little as a single step, no DNA purification required
- More sensitive detection, reporting success rates as low as 1%
- More reliable quantitation based on observed Ct instead of gel imaging
- Lower sample input requirements—works on as little as 0.5 ng DNA

CRISPY Master Mix comes in a 1 mL aliquot, which provides sufficient master mix for 200 reactions of 10 µL each.

Understanding the CRISPY assay

The CRISPY assay relies on the use of a snapback tag that is homologous to the wildtype, unedited sequence and a DNA polymerase that lacks both 5′-to-3′ exonuclease and strand displacement activity (Figure 1).

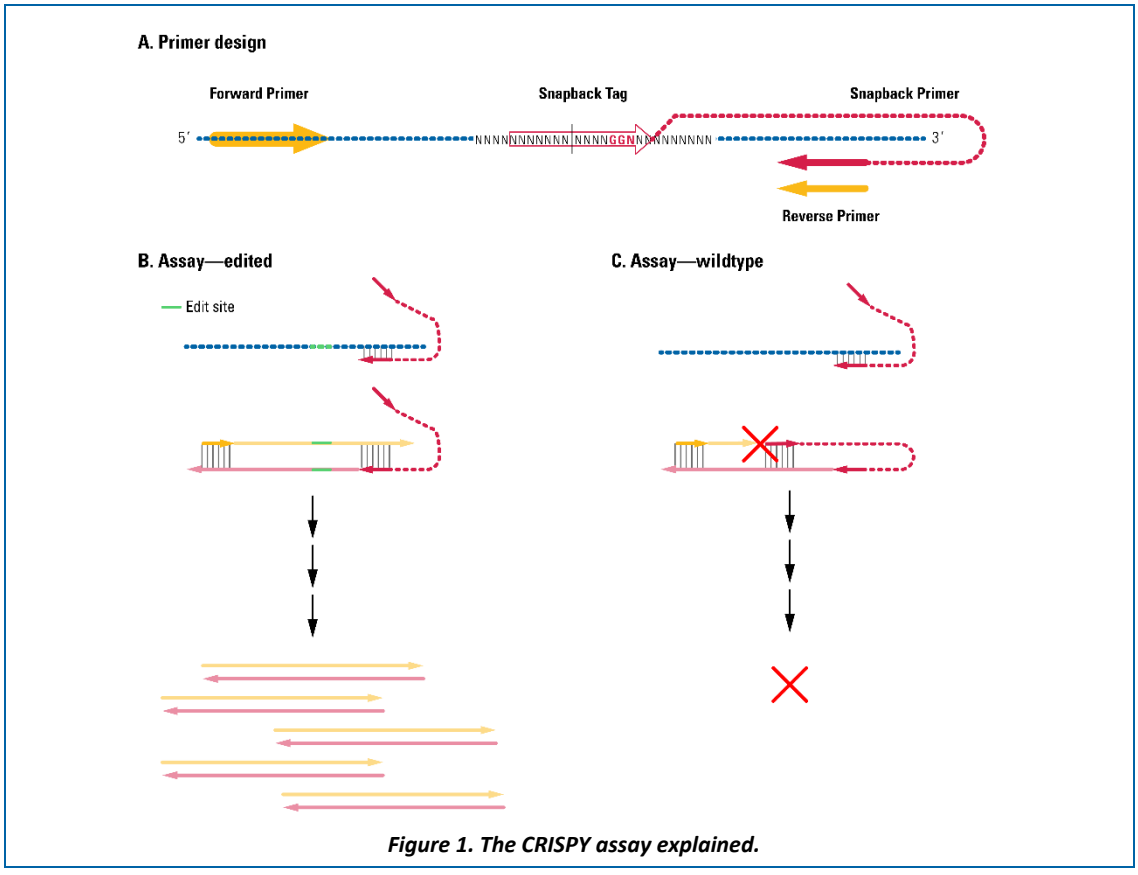

Figure 1. The CRISPY assay explained.

Primer design (Figure 1A)

Design forward and reverse primers (referred to here as standard primers) that will amplify across the gRNA locus. Place one of the primers within 60 nucleotides (nt) of the center of the gRNA annealing site—this will be part of your snapback primer and the other primer will be the common primer.

Design the snapback tag by selecting 14-16 nt centered around the third or fourth base upstream of the first base in the protospacer adjacent motif (PAM) sequence. For the snapback tag, use the sequence of the wildtype, unedited sequence.

> **NOTE**: The length of the snapback tag is flexible, and we recommend designing and screening several snapback primers with different tag lengths to identify the one with optimal selectivity and sensitivity. When screening different snapback primers, we recommend using the same amount of genomic DNA that you will use for both control and CRISPY assays, selecting the snapback primer that generates a sufficient Ct shift for quantitation. An optimal snapback primer will have a small ΔCt (snapback primer Ct – standard primer Ct) when using edited template and a ΔCt that is greater than 3.3 when using wildtype template. We have found that a snapback tag length of around 15 bases works well.
>
> **When using gene editing enzymes other than CRISPR/Cas9, the snapback tag can be selected by similarly centering at the prevalent cleavage site**.

Create the snapback primer by adding the snapback tag to the 5'-end of the designated primer (the one located within 60 nt of the center of the gRNA annealing site). If the loop formed by snapping back is shorter than 30 nt, add an optional non-homologous spacer sequence (TTTT) to ensure sufficient flexibility to form the loop.

We recommend using a primer design tool to design primers with a T_m = 60 ± 2°C (equivalent to 65°C when using fusion polymerases with a double-strand DNA binding motif) , making sure that your primer designs do not generate secondary structures and do not have 3'-end complementarity longer than 3 bases.

Only edited DNA gets amplified

During qPCR using the common and snapback primers, only target sites that have been edited will be amplified (Figure 1B) since the snapback primer will anneal intramolecularly to primer extension products from wildtype templates, preventing amplification by the DNA polymerase which cannot displace the self-annealed snapback tag (Figure 1C).

List of Components

Table 1. Components

Catalog number	Components	Qty/Volume	Storage Temperature
CRISPY100A-1	2X CRISPY Master Mix, 1mL (200 reactions)	200 Reactions/ 1 mL	-20˚C

Additional Required and Optional Equipment Not Included in Kit

- Forward, reverse, and snapback primers

  See the "Primer design" section in the Product Description above for our recommendations on primer design.

- Genomic DNA isolation kit (optional)
- qPCR instrument
- qPCR plate
- Pipettes and tips
- Low-binding microtubes
- Vortex mixer
- Microcentrifuge
- Plate spinner
- Pure water

Before you start:

We recommend using a clean area or a hood for qPCR setup to avoid contamination and calibrated pipettes and instruments for optimal performance. Multichannel and repetitive dispensing pipettes are highly recommended when running a large number of reactions to reduce the strain on hands and arms.

Protocol

Using purified genomic DNA

Set up your qPCR reaction using a control assay (standard primers) and the CRISPY assay (the common primer and the snapback primer).

NOTE: When evaluation of the specificity of the CRIPSY assay is needed, be sure to use wildtype genomic DNA as a reference. For CRISPY primers that have been tested and confirmed to have good suppression of wildtype template, running the wildtype template is optional.

1. First, prepare the primer and template mixture for each reaction in separate tubes or plate wells:

	Edited Template		Wildtype template (optional)	
	Control Assay	CRISPY Assay	Control Assay	CRISPY Assay
Common primer at 1 µM	1 µL	1 µL	1 µL	1 µL
Standard primer* at 1 µM	1 µL	0 µL	1 µL	0 µL
Snapback primer	0 µL	1 µL	0 µL	1 µL
Edited template	A µL	B µL	0 µL	0 µL
Wildtype template	0 µL	0 µL	C µL	D µL
Water	3-A µL	3-B µL	3-C µL	3-D µL
Total volume	5 µL	5 µL	5 µL	5 µL

* If your Common primer is the forward primer, use the reverse primer as the Standard primer. If your Common primer is the reverse primer, then use the forward primer as the Standard primer.

2. Add 5 µL of 2x CRISPY Master Mix to the above primer and template mixture, and mix well by pipetting up and down.

3. Run a real-time PCR and melting curve program on your qPCR instrument, collecting fluorescence in the SYBR Green channel for both qPCR and melting curve stages. Select ROX as passive reference. Thermocycle as follows:

1 cycle:	95°C x 5 minutes
35 Cycles:	95°C x 15 seconds
	65°C x 20 seconds

Follow the qPCR stage with a melting curve analysis, collecting fluorescence from 60°C to 95°C.

NOTE: We recommend running replicate reactions to improve the precision of your results.

Using cells

For adherent cells:

4. Trypsinize and wash cells in PBS buffer.
5. Resuspend cells in PBS at a concentration of 5 x 10^5 cells/mL.
6. Use 1 µL of resuspended cells/well of the qPCR plate, and continue with step 1, above.

For suspension cells

7. Pellet the cells by centrifuging at 300 x *g* for 5 minutes.
8. Resuspend cells in PBS at a concentration of 5 x 10^5 cells/mL.
9. Use 1 µL of resuspended cells/well of the qPCR plate, and continue with step 1, above.

Calculations

To calculate the percentage of successful editing events:

1. Set a threshold at 0.1 and output the Ct values of the control assay ($Ct_{Control}$) and CRISPY Assay (Ct_{CRISPY}) for the reactions using wildtype template (wt) and edited template (Edited).
2. Calculate ΔCt for the reaction using the wildtype template:

$$Ct_{CRISPY} - Ct_{Control} = \Delta Ct_{wt}$$

3. Calculate ΔCt for the reaction(s) using edited template:

$$Ct_{CRISPY} - Ct_{Control} = \Delta Ct_{Edited}$$

4. Calculate the percentage of successful editing events:
    a. If $\Delta Ct_{wt} \geq 3.3$ the percentage of successful editing events is:

$$\% \text{ Success} = (1/(2\text{^}\Delta Ct_{Edited})) * 100$$

    b. If $\Delta Ct_{wt} \leq 3.3$ and $\Delta Ct_{Edited} < \Delta Ct_{wt}$, the percentage of successful editing events is:

$$\% \text{ Success} = (1/(2\text{^}\Delta Ct_{Edited})) * 100$$

NOTE: If $\Delta Ct_{Edited} \leq \Delta Ct_{wt}$, we recommend redesigning the primers by any or all of the following (1) increase the length of the snapback tag by 1 or 2 nucleotides at both 5' and 3' ends, (2) move the common primer farther away from the edited locus, or (3) reduce the concentration of the common primer.

Example Data and Applications

The CRISPY assay specifically quantifies successful CRISPR/Cas9 editing events when performed on isolated genomic DNA

We edited the DNA (cytosine-5-)-methyltransferase 3 beta (DNMT3B) gene in HEK293 cells using an All-in-one Cas9 construct, isolated genomic DNA from the edited cells, and then performed a CRISPY assay to quantify editing success using standard (yellow curve) and snapback primers (red curve, Figure 2A). We also ran a CRISPY assay on DNA isolated from cells that had not been edited, again using standard (yellow curve) and Snapback primers (red curve, Figure 2B). As expected, the standard primers provided robust amplification in both edited and unedited DNA, however the snapback primers only provided appreciable amplification in the DNA isolated from edited cells. The ΔCt between the standard and snapback primers provides a direct measurement of editing success, and the difference between the shapes of the melt curves demonstrates that a deletion is present in the edited DNA (Figure 2C, boxed area).

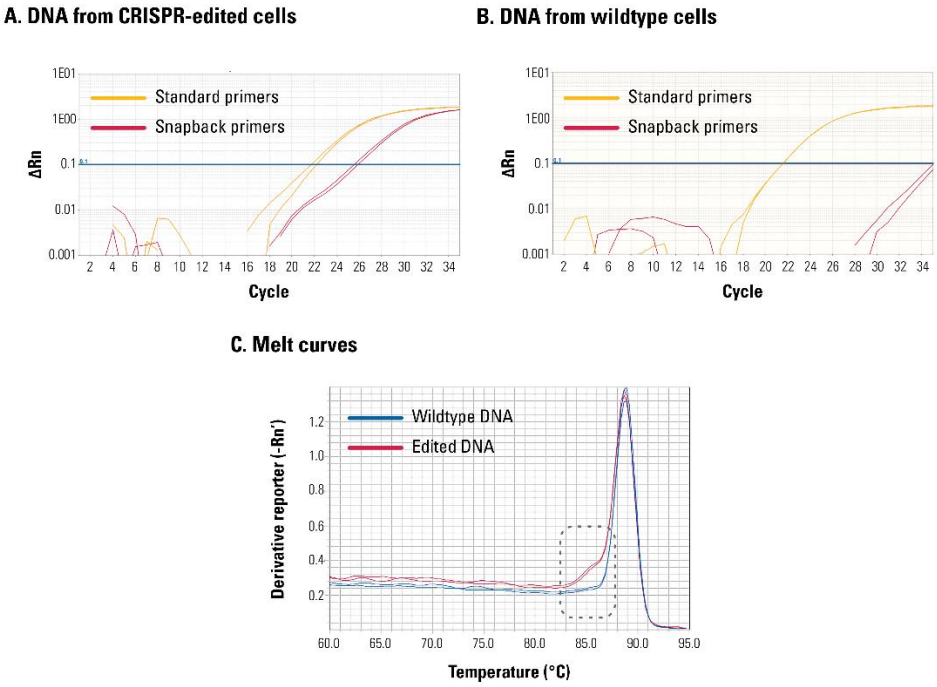

Figure 2. The CRISPY assay specifically quantifies successful CRISPR/Cas9 editing events when performed on isolated genomic DNA. *Amplification curves from (A) DNA isolated from CRISPR-edited cells and (B) DNA isolated from wildtype cells. (C) Melt curves from A and B.*

The CRISPY assay requires SBI's optimized CRISPY Master Mix

The CRISPY assay is a highly robust assay that requires the use of SBI's optimized CRISPY Master Mix—for example, ThermoFisher's SYBR Green master mix will amplify both edited (Figure 3A) and wildtype (Figure 3B) templates and provides indistinguishable melt curves (Figure 3C).

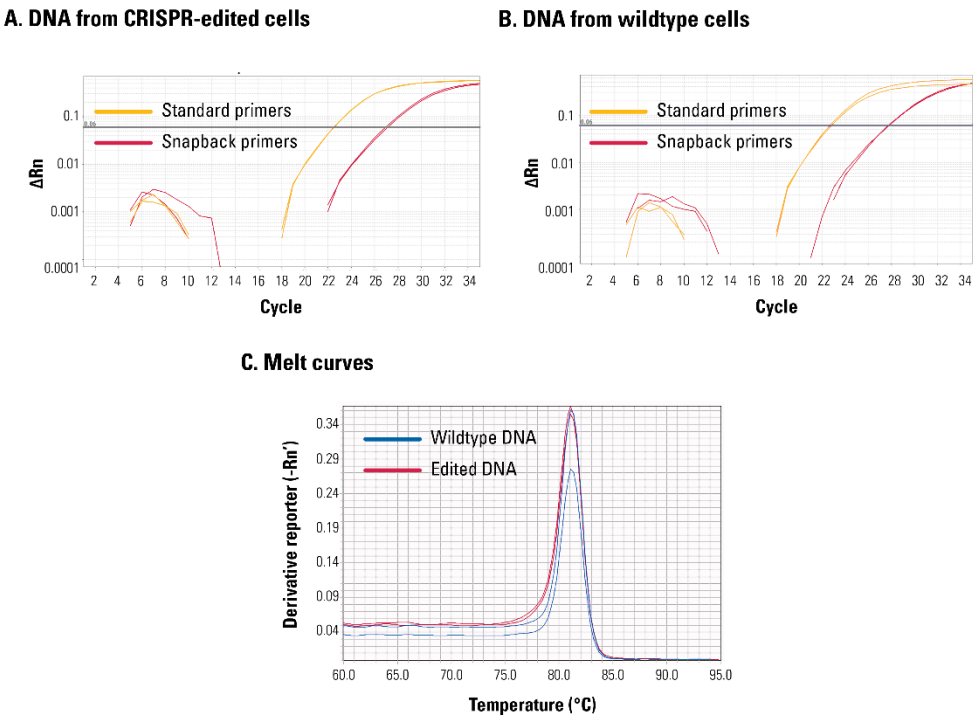

Figure 3. The CRISPY assay requires SBI's optimized master mix. *Amplification curves from qPCR reactions run using ThermoFisher SYBR Green master mix and (A) DNA isolated from CRISPR-edited cells and (B) DNA isolated from wildtype cells. (C) Melt curves from A and B.*

The CRISPY assay delivers excellent quantitation when performed directly on cells

To demonstrate the robustness of the CRISPY assay when using cells, we designed a new set of primers to assess the success of editing the same cells used in Figure 2. The ΔCt between standard and snapback primers in a CRISPY assay done directly on cells (Figure 4A) is virtually identical to the ΔCt in a CRISPY assay done on genomic DNA isolated from the same cells (Figure 4B), demonstrating that direct amplification of cells mirrors the results from purified DNA. This finding is also supported by comparing the melt curves performed on cells (Figure 4C) and isolated DNA (Figure 4D).

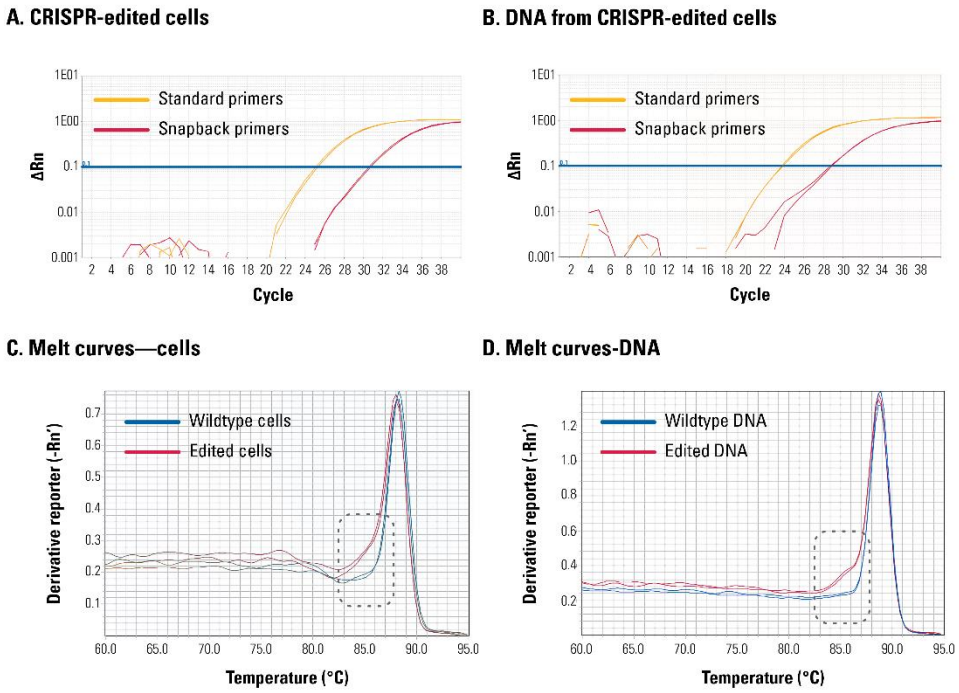

Figure 4. The CRISPY assay delivers excellent quantitation when performed directly on cells. *Amplification curves from (A) CRISPR-edited cells and (B) DNA isolated from CRISPR-edited cells. Melt curves from A (C) and B (D).*

Technical Support

For more information about SBI products and to download manuals in PDF format, please visit our web site: http://www.systembio.com

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