On- and off-target detection in a CRISPR-edited mammalian cell line using a single-step 3-color Crystal Digital PCR™ assay

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Assessing CRISPR-edited mammalian cell lines using a single-step 3-color Crystal Digital PCR™ assay

The generation and usage of homozygous fluorescent knock-in mammalian cell lines using sequence-specific Cas-endonucleases has become a commonly used molecular biology procedure.[1-8] Nevertheless, to identify the desired genetic modification and reject clones with off-target events, several hundred monoclonal colonies still need to be screened. The maintenance and screening of high numbers of clones is a laborious, time-consuming, and error-prone process. To facilitate colony screening, we implemented a single-step 3-color assay using Crystal Digital PCR™ to assess on-target copy number and detect off-target events simultaneously. Because digital PCR (dPCR) is highly sensitive, it requires low amounts of biological samples. Thus, the screening assay was integrated early in the genome engineering pipeline. In addition, because the single-step 3-color Crystal Digital PCR™ assay is achieved in a single assay in about three hours, the culture of incorrectly edited clones can be discontinued on the same day and early in the pipeline.

Highlights

- Crystal Digital PCR™, provides a robust and quantitative tool to assess on-target copy number and detect off-target events in a single 3-color assay.
- Crystal Digital PCR™ allows robust copy number assessment of large insertions such as *mEGFP*.
- Crystal Digital PCR™ uses small amounts of biological material and can be introduced early in the CRISPR screening pipeline, thus saving time.

Single-step 3-color digital PCR assay design (Fig. 1A)

- ***Total tag integration design*** | The "total-*mEGFP*" assay detects the presence of the *mEGFP* sequence in the genome. PCR primers and a green HEX fluorescent probe anneal to the *mEGFP* sequence and are designed to detect only the *mEGFP* transgene.
- ***On-target integration design*** | The "on-target-tag" assay detects the junction between the last exon of the endogenous *NUP93* editing site and the 5'- sequence of the integrated *mEGFP* transgene (tag). PCR primers and a blue FAM fluorescent probe are designed to amplify and detect the "on-target tag" for the determination of scar-free 5' integration.
- ***Reference design*** | The "reference" assay detects a sequence roughly 8-10 kb downstream of the target site, on the same chromosome. PCR primers and a red Cy®5 fluorescent probe anneal to the reference sequence and are designed to detect only the "reference" product, which is comparable in size to the "on-target-tag" and "total-*mEGFP*" amplicons.
- ***Computation of total mEGFP copies*** | The number of integrated *mEGFP* copies was obtained by normalizing the copies (cp)/µL of the "total *mEGFP*" assay with the cp/µL of the "reference" assay.
- ***Computation of on-target mEGFP copies*** | The number of on-target integrated *mEGFP* copies was obtained by normalizing the cp/µL of the "on-target-tag" assay with the cp/µL of the "reference" assay.

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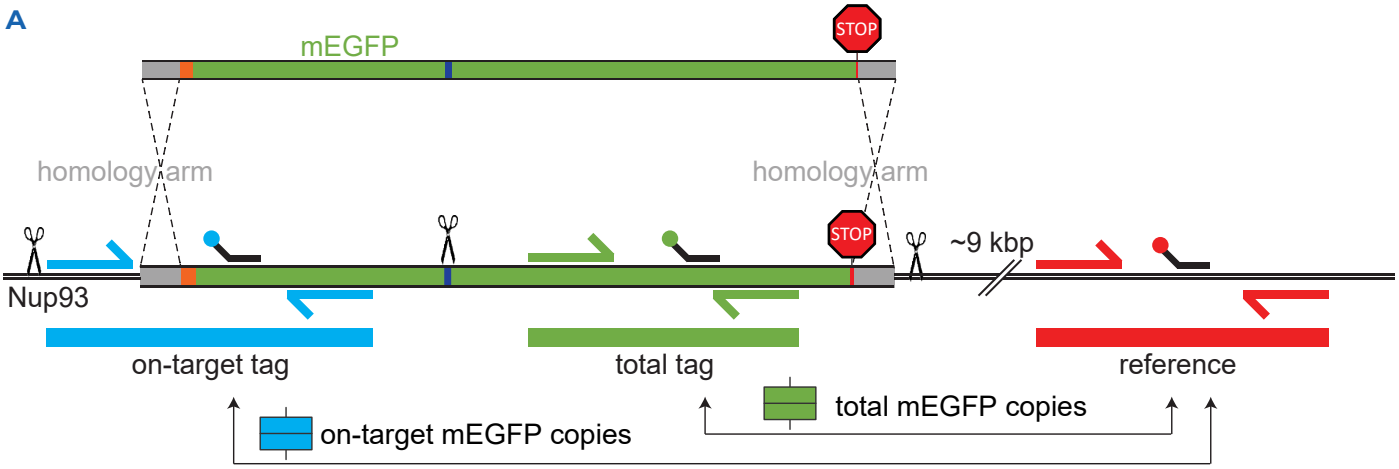
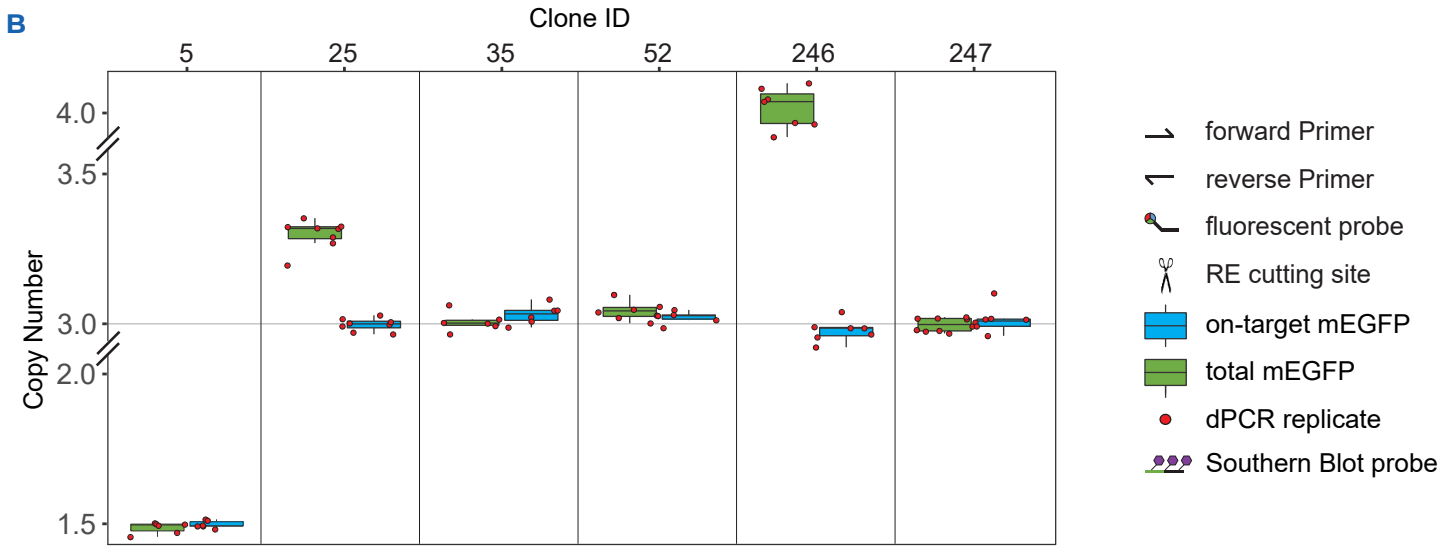
Figure 1 | Single-step 3-color dPCR assay validation using CRISPR-edited U-2 OS Nup93-*mEGFP* clones.

Modified from BioRxiv: https://www.biorxiv.org/content/10.1101/2021.06.23.449557v1





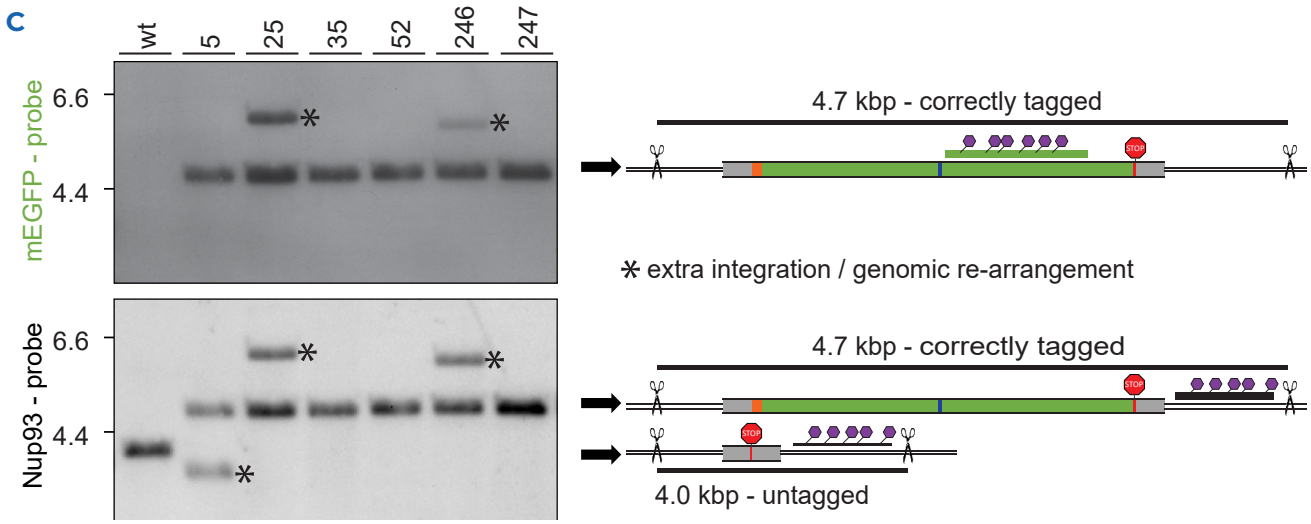


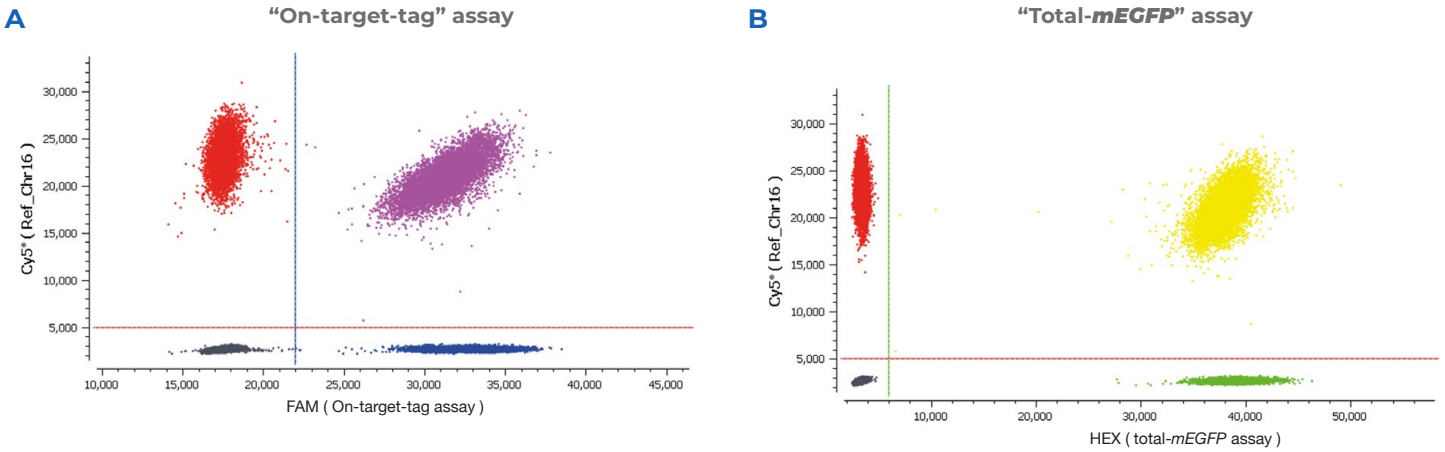
Figure 1 *continued* | Single-step 3-color dPCR assay validation using CRISPR-edited U-2 OS Nup93-*mEGFP* clones

1A ***Donor design*** (top) | A PCR product, amplified from a synthetic dsDNA template, is used as donor. It includes a short sequence (~50 bp homology arm) homologous to the 5'-region of *NUP93* (in grey), a linker (~5-10 amino acids, orange square), a STOP codon and a short sequence (50bp homology arm) homologous to the 3'-UTR of *NUP93* (in grey). Notably, the *mEGFP* sequence contains a silent mutation to generate a restriction to have independent templates during digital PCR (deep blue bar). A STOP codon and a short sequence (50bp homology arm) homologous to the 3'-UTR of *NUP93* (in grey). ***Single-step 3-color Crystal Digital PCR™ assay design*** (bottom) | Dashed line indicates homology-directed repair (HDR) after the targeted spCas9-mediated double-strand break (DSB). Primer positions are shown to generate products corresponding to "on-target tag," "total *mEGFP*," and "reference" assays. Arrows point to the ratios of products representing total *mEGFP* copies and on-target *mEGFP* copies.

1B ***Crystal Digital PCR™ measurements of six representative clones*** | Boxes represent on-target (blue) and total *mEGFP* (green) copy numbers. Every red dot represents an individual dPCR measurement (technical replicates). Allelicity of the *NUP93* gene in U-2 OS cells is three. Clones (clone ID 35, 52 and 347) scoring at 3 on-target and at 3 total *mEGFP* copies are homozygous for *NUP93-mEGFP*. A total number of *mEGFP* copies higher than 3 suggests extra integrations (clone ID 25 and 246). Estimates lower than 3 indicate either fewer tag integrations (monoallelic or biallelic clones) or potential genomic rearrangements (clone ID 5).

1C ***Southern Blot performed with the same clones as in B*** | The band at ~4.7kb indicates a correctly tagged *NUP93-mEGFP* allele detected with a *mEGFP*-specific probe. Any additional band detected using the *mEGFP*-specific probe indicates an extra integration of the tag in the genome. The *Nup93*-specific probe reveals the correctly tagged *NUP93-mEGFP* alleles at ~4.7 kb and the untagged alleles at ~4.0 kb. Bands marked with an asterisk correspond to unexpected, extra integrations or major genomic re-arrangements.

Figure 2 | Representative 2D dot plots from Crystal Miner software



2A "On-target-tag" assay detected by the FAM probe (On-target-tag assay) that binds to the junction between the last exon of the endogenous *NUP93* editing site and 5' sequence of the integrated *mEGFP* transgene.

2B "Total-*mEGFP*" assay detected by the HEX probe (total-*mEGFP* assay) that binds to the *mEGFP* sequence and is designed to detect only the *mEGFP* transgene. The reference assay is detected by a Cy®5 probe and binds to a sequence downstream of the target site on the same chromosome (Chr16).

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- ***Conclusion about the CRISPR-driven integration event (homozygous, monoallelic, biallelic, off-targets integration, error-prone integration)*** | If the ratio of the scores retrieved from the two assays (total *mEGFP* and on-target-tag assay) is not equal to 1, then an adverse editing event occurred (such as off-target integration, mis-editing, or incomplete editing).
- An in-house developed "R" notebook allows automated copy number assessment of clones and is available at https://github.com/beatrizserrano/CRISPR_updated_protocol.
- The internal probes of the three assays were labeled with **FAM**, **HEX** or **Cy®5**.

Notes

- The forward oligo of the "on-target-tag" assay should anneal outside of the homology arm of the CRISPR donor template.
- Short homology arms should be designed (≤50bp) to optimize Crystal Digital PCR™ performance and accuracy.
- All Crystal Digital PCR™ templates must be separated molecules. This can be achieved by restriction digestion of the genomic DNA. If a specific restriction site is not available within the donor template, a silent mutation can be generated to obtain an extra cutting site, as for *mEGFP*.
- The "reference" assay should be designed on the same chromosome as the on-target assay. Although unlikely to occur, an off-target event happening within the reference locus would lead to incorrect copy number estimates.
- A positive control for the "on-target-tag" and the "total-*mEGFP*" assays can be performed using wildtype genomic DNA – that is, unmodified – plus a donor plasmid carrying the desired genomic target region.
- See also the technical note: Guidelines for 3-color multiplex assay design for optimized performance with Crystal Digital PCR™ https://www.stillatechnologies.com/guidelines-for-3-color-multiplex-assay-design-for-optimized-performance-with-crystal-digital-pcr/

Results

We have developed a single-step 3-color Crystal Digital PCR™ assay and assessed its predictive power by comparing it to a conventional Southern blot. There are three *NUP93* alleles in U-2 OS cells. This quantitative assay showed that clones 35, 52 and 247 scored 3 on-target and total *mEGFP* copies, meaning that all three *NUP93* alleles were successfully tagged (i.e. homozygous clones), with no detectable off-target integrations. The Southern blot confirmed this conclusion. Clone 5 shows an equal number of *mEGFP* copies for both assays, though without matching the expected number of *NUP93* loci. The copy number assessment may indicate a single, correct editing event accompanied by a major genomic re-arrangement, as suggested by Southern Blot analysis. Clone 25 and 246 show a high number of total *mEGFP* integrations vs on-target integrations, indicating potential off-target editing or incomplete HDR. Southern Blot displays extra bands detected with the *mEGFP* and *NUP93* probes, suggesting genome rearrangements. Results obtained by 3-color Crystal Digital PCR™, *a straightforward one-step detection assay*, were perfectly concordant with the more labor-intensive southern blot technique.

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To learn more about digital PCR, please visit Stilla Technologies' Learning Center at www.gene-pi.com

Check out our joint webinar: https://www.youtube.com/watch?v=ErEr5xyCWek

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