

# Fast and Efficient Workflow to Monitor CRISPR-Cas9 Gene Editing of Haploid Cell Lines

Laura Moriarty, PhD; Meiyue Wu, PhD; and Elizabeth Jordan, PhD  
Bio-Rad Laboratories, Inc., 2000 Alfred Nobel Drive, Hercules, CA



## Drug Discovery and Development

Bulletin 7079

### Abstract

A great deal of excitement surrounded the introduction of CRISPR as a simplified gene editing technique. Its speed, low cost, and ability to be used in many model systems redefine what is possible in drug discovery and development. However, current workflows for generating knockout cell lines using CRISPR-Cas9 can be complex and inefficient. In this study, we demonstrate a novel workflow that includes Droplet Digital™ PCR, high resolution melt analysis, western blotting, and real-time PCR to generate and validate a knockout haploid cell line in less than five weeks.

### Introduction

CRISPR-Cas9 is the simplest gene editing technique discovered to date. Based on the adaptive immune system of bacteria and archaea, the CRISPR-Cas9 system uses short RNAs to direct Cas9 nuclease to target DNA sequences. Because it uses RNA rather than a protein to target nuclease activity, CRISPR-Cas9 can be retargeted by simply synthesizing or ordering a new guide RNA (gRNA), which also makes this technique amenable to high order multiplexing. These attributes make CRISPR-Cas9 ideal for introducing mutations in protein-encoding genomic DNA regions, which allows protein function to be studied in its native cellular context.

In this study, we report a novel workflow that incorporates Droplet Digital PCR (ddPCR™), high resolution melt (HRM) analysis, western blotting, and RT-PCR to generate Sanger sequencing validated knockout haploid human cell lines. For this experiment we targeted the *BUB1B* gene, which encodes a kinase involved in spindle checkpoint function and is implicated

in cancer, as the loss or reduced function of the spindle checkpoint has been found in many forms of cancer. The knock out or knock down of genes implicated in cancer is one of the many ways gene editing is being used to further study disease, as it allows researchers to model the effects of different mutations.

Our Haploid Gene Editing Workflow (Figure 1) combines multiple techniques to provide edited cells in less than five weeks by allowing easy assessment of transfection protocols and quick confirmation of edits, and through the inclusion of validated tools to analyze both gene and protein expression. By using haploid cells in this workflow we were able to simplify the workflow and associated data analysis because only a single inactivated allele is required to generate a loss-of-function phenotype. The resulting edited haploid cell lines can be used in many downstream assays such as target identification, mechanism of action studies, and cell-based toxicity assays.

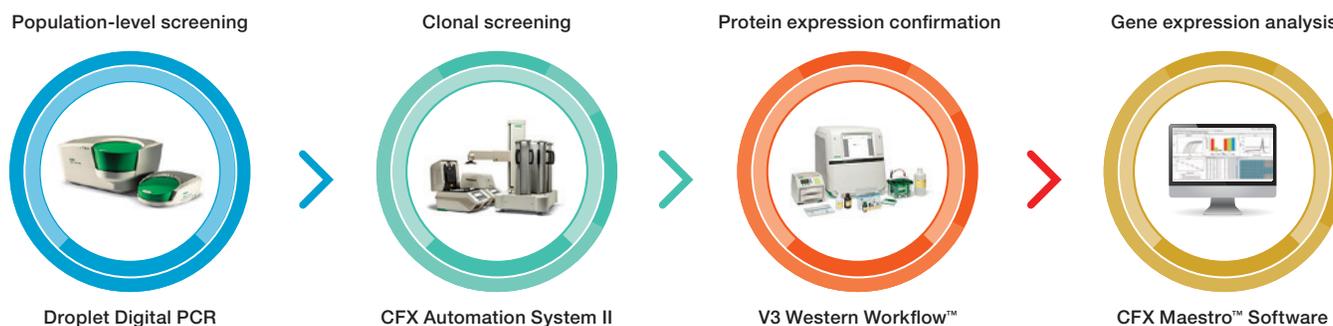


Fig. 1. Haploid Gene Editing Workflow.

## Materials and Methods

### Gene Editing

eHAP-1 cells (Horizon Discovery) were transfected with CRISPR-Cas9 targeting *BUB1B*. The clones were sent out for Sanger sequencing to confirm edits.

### Non-Homologous End Joining (NHEJ) Assay

The ddPCR NHEJ Genome Edit Detection Assay specific for our *BUB1B* mutation was designed on [bio-rad.com/digital-assays](http://bio-rad.com/digital-assays) (catalog #12002314). The assay was run on the QX200™ ddPCR System and the fractional abundance of NHEJ DNA was determined using QuantaSoft™ Analysis Pro Software as described in the ddPCR NHEJ Genome Edit Detection Assay protocol (#10000065278).

### High Resolution Melt (HRM) Assay

A high resolution melt assay was designed and run as per the Precision Melt Analysis™ Software Instruction Manual (#10000080911). Briefly, cells were lysed using the SingleShot™ Cell Lysis Kit (catalog #1725080) to generate gDNA directly from cell culture, and HRM analysis was performed using Precision Melt Supermix for High Resolution Melt Analysis (catalog #1725110) on the CFX384 Touch™ Real-Time PCR Detection System (catalog #1855485). Data analysis was performed using Precision Melt Analysis Software (catalog #1845015).

### Protein Expression Analysis

The V3 Western Workflow was used to determine protein expression. Briefly, 25 µg of cell lysate was applied to 12% Mini-PROTEAN® TGX™ Gels (catalog #4561043). After protein separation and blotting, the presence or absence of BUB1B protein was confirmed using PrecisionAb™ Validated Western Blotting Antibodies directed against BUB1B (monoclonal antibody, catalog #vma00256). Actin was visualized with a rhodamine-conjugated anti-actin human Fab fragment antibody (catalog #12004163). Chemiluminescence detection was performed using Clarity™ Western ECL Substrate (catalog #1705061). Western blot image analysis was performed using the ChemiDoc™ MP Imaging System (catalog #17001402).

### Gene Expression Analysis

The PrimePCR™ Cell Cycle Tier 1–4 Panel with 379 genes and 5 controls was used to quantify changes in gene expression between the edited and wild-type eHAP-1 cell lines. cDNA was prepared from control wild-type and *BUB1B*-edited eHAP1 RNAs using iScript™ gDNA Clear cDNA Synthesis Kit (catalog #1725034). SsoAdvanced™ Universal SYBR® Green Supermix (catalog #1725270) was used to analyze 10 ng cDNA per reaction on a total of six PrimePCR Cell Cycle Tier 1–4 Panel plates with samples run in triplicate. Plates were run using one CFX Automation System II (catalog #1845075, robotic plate handler) connected to two CFX384 Touch™ Real-Time PCR Detection Systems (catalog #1855485). Data were analyzed using CFX Maestro Software v1.1 (catalog #12004110).

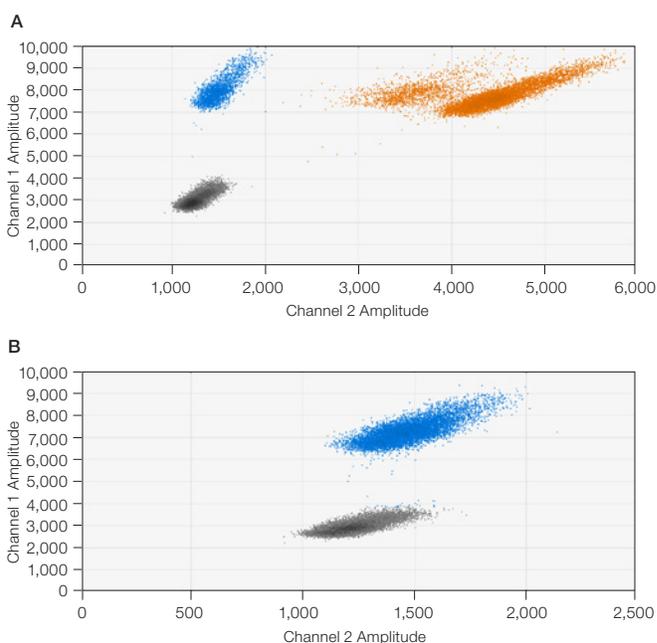
## Results and Discussion

Our Haploid Gene Editing Workflow provides a fast and efficient method for the generation, analysis, and confirmation of edits for downstream cell-based assays.

### Population-Level Screening

Droplet Digital PCR (ddPCR) NHEJ drop-off assays allowed us to identify and quantify the presence of edits and determine the percentage of edited cells using population-level screening. This is an alternative to incorporating markers, such as GFP, into editing strategies and enriching for edited cells by selecting only those cells that show fluorescence. The rapid readout enabled by ddPCR technology allows quick optimization of transfection assay conditions by adjusting the amount of cells used and switching from electroporation to lipid-based transfection methods to achieve the most efficient transfection workflow.

Using the NHEJ drop-off assay, we were able to determine that after initial transfection none of our clones contained 100% edited cells; data shown for one of our clones, *BUB1B-1*, demonstrates 25% edited cells (Figure 2A). To achieve a clonal population, single cells from edited clones were isolated and further expanded. The initial fractional abundance of edits was used to identify how many single cells to pick and carry forward for each clone. For example, for *BUB1B-1* with 25% edited cells, six clones were picked to ensure at least one clone that contained the desired edits was carried forward. This reduces the number of clones to carry forward, thereby reducing costs of plastics and media.

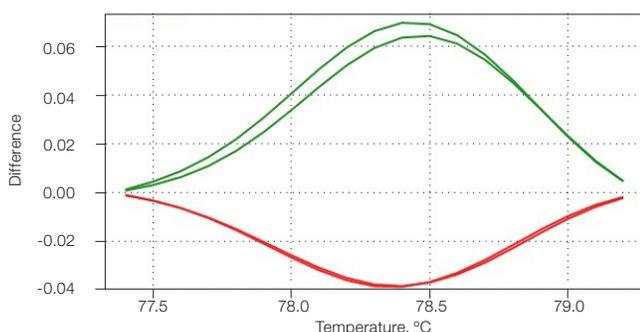


**Fig 2. 2-D plots of NHEJ ddPCR drop-off assay results.** Droplets that contain unedited DNA are positive for both FAM and HEX signal (shown in red). Droplets that contain NHEJ-edited DNA are positive for FAM only (shown in blue). **A**, edited DNA from a nonclonal population of cells (clone *BUB1B-1*) and **B**, edited DNA from a clonal population of edited cells (clone *BUB1B-2*).

After isolation and enrichment, the NHEJ assay was performed again, and clone *BUB1B-2* was found to be a fully clonal population with 100% edited cells (Figure 2B).

### Clonal Screening

Further confirmation of edits was performed at the clonal level using HRM analysis. HRM provides orthogonal data to confirm differences in genetic sequences as a result of gene editing. We used HRM in place of mismatch assays, which are more laborious to perform and not as sensitive. The HRM curves generated from edited and wild-type *BUB1B* clones showed significant differences (Figure 3). These differences suggest changes in target sequences and are indicative of successful gene editing.



**Fig. 3. HRM analysis of single-cell clones.** HRM analysis of single-cell clones harvested in SingleShot Lysis Buffer without DNase. Precision Melt Analysis Software was used to plot the melt curves. Wild-type clone (—); *BUB1B-2* clone (—).

### Genetic Sequence Analysis

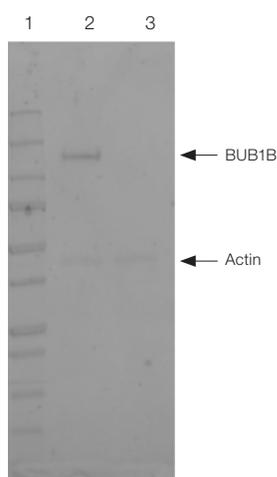
Sanger sequencing results (Figure 4) validated the NHEJ and HRM assay results by providing the sequence of the edited clones. In this example, the edited *BUB1B-2* clone had a 5 bp deletion in exon 3, which inserted a stop codon after 231 nucleotides of the mRNA.

Wild type												
68	69	70	71	72	73	74	75	76	77	78	79	80
TTT	TAC	ACT	GGA	AAT	GAC	CCT	CTG	GAT	GTT	TGG	GAT	AGG
Phe	Tyr	Thr	Gly	Asn	Asp	Pro	Leu	Glu	Val	Trp	Asp	Arg
<i>BUB1B-2</i>												
68	69	70	71	72	73	74	75	76	77	78	79	80
TTT	TAC	ACT	GGA	AAC	TCT	GGA	TGT	TTG	GGA	TAG		
Phe	Tyr	Thr	Gly	Asn	Ser	Gly	Cys	Leu	Gly	*		

**Fig. 4. Sanger sequencing data for *BUB1B-2* clone.** Sequencing data show a 5 bp deletion that results in a frameshift with a premature stop codon. Deleted 5 bp are shown in blue. Changes in the *BUB1B-2* nucleotide and protein sequence, including the early stop codon (\*) that results from this frameshift mutation, are shown in red.

### Protein Expression Confirmation

In the event of an in-frame edit instead of complete removal of a gene, some protein expression may still occur. Western blot analysis is a powerful tool for assessing the extent of protein expression knockdown in edited cells before the cell line is used in downstream assays. We were able to streamline this process by using the V3 Western Workflow, which quantifies protein expression levels in hours rather than days. We used PrecisionAb Western Blotting Antibodies, which have been validated, thereby reducing the need to perform additional assays to confirm their activity and specificity; however, if required, control lysates are provided as positive controls for such assays. We saw no expression of the *BUB1B* protein in the edited *BUB1B-2* clone (Figure 5).



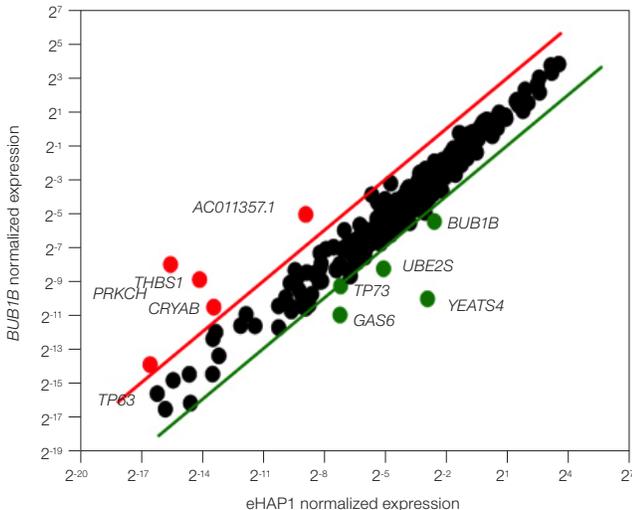
**Fig. 5. Western blot analysis of wild-type and knockout *BUB1B-2* cells.** Lane 1, Precision Plus Protein™ All Blue Prestained Protein Standards (catalog #1610373); lane 2, wild-type *BUB1B* clone; lane 3, *BUB1B* knockout clone *BUB1B-2*; wild-type *BUB1B*, 120 kD; actin loading control, ~42 kD.

### Gene Expression Analysis

Many assays can be run downstream of edited cell line production. In knockout studies it is common to explore gene expression profiles not only of the affected gene(s) but also of related genes or pathways to look for interdependencies.

By using two CFX Real-Time PCR Detection Systems connected to the CFX Automation System II we were able to collect data from twelve 384-well plates and measure the expression of genes from three different screening panels in under 4.5 hours. We compared gene expression of the wild type with the *BUB1B* knockout across 379 genes using the PrimePCR Cell Cycle Tier 1–4 Panel (Figure 6). Analysis using CFX Maestro Software identified five genes that were upregulated in the *BUB1B* knockout: *TP63*, *CRYAB*, *PRKCH*, *THBS1*, and *AC011257.1*. Additionally, five

genes were found to be downregulated: *TP73*, *UBE2S*, *GAS6*, *YEATS4*, and, as expected, *BUB1B*. Interestingly, *PRKCH*, a gene encoding a serine- and threonine-specific protein kinase similar to *BUB1B*, is upregulated in the knockout cell line, suggesting that the upregulation of this kinase may be upregulated to compensate for loss of *BUB1B* function.



**Fig. 6. Gene expression analysis of *BUB1B*-2 knockout cells.** A scatter plot provides a view of changes in expression. The red line represents a fourfold increase and the green line a fourfold decrease in expression compared to control cells (eHAP-1 wild type).

## Conclusion

We have demonstrated a simplified workflow that generated a *BUB1B* human haploid knockout cell line in less than five weeks. We were able to obtain robust data in a compressed timeline by making the following adjustments to traditional workflows:

- Using Droplet Digital PCR NEJ drop-off assays to provide a quick readout of the fractional abundance of edited cells instead of using reporter genes/markers
- Replacing more laborious, less sensitive mismatch assays with HRM analysis for confirmation of changes to genetic sequences
- Employing the V3 Western Workflow for protein quantitation to save up to a day in assay time and to provide confirmatory data at each stage of the western blotting workflow

- Utilizing validated antibodies that eliminate additional assay time and cost by removing the need to perform in-house confirmation of antibody activity and specificity
- Pairing the CFX Real-Time PCR Detection System with the CFX Automation System II to reduce hands-on time
- Taking advantage of CFX Maestro Software to allow easy identification of changes in gene expression in response to edits to the genome

Edited cell lines are key to many stages of drug development, from basic research to understanding the key pathways and associations between genes and/or proteins implicated in disease to downstream assays where the efficacy of potential drugs is being measured in cell-based models. The ability to generate edited cell lines within a shorter time frame using our Haploid Gene Editing Workflow can help accelerate these processes and support faster drug discovery and development.

## Acknowledgements

We acknowledge the critical work of Meiyue Wu, PhD; Steven Okino, PhD; Gerald Uy; Deanna Woo; Mark Shulewitz, PhD; and Yan Wang, PhD from Reagent R&D, LSG, Bio-Rad Laboratories, Inc. for the generation of the haploid cell lines.

Visit [bio-rad.com/web/GeneEditingHaploid](http://bio-rad.com/web/GeneEditingHaploid) for more information.

The QX200 Droplet Digital PCR System and/or its use is covered by claims of U.S. patents, and/or pending U.S. and non-U.S. patent applications owned by or under license to Bio-Rad Laboratories, Inc., including, but not limited to, U.S. Patent Nos. 9,089,844; 9,126,160; 9,216,392; 9,347,059; 9,500,664; 9,636,682; and 9,649,635. Purchase of the product includes a limited, non-transferable right under such intellectual property for use of the product for internal research purposes in the field of digital PCR only. No rights are granted for diagnostic uses. No rights are granted for use of the product for commercial applications of any kind, including but not limited to manufacturing, quality control, or commercial services, such as contract services or fee for services. Information concerning a license for such uses can be obtained from Bio-Rad Laboratories. It is the responsibility of the purchaser/end user to acquire any additional intellectual property rights that may be required.

Bio-Rad's thermal cyclers and real-time thermal cyclers are covered by one or more of the following U.S. patents or their foreign counterparts owned by Eppendorf AG: U.S. Patent Numbers 6,767,512 and 7,074,367.

Precision Plus Protein Standards are sold under license from Life Technologies Corporation, Carlsbad, CA for use only by the buyer of the product. The buyer is not authorized to sell or resell this product or its components.

FAM and HEX are trademarks of Life Technologies Corporation. SYBR is a trademark of Life Technologies Corporation. Bio-Rad Laboratories, Inc. is licensed by Life Technologies Corporation to sell reagents containing SYBR Green I for use in real-time PCR, for research purposes only.

**BIO-RAD**

**Bio-Rad  
Laboratories, Inc.**

Life Science  
Group

Web site [bio-rad.com](http://bio-rad.com) USA 1 800 424 6723 Australia 61 2 9914 2800 Austria 43 01 877 89019 Belgium 32 03 710 53 00 Brazil 55 11 3065 7550  
Canada 1 905 364 3435 China 86 21 6169 8500 Czech Republic 36 01 459 6192 Denmark 45 04 452 10 00 Finland 35 08 980 422 00 France 33 01 479 593 00  
Germany 49 089 3188 4393 Hong Kong 852 2789 3300 Hungary 36 01 459 6190 India 91 124 4029300 Israel 972 03 963 6050 Italy 39 02 49486600  
Japan 81 3 6361 7000 Korea 82 2 3473 4460 Mexico 52 555 488 7670 The Netherlands 31 0 318 540 666 New Zealand 64 9 415 2280 Norway 47 0 233 841 30  
Poland 36 01 459 6191 Portugal 351 21 4727717 Russia 7 495 721 14 04 Singapore 65 6415 3188 South Africa 36 01 459 6193 Spain 34 091 49 06 580  
Sweden 46 08 555 127 00 Switzerland 41 0617 17 9555 Taiwan 886 2 2578 7189 Thailand 66 2 651 8311 United Arab Emirates 971 4 8187300  
United Kingdom 44 01923 47 1301

