

Protocol

Detection of Indel Mutations in *Drosophila* by High-Resolution Melt Analysis (HRMA)

Benjamin E. Housden^{1,2} and Norbert Perrimon^{1,2,3}

¹Department of Genetics, Harvard Medical School, Boston, Massachusetts 02115; ²Howard Hughes Medical Institute, Harvard Medical School, Boston, Massachusetts 02115

Although CRISPR technology allows specific genome alterations to be created with relative ease, detection of these events can be problematic. For example, CRISPR-induced double-strand breaks are often repaired imprecisely to generate unpredictable short indel mutations. Detection of these events requires the use of molecular screening techniques such as endonuclease assays, restriction profiling, or high-resolution melt analysis (HRMA). Here, we provide detailed protocols for HRMA-based mutation screening in *Drosophila* and analysis of the resulting data using the online tool HRMAAnalyzer.



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.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at <http://cshprotocols.cshlp.org/site/recipes>.

Reagents

Agarose

Buffer SB <R>

Chemically competent *E. coli* and reagents for transformation

DNA sequencing primers and reagents

M13F: 5'-GTAAAACGACGGCCAGT-3'

M13R: 5'-CAGGAAACAGCTATGACC-3'

dNTPs (10 mM)

Drosophila for HRMA (anesthetized adult flies or cells), with wild-type controls

Gel purification kit (e.g., QIAGEN) (as needed; see Step 18)

High-fidelity polymerase enzyme and buffer (e.g., Phusion with 5× HF buffer; New England BioLabs)

LB liquid medium <R> with ampicillin for selection

In addition, prepare plates containing LB medium solidified with agar (plus ampicillin).

³Correspondence: bhousden@genetics.med.harvard.edu; perrimon@receptor.med.harvard.edu

MgCl₂ (100 mM)

Oligonucleotide primers for polymerase chain reaction (PCR) and high-resolution melt analysis (HRMA)

We recommend using Primer3 (<http://bioinfo.ut.ee/primer3>) or a similar program to design primers for PCR and HRMA. Design one set of PCR primers to amplify a 300- to 600-bp fragment surrounding the sgRNA target site (Step 2). Design a second set of HRMA primers to amplify a 45- to 150-bp fragment contained within the sgRNA PCR product (Step 5).

Plasmid DNA miniprep kit (e.g., QIAGEN)

Precision Melt Supermix (Bio-Rad)

Zero Blunt TOPO PCR cloning kit (Invitrogen)

Equipment

Bullet blender or pestles for homogenization in 1.5-mL microcentrifuge tubes (for adult flies)

Centrifuge

Heat blocks or water baths at 50°C and 95°C

HRMANalyzer (<http://www.flyrnai.org/hrma>)

Microcentrifuge tubes (1.5 mL)

PCR tubes

RT-PCR (reverse transcription-polymerase chain reaction) machine capable of reading at temperature intervals of 0.1°C

Thermocycler

METHOD

Performing the HRMA Reaction

To generate P-values from HRMA indicating the likelihood of experimental samples containing different sequence versus the controls, at least three control samples (i.e., using wild-type DNA) must be present; however, the quality of output data is improved with additional controls. For the following protocol, we recommend performing at least three control reactions for simple samples (e.g., from diploid flies) and at least eight controls for complex samples (e.g., from mixed cell populations).

1. Prepare genomic DNA from adult flies or cells as follows.

Adult Flies

- i. Place an anesthetized fly into a 1.5-mL tube with 50 µL of buffer SB.
- ii. Thoroughly homogenize the sample using a bullet blender or pestle.
- iii. Centrifuge the sample at maximum speed for 1 min to pellet the remaining tissue fragments. Transfer the supernatant to a PCR tube.
- iv. Incubate the supernatant for 1 h at 50°C followed by 10 min at 95°C.

Cells

- v. Collect 10,000–100,000 cells in a 1.5-mL microcentrifuge tube. Pellet the cells by centrifugation at 5000g for 2 min.
 - vi. Resuspend the cells in 100 µL of buffer SB.
 - vii. Incubate the sample for 1 h at 50°C followed by 10 min at 95°C.
2. Assemble the following PCR to amplify the 300- to 600-bp sequence surrounding the sgRNA target locus.

Reagent	Amount to add
Genomic DNA	1 μ L
Phusion HF buffer	10 μ L
dNTPs (10 mM)	1 μ L
Forward PCR primer (10 μ M)	1 μ L
Reverse PCR primer (10 μ M)	1 μ L
Phusion polymerase	0.5 μ L
MgCl ₂ (100 mM)	1.25 μ L
H ₂ O	33.25 μ L

3. Transfer the reaction to a thermocycler and run the following program.

	Temperature	Time
Start	98°C	3 min
35 cycles	98°C	30 sec
	50°C	30 sec
	72°C	30 sec
	10°C	
Hold	10°C	

This PCR is designed to amplify products with low specificity using a wide range of primer pairs and will likely produce multiple nonspecific bands. In general, it is not necessary to check the product on a gel, as low concentrations are sufficient for the HRMA reaction and nonspecific products are not a concern.

4. Dilute the PCR products 1:10,000 using H₂O.
 5. Assemble the HRMA reaction using primers that amplify a 45- to 150-bp fragment contained within the sgRNA PCR product.

Reagent	Amount to add
DNA template from Step 4	1 μ L
Precision Melt Supermix	5 μ L
Forward HRMA primer (10 μ M)	0.3 μ L
Reverse HRMA primer (10 μ M)	0.3 μ L
H ₂ O	3.4 μ L

6. Transfer the reaction to an RT-PCR machine and run the following program.

	Temperature	Time
Start	95°C	3 min
50 cycles	95°C	18 sec
	50°C	30 sec
	Fluorescence read	
1 cycle	95°C	2 min
	25°C	2 min
	4°C	2 min
	Melt curve	55°C to 95°C

See Troubleshooting.

Analyzing Data Using HRMA Analyzer

7. Export the HRMA data from the RT-PCR machine with the following format. (See the help file at www.flyrnai.org/HRMAhelp.html.)



Column 1	Temperature
Columns 2–End	Fluorescence readings
Row 1	Headings (Temperature; Sample name 1; Sample name 2; etc.)

All control sample names must contain the word “control.”

Many RT-PCR machines automatically convert fluorescence readings to $-dF/dT$. The analysis will not work using this format; thus, be sure to export the raw data (usually called RFU data).

- Convert the file format to .xlsx or .txt
- Upload the melt data file to HRMAAnalyzer at: <http://www.flyrnai.org/hrma> (Fig. 1A).
- Click “Generate Figures.”
- Use the “Data quality cutoff” slider to remove any samples that were not amplified efficiently and click “next.”

Samples not amplified efficiently will have considerably lower fluorescence values than other samples and will often lead to false positive results.

- Select the upper and lower normalization ranges by dragging the vertical bars or entering values directly into the relevant boxes. Click “Generate Figures” and then “Next” (Fig. 1B).

The upper normalization range should be positioned to the left of the melting region and the lower range below the melting region (Fig. 1B). Both ranges should cover 3°C–8°C, although this parameter is relatively flexible.

The “Overlay” option can be used to shift all melt curves along the horizontal axis such that they cross at a point defined by the “Overlay position” slider. This allows comparison of curve shape independent of the melting temperature and can be useful for separation of heterozygous and homozygous samples. It is generally not required and is not recommended for analysis of samples from complex backgrounds, such as in cell culture experiments in which many different sequences may be present.

- Click “Generate Figures” to obtain a summary graph of the results with hits shown with blue lines and nonhits with black lines (Fig. 1C).

The clustering option can be used to group samples likely to have the same composition (e.g., carrying the same mutation). However, this option does not perform well on complex samples in which many sequences are present.

- Click “Download Data” to retrieve data from each step of the analysis process.

Several files will be downloaded, including the data plotted on the graphs at each analysis step. In addition, a file named “Data_significance.txt” will include a P-value representing the likelihood of each sample containing sequence different from control samples, and a value labeled “Total area” which can be used to rank the results (high values indicate greater difference from control melt curves).

See Troubleshooting.

Analyzing Hits by Sequencing

HRMAAnalyzer provides a list of samples likely to have differing sequences from the controls. When working with relatively simple samples, such as those derived from heterozygous mutant flies, this analysis is relatively reliable. However, in cell lines in which many different mutations may be present at potentially low levels, HRMA signals may be weak. It is therefore advisable in these cases to confirm the presence and nature of mutations by sequencing following HRMA.

- Assemble the following PCR to amplify the region surrounding the mutation.

Reagent	Amount to add
Genomic DNA	1 μ L
Phusion HF buffer	10 μ L
dNTPs (10 mM)	1 μ L
Forward PCR primer (10 μ M)	1 μ L
Reverse PCR primer (10 μ M)	1 μ L
Phusion polymerase	0.5 μ L
H ₂ O	34.5 μ L

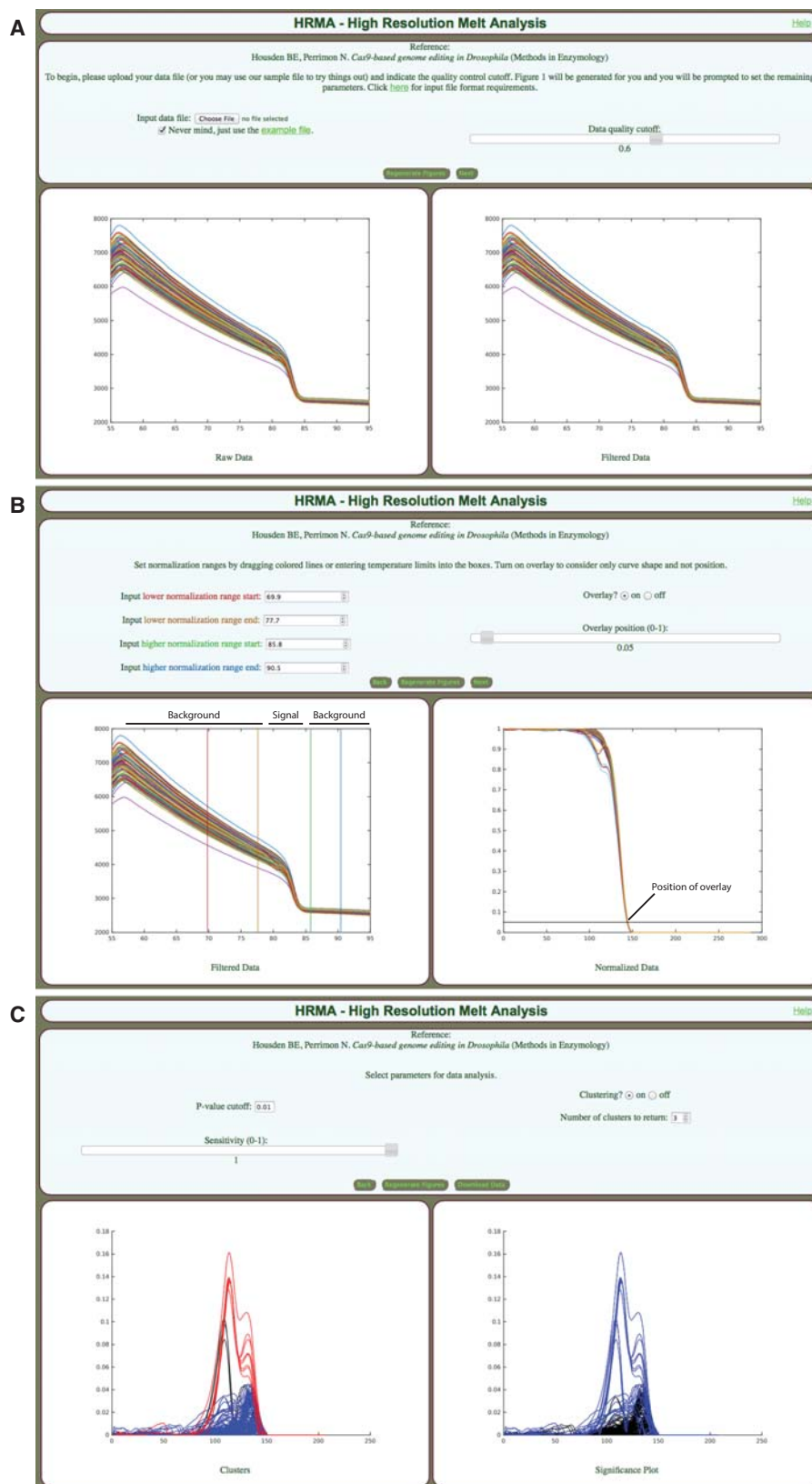


FIGURE 1. HRMAAnalyzer interface: Screenshots from the HRMAAnalyzer tool. (A) Stage 1: Uploading data and removal of poor quality samples from analysis. (B) Stage 2: Data normalization. (C) Stage 3: Analysis of normalized data.

16. Transfer the reaction to a thermocycler and run the following program.

	Temperature	Time
Start	98°C	3 min
30 cycles	98°C	30 sec
	60°C	30 sec
	72°C	30 sec
	10°C	
Hold	10°C	

This PCR may require optimization to produce a specific product.

17. Run a 5- μ L aliquot of the PCR product on an agarose gel to check that a band of the correct size was produced.
18. If necessary, gel-purify the correct product and elute in 30 μ L of H₂O
19. Assemble a TOPO cloning reaction using the Zero Blunt reagents as follows.

Reagent	Amount to add
Purified PCR product	2 μ L
Salt solution	0.5 μ L
TOPO vector	0.5 μ L

20. Incubate the reaction for 5 min at room temperature.
21. Transform the DNA into chemically competent *E. coli* using standard methods. Spread the cells onto plates containing solid LB agar with ampicillin. Incubate overnight at 37°C.
22. Select individual colonies and culture in 2 mL of LB liquid medium with ampicillin selection overnight at 37°C. Prepare plasmid DNA minipreps from the overnight cultures using standard methods.

The recommended number of picked colonies is dependent on the source of the samples. When using diploid flies, relatively few colonies can be analyzed to identify all sequences present. Five colonies per sample should be sufficient. When sequencing from cultured cells, many more sequences may be present, so more colonies should be picked and sequenced.

23. Sequence the plasmid DNA constructs using M13F and M13R primers.
24. Perform a local alignment to the experimentally determined starting genomic sequence (if determined), or to a reference genomic sequence, using standard sequence analysis software.

See Troubleshooting.

TROUBLESHOOTING

Problem (Step 6): The HRMA fluorescence signal is low.

Solution: This result suggests that either the first PCR reaction was inefficient or the HRMA amplification step failed. Run products from the first PCR on a gel and check for a band of the correct size. If a band is not present, optimize the reaction or redesign the primers. If a band is present, redesign primers for the HRM reaction and repeat the assay.

Problem (Step 14): No significance values are produced during analysis.

Solution: This is likely due to insufficient controls. Even if multiple controls are included, some may be filtered due to low data quality. Check that three or more controls are still present in the output data. If not, repeat the assay with additional control samples.

Problem (Step 14): None of the samples are significantly different from controls.

Solution: This may simply mean that no mutations are present. Alternatively, it could be due to noise in the control samples. Inspect the data in file "Data_normalized.txt" for control samples to check

B.E. Housden and N. Perrimon

that the curves are very similar. If they are not, repeat the assay using fresh control DNA to improve quality.

Problem (Step 24): Significantly different samples do not contain mutations when analyzed by sequencing.

Solution: This can occur when HRMA primers produce nonspecific bands. Run HRMA reaction products on a gel to ensure that a single band is produced.

DISCUSSION

The detection of indel mutations induced using CRISPR requires the use of molecular screening techniques, as no markers are introduced to indicate the mutation event. Several methods are available to screen for indel mutations. For example, where an indel mutation is likely to disrupt a restriction site, restriction profiling can be used to determine the presence and frequency of mutations (Nekrasov et al. 2013; Liang et al. 2014). Alternatively, endonuclease assays have been developed to detect the heteroduplexes formed when mutant DNA sequences are mixed with wild-type (Cho et al. 2013; Cong et al. 2013; Vouillot et al. 2015). Finally, HRMA can be used to detect the presence of sequence alterations such as indel mutations (Dahlem et al. 2012; Xing et al. 2012; Bassett et al. 2013). One advantage of HRMA over other assays is that many samples can be analyzed in parallel with little increase in workload. In addition, HRMA is extremely sensitive and can detect rare mutations or complex mixtures of mutations that may arise from genome editing in cultured cells and might be missed using endonuclease assays. Methods for application of these approaches in *Drosophila* have been described elsewhere (Bassett and Liu 2014; Beumer and Carroll 2014; Housden et al. 2014). Here we have focused on HRMA and data analysis using the HRMAlyzer tool, as this technique can initially appear complex and is covered less extensively in previous literature.

RECIPES

LB Liquid Medium

In 1 L H₂O, dissolve 10 g of Bacto Tryptone, 5 g of yeast extract, and 10 g of NaCl. Adjust the pH to 7.5 with NaOH. Sterilize by autoclaving.

Buffer SB

10 mM Tris-HCl (pH 8.2)
1 mM EDTA
25 mM NaCl
400 µg/mL Proteinase K (added fresh before use)

ACKNOWLEDGMENTS

We thank David Doupé and Stephanie Mohr for helpful discussions during the preparation of this manuscript. Work in the Perrimon Laboratory is supported by grants from the National Institutes of Health and the Howard Hughes Medical Institute.

REFERENCES

- Bassett A, Liu JL. 2014. CRISPR/Cas9 mediated genome engineering in *Drosophila*. *Methods* 69: 128–136.
- Bassett AR, Tibbit C, Ponting CP, Liu JL. 2013. Highly efficient targeted mutagenesis of *Drosophila* with the CRISPR/Cas9 system. *Cell Rep* 4: 220–228.
- Beumer KJ, Carroll D. 2014. Targeted genome engineering techniques in *Drosophila*. *Methods* 68: 29–37.
- Cho SW, Kim S, Kim JM, Kim JS. 2013. Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. *Nat Biotechnol* 31: 230–232.



- Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, et al. 2013. Multiplex genome engineering using CRISPR/Cas systems. *Science* 339: 819–823.
- Dahlem TJ, Hoshijima K, Jurynek MJ, Gunther D, Starker CG, Locke AS, Weis AM, Voytas DF, Grunwald DJ. 2012. Simple methods for generating and detecting locus-specific mutations induced with TALENs in the zebrafish genome. *PLoS Genet* 8: e1002861.
- Housden BE, Lin S, Perrimon N. 2014. Cas9-based genome editing in *Drosophila*. *Methods Enzymol* 546: 415–439.
- Liang Z, Zhang K, Chen K, Gao C. 2014. Targeted mutagenesis in *Zea mays* using TALENs and the CRISPR/Cas system. *J Genet Genomics* 41: 63–68.
- Nekrasov V, Staskawicz B, Weigel D, Jones JD, Kamoun S. 2013. Targeted mutagenesis in the model plant *Nicotiana benthamiana* using Cas9 RNA-guided endonuclease. *Nat Biotechnol* 31: 691–693.
- Vouillot L, Thelie A, Pollet N. 2015. Comparison of T7E1 and surveyor mismatch cleavage assays to detect mutations triggered by engineered nucleases. *G3 (Bethesda)* 5: 407–415.
- Xing L, Hoshijima K, Grunwald DJ, Fujimoto E, Quist TS, Sneddon J, Chien CB, Stevenson TJ, Bonkowsky JL. 2012. Zebrafish foxP2 zinc finger nuclease mutant has normal axon pathfinding. *PLoS One* 7: e43968.



Cold Spring Harbor Protocols

Detection of Indel Mutations in *Drosophila* by High-Resolution Melt Analysis (HRMA)

Benjamin E. Housden and Norbert Perrimon

Cold Spring Harb Protoc; doi: 10.1101/pdb.prot090795

Email Alerting Service

Receive free email alerts when new articles cite this article - [click here](#).

Subject Categories

Browse articles on similar topics from *Cold Spring Harbor Protocols*.

- [Bioinformatics/Genomics, general](#) (172 articles)
- [Computational Biology](#) (98 articles)
- [Drosophila](#) (235 articles)
- [Drosophila Transgenics](#) (31 articles)
- [Genetics, general](#) (343 articles)
- [Genome Analysis](#) (145 articles)
- [Polymerase Chain Reaction \(PCR\)](#) (76 articles)
- [RT-PCR](#) (44 articles)

To subscribe to *Cold Spring Harbor Protocols* go to:
<http://cshprotocols.cshlp.org/subscriptions>