High Resolution Melt Analysis (HRMA) Protocol

High Resolution Melting analysis (HRMA) is a PCR based analysis method used to detect small variations in nucleic acid sequences. HRMA requires three steps: 1) production of a small PCR amplicon flanking the region of interest (i.e. a disease associated SNP or a CRISPR target site); 2) heteroduplex formation in the presence of a fluorescent DNA binding dye and; 3) detection of small differences in PCR melting (dissociation) curves by analyzing the loss of fluorescence as hetroduplexes are melted. This protocol utilizes a standard thermocycler in conjunction with an Idaho Technology LightScanner instrument to perform the melt analysis. Most real-time PCR instrumentation that have precise temperature ramp control and advanced data capture capabilities are suitable for this analysis and may be more widely available at other universities. Data is analyzed and manipulated using software designed specifically for HRMA analysis.

DNA isolation: This can change based on your preference for lysis buffer, but the same lysis buffer must be used for each sample so as to compare them directly to each other, especially WT samples. The different salt concentration of lysis buffers can shift the temperature at which amplicons melt making it difficult to normalize and compare data.

Sequence genomic region to identify endogenous SNPs (optional):
HRMA was developed to scan genomes for SNP and other unknown mutations. Some genomes (i.e. the zebrafish genome) are full SNPs in exonic and intronic regions. Therefore, you must sequence the target region to make sure that there are no existing SNPs that could interfere with your HRMA.

Alternative to sequencing genomic region (optional):

1. Perform HRMA (as described below) on 8-16 separate samples.
2. If all the samples melt the same pick one amplicon and sequence it to compare to the reference genome or simply proceed with HRMA assay.
3. If separate samples melt differently sequence a representative from each sample group and compare to the reference genome.If this is the case:
    a. Design primers such that variable SNPs are not included in the HRMA amplicon. If this is not possible, samples will need to divided into groups based on genotype prior to analysis.
    b. When working with models such as zebrafish you can isolate a population with the same genotype for CRISPR editing.

HRMA primer design:

1. Use Primer3Plus or any other primer design program
2. Optimal Conditions Range
    a. 55% GC 40-65% GC
    b. 62°C Tm 59-65°C
    c. 22nt 20-27nt
    d. ~90bp 50-125bp amplicon (smaller is better)
    e. GC clamps at ends (low priority)
    f. Try to match Tm temps of the primer pairs (high priority)
    g. Keep primers in exon around target site if can, there usually is less sequence variation. Primers in intronic region will work.

h. Design 2 forward and 2 reverse primers that you can mix and match for each target
i. Check primers against genome (blast zebrafish genome) and check primer pairs for primer dimer, etc. This can be easily done through Primer3Plus.

Optimization of HRMA Primer Mix (PM):

j. Run a PCR for each PM at 66, 68, 70°C (this is a good starting point and Tm may be as low as 62°C and require a second gradient)
    i. This is easily done using the gradient feature on PCR machines
    ii. Set the gradient to 66-70°C
    iii. Column 1 will be at 66°C, column 6 or 7 will be close to 68°C, and column 12 with be 70°C
    iv. Run samples for each PM in the three columns and compare results
k. Then run replicates at the optimized temperature along with a water control to check that the wildtype melt is consistent and that there are no primer dimers (indicated as a signal in the no template water control).
l. Use the HRMA analysis protocol listed below

3. Best Primer set
    a. Clean melt curve: i.e.-just one melt point
    b. Sharp transition to melt
    c. Cannot have primer dimer products in dH2O with the same melt temp as the target amplicon
    d. Want the most stringent condition you can possible use

HRMA analysis:

1. Using Idaho Technologies LightScanner Master Mix (includes Taq, dNTPs, buffer)
2. Reaction

		Final
a. Master mix4ul	1X	
b. 2uM PM	1	0.2uM
c. DNA	1	10-60ng (less DNA may be better)
d. dH2O	4	

3. Aliquot 20ul sterile mineral oil into each well of a 96-well black hard shell with white well plate (no matter if using full plate or not, always put mineral oil into each well to ensure equal heating and cooling)
4. Make master mix of buffer, PM, and dH2O
5. Aliquot 9ul into each well (filter pipette tip, can use multi-pipettor and 8-well PCR strips to increase efficiency)
6. Add 1ul of gDNA to each well (filter tip)
7. Cover with optical grade PCR sealing tape
8. Spin for 3min at 3000rpm 4°C in table top centrifuge (gets reagents to bottom of wells)
9. Place in 96-well PCR machine
10. Program
    a. 94°C 3 min
    b. 50cycles

i. 94°C 30sec
ii. 66, 68, or 70°C 15-20sec
shorter times increase specificity
these are guideline temps and times, lower or higher is ok

c. 1 cycle—heteroduplex formation
i. 94°C 30sec
ii. 25°C 30sec Cool at fastest rate possible to increase heteroduplex formation

11. Spin for 3min at 3000rpm 4°C in table top centrifuge (gets rid of air bubbles)
12. Place in Idaho Technology LCscanner machine and run melt curve analysis from ~65-95°C using standard settings
13. Analyze results using LCscanner software package

Notes

1. Must have 3-6 WT samples in the HRMA analysis to set the baseline
2. WT samples should be in the range of 0.01-0.02 from each other when viewed on a difference curve. This is only an empirically derived guideline.
3. Mutants will vary in their deflection from the baseline when viewed on a difference curve: a range of 0.04-5.0 has been seen.

Materials:

Bio-Rad
www.bio-rad.com
-Hard-Shell Thin-Wall 96-Well Skirted PCR Plates: black shell/white well
HSP-9665
-Optical Sealing Tape
223-9444

Sigma
www.sigmaaldrich.com
-Mineral Oil Light for Molecular Biology: M5904
Some mineral oil have autofluorescence that will obscure your results

Idaho Technology Inc
www.idahotech.com
-LightScanner Master Mix
HRLS-ASY-0003

Citations/further resources:

https://anatomypubs.onlinelibrary.wiley.com/doi/full/10.1002/dvdy.22143

https://pubmed.ncbi.nlm.nih.gov/24561516/

https://www.jove.com/t/51138/rapid-efficient-zebrafish-genotyping-using-pcr-with-high-resolution