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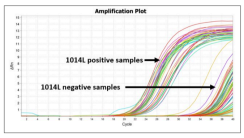
Detection of knockdown resistance mutations in Musca domestica by rhPCR



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We use this protocol and it's working

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Disclaimer

Abstract

This protocol details the step-by-step procedure for assessing *Musca domestica* knockdown resistance (*kdr*) mutations using RNAse H2 PCR (rhPCR). This procedure utilizes the specificity of rhPCR, the high throughput of 96-well plate-based sample handling, and the rapidity of SYBR based PCR assays to genotype for critical *kdr* mutations (D600N, M918T, T929I, and L1014H/F). Compared to existing assays for *kdr* assessment in *M. domestic*a, these assays reduce expense per sample by ~75% and time by ~80%.

Guidelines

None

Materials

Consumables needed for assays:

	A	B	C
	Item	Product Number	Vendor
	96 well cap	276002	Thermo
	2ml 96 well homogenization plate	278743	Thermo
	2.3-mm-diameter zirconia silica beads	11079124ZX	BioSpec
	tips		
	dH2O		
	Microamp 384-well plate	4309849	AB
	Sybr Select Master Mix	4472919	Invitrogen
	rhPCR F primer*		Integrated DNA technologies
	rhPCR R primer*		Integrated DNA technologies
	RNAse H2	11-03-02-02	Integrated DNA technologies
	NFW	AM9937	AB
	tips		

Safety warnings

Always use proper PPE.
Follow all laboratory safety procedures.
Follow all institutional and manufacturer guidelines for the safe and proper use of laboratory equipment and reagents.

Ethics statement

None

Before start

None

Sample preparation

3m

1 Room temperature Add cubic zirconium beads to Omni Products 96-deep well plate using Biospec bead loader

2 Room temperature Add 400 µL of deionized water using Eppendorf Repeatter E3

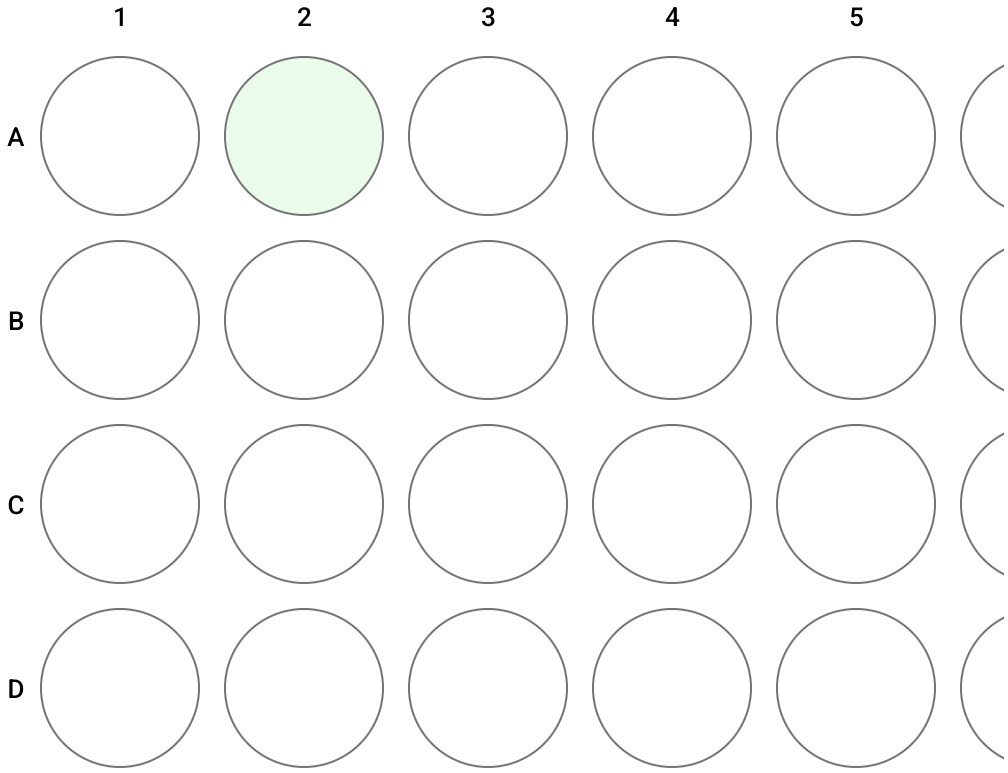
3 Clean forceps with 70% ethanol solution

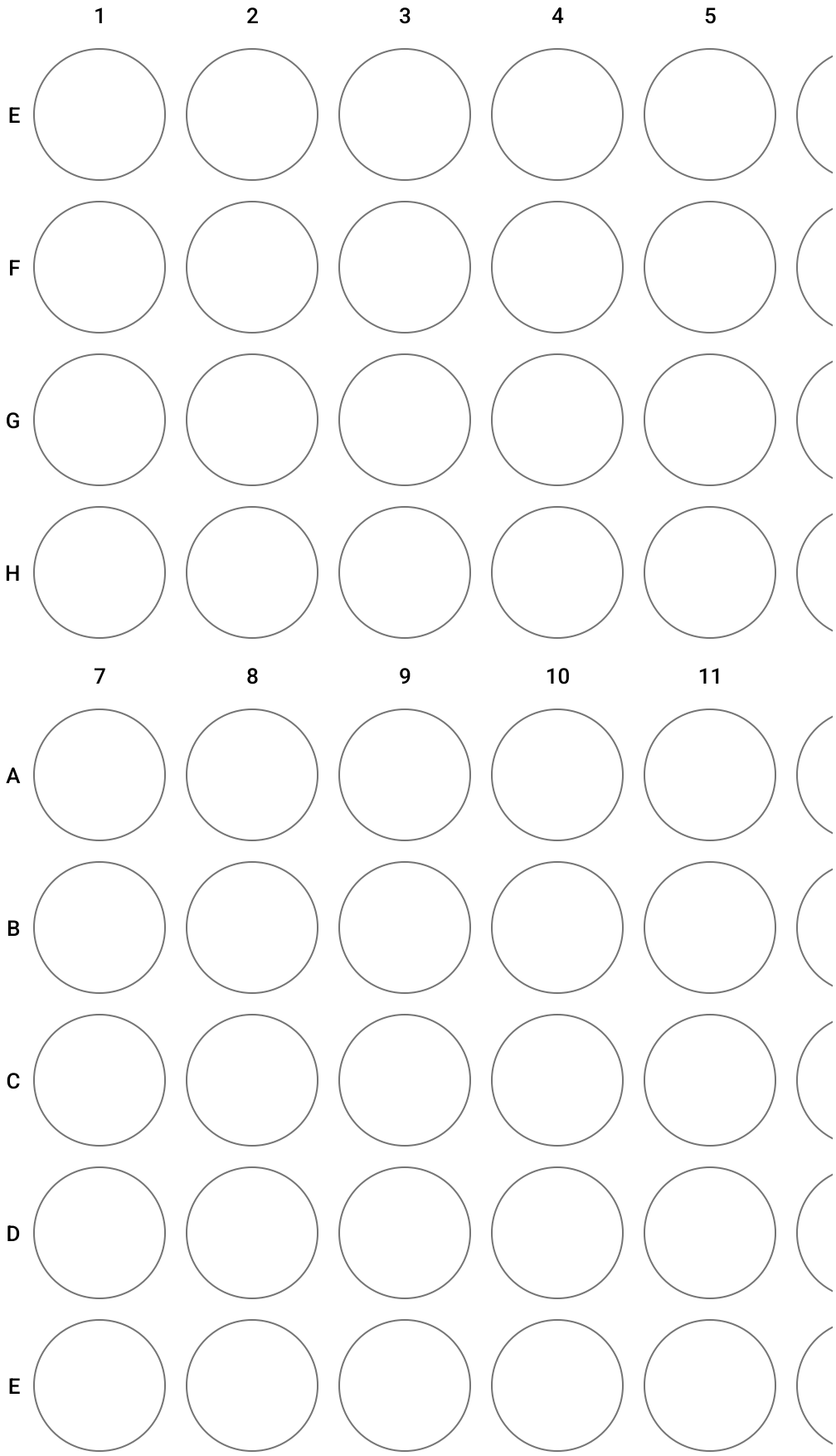
4 On ice

Note

Save column 1 for addition of controls.
Maintain samples and plates on ice while preparing samples.

Remove 2-3 legs from a single organism and place into well A2. Place carcass into well A2 of a 96-well storage plate.

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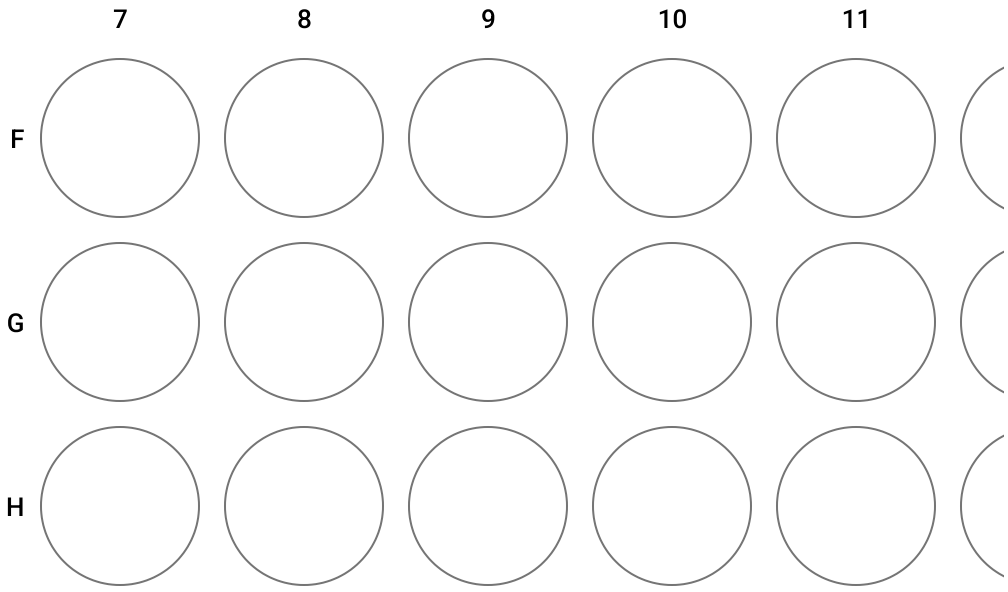


5 go to step #3 On ice Repeat process of filling plate for columns 2 through 12. Load samples by working down columns rather than across rows. This results in reduced reagent consumption for automated reaction assembly of partial plates.

6 On ice Using same procedure as in steps 4-6, add controls to Wells A1-H1. Alternatively, purified synthetic DNA can be used as controls.
A1: 1014L allele/918M allele/929T allele/600D allele (no *kdr* CAR21 strain- Carolina Biological Supply)
B1: 1014H allele/918M allele/929T allele/600D allele (NCHis strain- Jeff Scott Cornell University)
C1: 1014F allele/918M allele/929T allele/600D allele (ALkdr strain- Jeff Scott Cornell University)
D1: 918T allele/929T allele/600D (JPSkdr strain- Jeff Scott Cornell University)
E1: 918M allele/929I allele/600D allele (kdr1b strain- Jeff Scott Cornell University)
F1: 918T allele/929T allele/600N (TypeN strain- Jeff Scott Cornell University)
G1: no sample blank
H1: no sample blank

7 On ice Seal leg plate with Omni Products silicon sealing mat. Seal carcass plate with foil seal. Freeze carcass plate at -80C to maintain RNA.

8 Homogenize sealed leg plate for 1 min at 30 hertz on Omni plate homogenizer. 1m
00:01:00

Equipment	
BEAD RUPTOR 96	NAME
homogenizer	TYPE
Omni International	BRAND
27-0001	SKU

9 2000 rpm, Room temperature, 00:02:00 2m

Equipment	
Centrifuge	NAME
Eppendorf	BRAND
5804	SKU

Centrifuge leg sample plate.

10 On ice Return plate to ice.

Reagent Preparation 3m

11 Label 9 - 1.7ml microcentrifuge tubes (as 1-9) to prepare allele specific rhPCR reactions.

12 On ice Prepare 300 ul of 20 mU/ul H2 enzyme by diluting 3ul of 2U/ul H2 enzyme in 297ul of H2 buffer (IDTDNA).

13 Prepare 9 allele-specific H2 PCR reactions following the table below:

Note

Primers and H2 enzyme can be ordered from IDT DNA. SYBRGreen is from ThermoFisher. All values are in microliters.

Reaction tube #:	1	2	3	4	5	6	7	8	9
Allele:	1014 L	1014 H	1014 F	918M	918T	929T	929I	600D	600N
SYBR	560.00	560.00	560.00	560.00	560.00	560.00	560.00	560.00	560.00
NFW	283.36	312.48	283.36	320.32	320.32	283.36	283.36	319.76	319.76
20mU H2	43.68	14.56	43.68	6.72	6.72	43.68	43.68	7.28	7.28
rhPCR primer: 1014L	4.48								
rhPCR primer: 1014H		4.48							
rhPCR primer: 1014F			4.48						
rhPCR primer: 1014r	4.48	4.48	4.48						
rhPCR primer: 918M				4.48					
rhPCR primer: 918T					4.48				
rhPCR primer: 918r				4.48	4.48				
rhPCR primer: 929T						4.48			
rhPCR primer: 929I							4.48		
rhPCR primer: 929r						4.48	4.48		

	rhPCR primer: 600D								4.48	
	rhPCR primer: 600N									4.48
	rhPCR primer: 600r								4.48	4.48

14 Seal tubes, vortex briefly to mix and then spin.

15 Place V-bottom PCR plate into Eppendorf plate rack. Aliquot 108ul of reaction 1 to each well of column 1.

16 go to step #15 Repeat step above for reactions 2-9.

Reaction Assembly on Eppendorf 5750

3m

17 Start Eppendorf 5750 system.

18 Load (or write) program USDA/Musca domestica/20210925.

> **Note**
>
> This program aliquots each of the 9 allele-specific mastermixes into 384-well plates and then adds homogenate from the homogenized sample plate.

Program is as follows:

Procedure

Procedure Start

1. Number of samples
Name: 1014L_Column1
Variable, maximum: 96

2. Reagent transfer
Pipette: 8.0 µl
rack_1 to pcr384_1

3. Number of samples
Name: 1014H_Column2
Variable, maximum: 96

4. Reagent transfer
Pipette: 8.0 µl
rack_1 to pcr384_1

5. Number of samples
Name: 1014F_Column3
Variable, maximum: 96

6. Reagent transfer
Pipette: 8.0 µl
rack_1 to pcr384_1

7. Number of samples
Name: 918M_Column4
Variable, maximum: 96

8. Reagent transfer
Pipette: 8.0 µl
rack_1 to pcr384_2

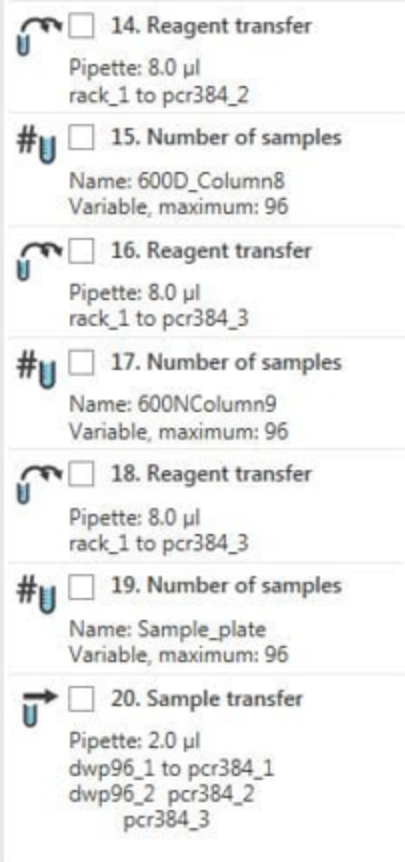
9. Number of samples
Name: 918T_Column5
Variable, maximum: 96

10. Reagent transfer
Pipette: 8.0 µl
rack_1 to pcr384_2

11. Number of samples
Name: 929T_Column6
Variable, maximum: 96

12. Reagent transfer
Pipette: 8.0 µl
rack_1 to pcr384_2

13. Number of samples
Name: 929I_Column7
Variable, maximum: 96



19 Setup platform of Ep5750 workstation as shown below.
Place tips in positions A2, A3 and B2.
Place labelled 384-well plates in positions C2, C3 and C4.
Place aliquoted reagent plate in B3.
Place homogenized sample plate in B4.

20 Close safety cover of Ep5750 workstation.

21 Start program and allow to run until reaction setup is complete.

22 Exit Ep5750 program and open cover.

23 Seal 384-well plates with Eppendorf Optical covers. Seal tightly around edges with sealing tool.

24 Spin plates for 30 seconds in plate centrifuge and place covered in refrigerator until amplification.

25 Clean Ep5750 worksurface and shutdown workstation.

25.1 Remove empty tip racks.

25.2 Recover partial tip rack.

25.3 Remove sample homogenate plate from Ep5750 platform and reseal with silicone sealing mat. Freeze immediately at -80C to preserve RNA and reduce degradation.

25.4 Empty platform waste container.

25.5 Wipe platform with a water moistened paper towel.

25.6 Shutdown workstation computer and switch off Ep5750.

Thermocycling procedure

3m

26 Start Applied Biosystems QuantStudio6 Flex and open QuantStudio software

27 Select Open/Template/Mdom15State on Desktop

28 Open Mdom_Plate_1.edt, Mdom_Plate_2.edt and Mdom_Plate_3.edt

28.1 Add sample information and date to comments box for plates 1,2 and 3

28.2 Save each as a .eds file starting with date of test as: YYYYMMDD_Mdom_Plate_1

28.3 Extend plate carrier by selecting front panel virtual door button.

28.4 Load Plate_1 ensuring position A1 is at upper left as marked on plate holder.

28.5 Close door by pressing virtual door button on front panel.

28.6 01:04:00 Press start and allow approximately 64 minutes to complete run 1h 4m

28.7 go to step #28.3 Repeat substeps for Plate_2 and Plate_3

Data analysis

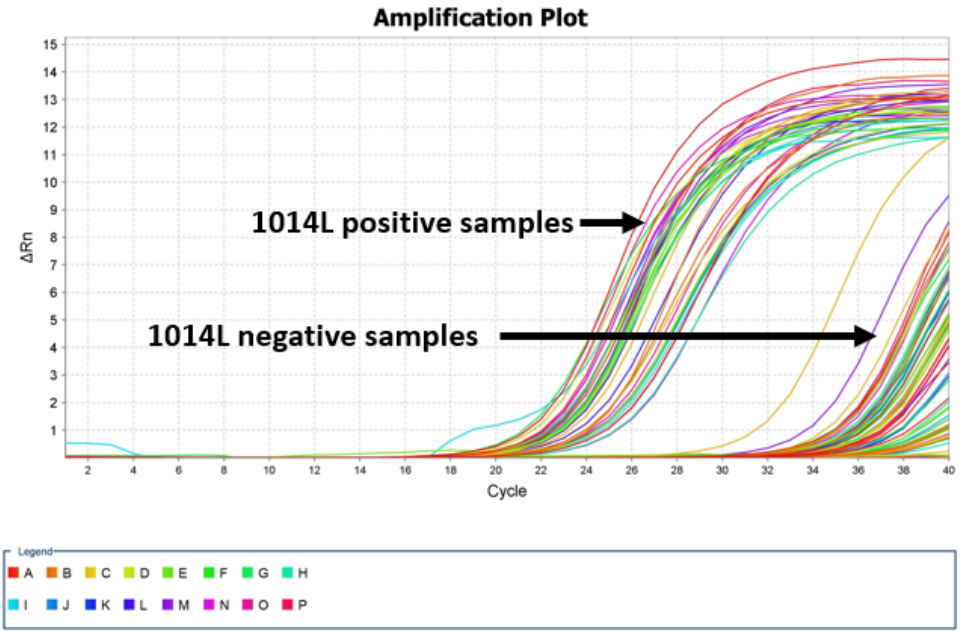
29

Note

Allele presence or absence for each sample can be done by visual inspection of the amplification curves directly in the QuantStudio6 software or by exporting an excel file and calling alleles based on cycling threshold values and melting temperature. This procedure details the visual inspection method for the presence or absence of the 1014L allele. Calls for the other 8 reactions are done the same way.

Assess overall assay validity by examining Wells M1 and O1. They should not amplify as they contain no template. Amplification indicates contamination and any assay with amplification in blank wells should be redone.

29.1 Select controls with known 1014L alleles (CAR21 in position A1) and control without 1014L alleles (C1, E1, G1, I1, K1). The 1014L positive samples will amplify within a small range of cycles and the 1014L negative samples will appear much later (note that they will usually appear with enough cycling time).



29.2 Examine each test samples to determine whether it is positive or negative for an allele based on the controls.

Note

Some results may be indeterminate (amplify between clear positive and negative groups) and will need to be rerun or verified with another method.

29.3 go to step #29.1 Repeat step 29 for each of the remaining 8 alleles.

Protocol references

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