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Protocol for detecting mutations conferring resistance to Chloroquine: PfCRT: K76T

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Detection of the mutations of interest requires successful completion of the steps listed here:

1. Chelex Extraction: Parasite DNA is extracted from blood blots collected from patients on filter paper.
2. Nested PCR: a specific DNA segment that may contain the mutation is amplified
3. Restriction Enzyme Digestion: the amplified DNA from is cut into smaller segments whose length is dependant on whether the mutation is present or absent
4. Gel Electrophoresis: the products of are run on a gel and the pattern of bands indicates a wild, mixed or mutant phenotype

For those familiar with the lab techniques required, an overview is provided in the following section containing the appropriate master mixes, primers and their sequences, PCR conditions, gels required, the electrophoresis band pattern of various mutations and recipes of commonly used reagents.

For those less familiar with the techniques involved, step-by-step instructions are provided for several of the necessary procedures.

PCR primers and Conditions

Gene and PCR round	PCR Primers (5' → 3')	PCR Conditions
PfCRT 76 1 st round [†]	CRT-1: (21bp) GACGAGCGTTATAGAGAATTA CRT-2: (20bp) CCAGTAGTTCTTGTAAGACC	<u>Initial denaturation</u> 94°C x 3m <u>PCR</u> 40 cycles; 94°C x30s, 47°C x1m, 72°C x1.5m <u>Final elongation</u> 72°C x 3 m <u>Hold @ 4 °C</u>
PfCRT 76 2 nd round	CQR-A: (21bp) TGTGCTCATGTGTTTAAACTT CQR-B: (23bp) CAAACTATAGTTACCAATTTTG	<u>Initial denaturation</u> 94°C x 5m <u>PCR</u> 30 cycles; 94°C x30s, 52°C x1m, 72°C x1m <u>Final elongation</u> 72°C x 3 m <u>Hold @ 4 °C</u>

[†] Developed by A. Myrick, 2005

PCR Controls and expected fragment sizes

Gene	Wild-type control	Mutant Control	Wild-type fragments (bp)	Mutant fragments (bp)
PfCRT 76	HB3	DD2	34, 100	134

Master Mix for 1st and 2nd round PfCRT 76 PCR Reactions

	Stock Concentration	Final Concentration	Volume (in uL)
H ₂ O	-	-	16.05
Forward primer	10 uM	0.2 uM	0.5
Reverse primer	10 uM	0.2 uM	0.5
Buffer	10X	1X	2.5
dNTP	2 mM	200 uM each	2.5
MgCl ₂	50 mM	1.5 mM	0.75
Taq (INV)	5 U/uL	1 U	0.2
Template	unknown	unknown	2.0
Final volume			25.0

RED for PfCRT 76 (50° C for 2 hours)

	Stock Concentration	Final Concentration	Volume (in uL)
H ₂ O	-	-	6.8
Neb3	10X	1X	2.0
BSA	100X	1X	0.2
Apo I (NEB)	10 U/uL	1 U	1.0
Template	unknown	unknown	10.0
Final volume			20.0

Commonly used reagents

5X TBE

Per liter of stock solution
54g tris base
27.5g boric acid
20 mL 0.5M EDTA pH 8

2 mM dNTP from stock

1 mL 100 mM dNTP from stock
11.5 mL H₂O
Aliquot into 1 mL volumes

2.5% agarose gel

5.0g agarose
200 mL 1x TBE
15 uL ethidium bromide

Makes 2 gels.
Microwave 4 ½ minutes for 2 gels, 6 minutes for 4 gels, then cool to 60° C and add ethidium bromide.

6X loading dye (10ml)

25 mg (0.25%) xylene cyanole
3 mL (30%) glycerol
7 mL H₂O

DNA ladder with dye (1ml)

50 uL of 50bp ladder (INV)
170 uL 6X bromphenol blue loading dye
780 uL H₂O to make 1 mL

First round PCR

1. Remove samples for analysis and appropriate reagents except Taq from the freezer.
2. Arrange samples on a plastic block in the desired order and record the position and complete sample identifier clearly in your lab book. Remember to include one negative and appropriate positive wild-type and mutant controls. It is very important to check periodically throughout the preparation of the first round that the order has not been disturbed since any misplaced samples will invalidate the entire experiment.
3. Copy the master mix into your lab book and multiply the volume for each reagent by the total number of samples plus about 4 extra for every 50 samples you plan to type. Remember that if the concentrations of any of your stock solutions are different than what is shown in the table, you will have to recalculate the actual volume added for your master mix.
4. Take lab book, thawed samples and reagents and PCR block to a clean work area, preferably under a hood.
5. Place an empty PCR tray in plastic PCR block and set aside.
6. Make your master mix in an appropriately sized tube: Put on gloves. Add all reagents but Taq. Pipette water first, then primers, buffer, dNTP, MgCl₂. Each reagent should be vortexed for 1-2 seconds before you add it.
7. Add the Taq: take the appropriate pipette and master mix back to the freezer where Taq is stored. Add the Taq and replace in the freezer. Take completed master mix back to the hood and vortex for 2-3 seconds.
8. Aliquot 23 uL of master mix into the appropriate number of wells.
9. Lift PCR tray from block and verify that all wells that should have aliquots have them.
10. Add template DNA: Do not add anything to the first well; this will be the negative control. Starting with your first + control, carefully add 2 uL of template DNA to the appropriate well. Each sample should be vortexed for 1-2 seconds before withdrawing the aliquot.
11. Cap all appropriate wells and label PCR tray with the round# (1st), gene marker, and the page in the lab book containing sample key and date.
12. Place samples in PCR machine using the appropriate conditions.
13. When reaction is complete, store the products in the refrigerator until they are needed.

Second round PCR

1. Remove appropriate reagents except Taq from the freezer and the 1st round PCR product from refrigerator.
2. Copy the master mix into your lab book and multiply the volume for each reagent by the total number of samples plus about 4 extra for every 50 samples you plan to type. Remember that if the concentrations of any of your stock solutions are different than what is shown in the table, you will have to recalculate the actual volume added for your master mix.
3. Take lab book, thawed samples and reagents and PCR block to a clean work area, preferably under a hood. Place an empty PCR tray in plastic PCR block and set aside.
4. Make your master mix in an appropriately sized tube: Put on gloves. Add all reagents but Taq. Pipette water first, then primers, buffer, dNTP, MgCl₂. Each reagent should be vortexed for 1-2 seconds before you add it.
5. Add the Taq: take the appropriate pipette and master mix back to the freezer where Taq is stored. Add the Taq and replace in the freezer. Take completed master mix back to the hood and vortex for 2-3 seconds.
6. Aliquot 23 uL of master mix into the appropriate number of wells.
7. Lift PCR tray from block and verify that you have placed aliquots in the correct wells.
8. Add template DNA: Using the 8 or 12-channel 0.5-10.0 uL pipette carefully transfer 2 uL of first round PCR product template DNA to the appropriate wells.
9. Cap all appropriate wells and label PCR tray with the round# (2nd), gene marker, and the page in the lab book containing sample key and date.
10. Place samples in PCR machine using the appropriate conditions described in table 1.
11. After the PCR has completed, run all controls, 3-4 samples and 100 bp ladder on a gel. Use only 8-10 uL of 2nd round product and 2-3 uL of dye per well. In order to proceed to restriction enzyme digestion, the negative control must be empty and both wild type and one mutant controls must have PCR product of the appropriate length visible. If the PCR works, move on to the RED.
12. Store the remaining products in the refrigerator until they are needed.

Restriction Enzyme Digestion

1. Remove the second round PCR product, and appropriate reagents including the enzyme from the freezer for thawing. Place the enzyme on ice.
2. Copy the reagents into your lab book and multiply each reagent by the total number of samples plus about 4 extra for every 50 samples you plan to PCR. This will be your “master mix”.
3. Take lab book, PCR product, thawed reagents and PCR block to your work area.
4. Place a PCR tray in plastic PCR block and set aside.
5. Make your master mix in an appropriately sized tube: Put on gloves. Each reagent except enzyme should be vortexed for 1-2 seconds before you add it. Pipette water first.
6. Aliquot 10 uL of master mix into the appropriate wells, then lift the PCR tray out of the block to verify you have done this correctly.
7. Add template DNA: Using the 8 or 12-channel 0.5-10.0 uL, carefully add the template DNA to the appropriate wells.
8. Cap all wells and label PCR tray with RED and the appropriate mutation, and the page in lab book containing sample key and date.
9. Place samples in PCR machine programmed for appropriate temperature and incubation time.
10. If you are unable to proceed to the next step, store completed reaction in the refrigerator.

Gel Electrophoresis

1. Remove samples (PCR or restriction enzyme digestion product), 6X loading dye, 50 base pair ladder and the appropriate number of gels from the refrigerator and bring them to your work area by the gel boxes.
2. Check the TBE in the gel boxes and replace it if necessary. TBE should be changed after every 5th gel; you should have a labeling system on the boxes themselves to be able to do this reliably. When not in use the gel boxes with TBE should be covered.
3. Cut a piece of paraffin film the approximate length of the gel box and place in front of the box; score the corners so it will stay down.
4. First pipette approximately 5 uL of dye per well onto paraffin.
5. Add 15-20 uL of RED product to each drop of dye. As always, keep careful track of the order of your samples to ensure that you can interpret the results with confidence.
6. Place the gel in the gel box. Skipping the first well, pipette the dye/RED product combination from the paraffin to the well. Place up to 18 samples per gel.
7. Add 10-15 uL of 50 bp ladder to the first well and the well next to your last sample.
8. Run the gel at 80-90 volts. For a RED products, the gel is ready to be removed when the light blue dye front (created by the loading dye) is approximately half way down the gel. This generally takes about 1 ½ - 2 hours but watch your first few gels to determine how long it takes for you.
9. Remove the gel, place it in a tray and bring it to the gel photography station.
10. After the gel has been visualized, make sure to save the resulting picture to a well labeled disk if you will be using the results. Print a hard copy to paste and label in your lab book.

Interpret the results: this is best done looking at the computer screen, not from the printed picture. A wild-type sample has all the wild type bands and none of the unique mutant type bands. A mutant sample has all the mutant bands and none of the unique wild type bands. A mixed sample has both unique wild type and mutant bands present. For the experiment to be valid the negative control must have no bands, and the wild type and mutant controls must have the appropriate bands. If a band is visible at all, it should be interpreted as being present. Sometimes small bp bands of non-specific products can be observed at the bottom of the gel. These can be ignored.