April, 2004

Protocols for detecting mutations conferring resistance to the antifolate class of antimalarial drugs: DHFR Ile-51, Arg-59, Asn-108, Leu-164, and DHPS Gly-437, Glu-540

Author: Jonathan Vlahos
Sunil Parikh
Chris Dokomajilar

Contributors: Grant Dorsey
Daniel Kyabayinze
Sam Nsobya
Sarah Staedke
Hakim Sendagire
Ben Hunt

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

1. Chelex Extraction: Parasite DNA is extracted from blood blots collected from patients on filter paper.
2. 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.

The chelex extraction of parasite DNA only needs to be done once for each sample of interest and is described in the attached document.

PCR primers and Conditions

Gene and PCR round	PCR Primers*	PCR Conditions
DHFR 51, 59, 108, 164 1st round†	A1: 5'-TTTATATTTTCTCCTTTTTA (20bp) A2: 5'-CATTTTATTATTCGTTTTCT (20bp) (these are modified primers, Durasingh uses M1 & M5)	Initial denaturation 94°C x 3m PCR 5 cycles 94°C x 45s, 54°C x 45s, 72°Cx 1.5m 35 cycles 94°C x 30s, 56°C x 30s, 72°C x 45s Final elongation 72°C x 10 m Hold @ 4 °C
DHFR 51, 108, 164 2nd round†	A3 5'- GTCTGCGACGTTTTCGATATTTATGC (26bp) F1 5'- AAATTCTTGATAAACAACGGAACCT**tt**TA (29bp) (these are modified primers, Durasingh uses M3 & F/)	Initial denaturation 94°C x 3m PCR 5 cycles 94°C x 45s, 50°C x 45s, 72°Cx 1.5m 35 cycles 94°C x 30s, 54°C x 30s, 72°C x 45s Final elongation 72°C x 10 m Hold @ 4 °C
DHFR 59 2nd round‡	F:5'- GAAATGTAATTCCCTAGATATG**g**AATATT (29bp) M4: 5'- TTAATTTCCCAAGTAAAACTATTAGA**g**CTTC (31bp)	Initial denaturation 94°C x 2m PCR 5 cycles 94°C x 45s, 48°C x 45s, 72°Cx 1.5m 30 cycles 94°C x 30s, 50°C x 30s, 72°C x 45s Final elongation 72°C x 10 m Hold @ 4 °C
DHPS 437/540 1st round‡	R1: 5'- AACCTAAACGTGCTGTTCAA (20bp) R2: 5'- AATTGTGTGATTTGTCCACAA (21bp)	Initial denaturation 94°C x 3m PCR 40 cycles 94°C x 1m, 45°C x 45s, 72°C x 1m Final elongation 72°C x 10 m Hold @ 4 °C
DHPS 437/540 2nd round‡	K: 5'- TGCTAGTGTTATAGATATAGG**at**GAG**c**ATC (30bp) K1: 5' – CTATAACGAGGTATT**g**CATTTAAT**g**CAAGAA (31bp)	PCR 5 cycles of 94°C x 2m, 45°C x 2m, 72°Cx 1.5m 35 cycles 94°C x 1m, 45°C x 1m, 72°C x 1m Final elongation 72°C x 10 m Hold @ 4 °C

* lower case nucleotides signify mismatches
† developed by Staedke and Sendagire
‡ from: Duraisingh MT, Curtis J and Warhurst DC. Plasmodium falciparum: detection of polymorphisms in the dihydrofolate reductase and dihydropteroate synthetase genes by PCR and restriction digestion. Exp Parasitol 1998;89:1-8.

PCR Controls and expected fragment sizes

Gene	Wild-type controls	Mutant Control	Wild-type fragments (bp)	Mutant fragments (bp)
DHFR 108	3D7 or FCR3	V1/S	522	190, 332
DHFR 51	3D7 or FCR3	V1/S	5,10,46,55,63,65,120,139	5,10,46,55,65,120,202
DHFR 59	3D7 or FCR3	V1/S	142, 184	22, 142, 162
DHFR 164	3D7 or FCR3	V1/S	107, 171, 245	28, 107, 143, 245
DHPS 437	Mali or FCR3	Peru	438	34, 404
DHPS 540	Mali or FCR3	Peru	7, 19, 412	7, 19, 99, 313

Master Mixes for PCR Reaction

Master Mix for 1st round DHFR 51, 59, 108, 164, DHPS 437 and 540

	Stock Concentration	Final Concentration	Volume (in uL)
H_2O	-	-	16.05
Buffer	10X	1X	2.5
dNTP	2 mM	200um each	2.5
$MgCl_2$	50 mM	1.5 mM	0.75
Primer 1	10 uM	0.2 uM	0.5
Primer 2	10 uM	0.2 uM	0.5
Taq	5 U/uL (INV)	1 U	0.2
Template	unknown	unknown	2.0
Final volume			**25.0**

Master Mix for 2nd round DHPS 437 & 540

	Stock Concentration	Final Concentration	Volume (in uL)
H_2O	-	-	34.3
Buffer	10X	1X	5.0
dNTP	2 mM	200um each	5.0
$MgCl_2$	50 mM	1.5 mM	1.5
Primer 1	10 uM	0.2 uM	1.0
Primer 2	10 uM	0.2 uM	1.0
Taq	5 U/uL (INV)	1 U	0.2
Template	unknown	unknown	2.0
Final volume			**50.0**

Master Mix for 2nd round DHFR 51, 59, 108, 164

	Stock Concentration	Final Concentration	Volume (in uL)
H_2O	-	-	34.3
Buffer	10X	1X	5.0
dNTP	2 mM	200um each	5.0
$MgCl_2$	50 mM	1.5 mM	1.5
Primer 1	10 uM	0.2 uM	1.0
Primer 2	10 uM	0.2 uM	1.0
Taq	5 U/uL (INV)	1 U	0.2
Template	unknown	unknown	2.0
Final volume			**50.0**

Restriction Enzyme Digestions

RED for DHFR 51 (65˚ C for 2 hours)

	Stock Concentration	Final Concentration	Volume (in uL)
Tsp 509I	10 U/uL (NEB)	7 U	0.7
Neb1	10X	1X	2.0
H_2O	-	-	7.3
Template	Unknown	unknown	10.0
Final volume			**20.0**

RED for DHFR 59 (37˚ C for 2 hours)

	Stock Concentration	Final Concentration	Volume (in uL)
XmnI	20 U/uL (NEB)	6 U	0.3
Neb2	10X	1X	2.0
BSA	100X	1X	0.2
H_2O	-	-	7.5
Template	unknown	unknown	10.0
Final volume			**20.0**

RED for DHFR 108 (65˚ C for 2 hours)

	Stock Concentration	Final Concentration	Volume (in uL)
BsrI	5 U/uL (NEB)	6 U	1.2
Neb3	10X	1X	2.0
H_2O	-	-	6.8
Template	Unknown	unknown	10.0
Final volume			**20.0**

RED for DHFR 164 (37˚ C for 2 hours)

	Stock Concentration	Final Concentration	Volume (in uL)
DraI	20 U/uL (NEB)	10 U	0.5
Neb4	10X	1X	2.0
H_2O	-	-	7.5
Template	Unknown	unknown	10.0
Final volume			**20.0**

RED for DHPS 437 (37˚ C for 2 hours)

	Stock Concentration	Final Concentration	Volume (in uL)
AvaII	10 U/uL (NEB)	20 U	2.0
Neb4	10X	1X	2.0
H_2O	-	-	6.0
Template	unknown	unknown	10.0
Final volume			**20.0**

RED for DHPS 540 (37˚ C for 2 hours)

	Stock Concentration	Final Concentration	Volume (in uL)
Fok I	4 U/uL (NEB)	2 U	0.5
Neb4	10X	1X	2.0
H_2O	-	-	7.5
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 H_20
Aliquot into 1 mL volumes

2.5% agarose gels

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 (use scale in room 409)
3 mL (30%) glycerol
7 mL H_20

DNA ladder with dye (1ml)

50 uL of 50bp ladder (INV)
170 uL 6X bromphenol blue loading dye
780 uL H_2O to make 1 mL

Autoclave: put items to be autoclaved in a large, plastic beaker, cover with foil and one strip of autoclave tape. Put contents in autoclave and shut firmly (have someone show you) and turn handle to “load” for 5 minutes. Then turn handle to “sterilize” for 25 minutes and finally to “vent/dry” for 5 minutes before removing items.

Commonly used numbers and websites

Cell culture facility: For ordering Taq, dNTP, agarose, www.ccf.ucsf.edu
IDT: for ordering oligos, www.idtdna.com
New England Biolabs: For ordering Restriction Endonlucleases, www.neb.com

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. Pippette water first, then primers, buffer, dNTP, $MgCl_2$. 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 <u>appropriate</u> 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 or 2nd), gene (DHFR or DHPS), 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. Pippette water first, then primers, buffer, dNTP, $MgCl_2$. 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 48 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-channel 0.5-10.0 uL pipette carefully add 2 uL of template DNA to the appropriate wells.
9. Cap all appropriate wells and label PCR tray with the round# (1st or 2nd), gene (DHFR or DHPS), 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 (see table 2) 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. Note: Because DHPS 437 and 540 utilize the same PCR product, it is often useful to prepare their digestions at the same time. If you choose to do this, remove both sets of reagents, make two master mixes etc. Make sure to label well so the two digestions do not get confused.
3. 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".
4. Take lab book, PCR product, thawed reagents and PCR block to your work area.
5. Place a PCR tray in plastic PCR block and set aside.
6. 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. Pippette water first.
7. 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.
8. Add template DNA: Using the 8-channel 0.5-10.0 uL or 8 channel p200 pipette as appropriate, carefully add the template DNA to the appropriate wells.
9. Cap all wells and label PCR tray with RED and the appropriate mutation, and the page in lab book containing sample key and date.
10. Using appropriate counter balance, centrifuge PCR tray briefly to 1000 RPM to remove bubbles that may be in wells.
11. Place samples in an incubator at 37° C for 2 hours for DHFR 59 or DHPS 437, 540. Place samples in PCR machine at 65° C for 1-2 hours for DHFR 51, 108, or 164.
12. 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. At SFGH this is on the 3rd floor. Have someone in the lab show you how to use your particular machine.
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.
11. 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.