

December 2006

# Protocol for Genotyping *P. falciparum*

Merozoite Surface Protein 1 (MSP-1)

Merozoite Surface Protein 2 (MSP-2)

Author: Chris Dokomajilar  
Bryan Greenhouse

Contributors: Adithya Cattamanchi  
Sunil Parikh  
Sam Nsohya  
Grant Dorsey

## Introduction

The primary purpose of the genotyping methodology described in this protocol is to distinguish new from recrudescence infections in antimalarial drug efficacy trials. Genotyping patterns for infecting parasites at the time of initial diagnosis and at the time that repeat therapy are compared. Recrudescence (treatment failure) is defined as repeat therapy due to parasites present at the time of initial therapy. A new infection (not a treatment failure) is defined as repeat therapy due to parasites not present at the time of initial therapy. This information is used to generate estimates of the true risk of treatment failure. Genotyping is especially useful in drug efficacy studies done in highly endemic areas where follow-up is extended beyond 14 days. Formulas for common reagents used here can be found in the appendices of this protocol and should be prepared ahead of time. The steps for genotyping are as follows:

### Sample Preparation

Venous blood is sampled and dried on Whatman 3MM filter paper and an alcohol sterilized hole punch is used to cut circles for testing. DNA is isolated from dried blood spots (DBS) by using the Chelex extraction method described in Appendix 1. The minimum recommended DBS size is one 6mm circle. Along with the extraction of unknown sample DBS, prepare and extract a set of positive controls for all alleles of the genes being tested in this experiment.

### PCR Amplification

Polymerase Chain Reaction (PCR) methods are adapted from Zwetyenga et al 1998 and Snounou et al 1999. The surface antigen loci *msp1* and *msp2* are amplified using sequence specific primers. Template DNA is amplified using nested PCR, with second round primers specific to allelic families: K1, MAD20, and RO33 for *msp1*, and IC3D7 and FC27 for *msp2*. Separate reactions are performed for each pair of primary and nested primers. For example, one 96-well plate of *msp2* primary PCR will become two 96-well plates of IC3D7 and FC27 PCR. A No Template Control (NTC) is used in all reactions and genomic DNA from cloned laboratory strains, described below, is used as a positive control for respective alleles.

### Detection

PCR products are stained by ethidium bromide and separated by gel electrophoresis on a 2.5% agarose gel. DNA size standards are separated alongside PCR products to allow sizing of discriminate bands. Upon completion of the gel electrophoresis, gels are placed in a gel imaging cabinet and digitally photographed under UV light. Gel images are saved in a format compatible with GelCompar II gel processing software and printed for placement in the lab notebook.

GelComparII software is used to analyze separated PCR product (bands) using densitometry. This analysis gives distinct band sizes for all strains in corresponding reactions and data are entered into a standard database for classification of infecting parasites. Alleles between paired pretreatment and recurrent parasitemia samples run on adjacent lanes are considered to be the same if PCR products are measured to be within 10 base-pairs in molecular weight.

# Experimental Procedure

## PCR Preparation

- All PCR reagents should be stored in the freezer (-20°C) when not in use and necessary reagents allowed to thaw completely in time for preparation. The polymerase enzyme, however, should remain in the freezer until use as it is temperature sensitive and does not freeze.
- Begin the experiment in a lab notebook by designing a sample identification table in the standard 12 x 8 format. List all controls (no template control and allelic positive controls) and samples to be tested. Please consider the following when arranging paired samples: PCR samples should remain in an even number across the PCR arrangement so they remain paired during gel electrophoresis. Therefore, controls should be an even number and inserting an extra control may be necessary. Arrange the controls and samples starting from the top left well A1 and working across the row to A12 and continuing to the next row. See the example below:

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC	+C 3D7	+C HB3	+C HB3	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8
B	Sample 9	Sample 10										

- Primers for both rounds of PCR are listed below along with respective annealing temperatures. Genomic DNA from cloned laboratory strains to be used as positive controls are listed alongside the allelic families: K1, MAD20, and RO33 for *msp1*, and IC3D7 and FC27 for *msp2*.

### Primary Round:

Gene	Primer Name	Sequence	Annealing Temp. (°C)
MSP-1	M1-OF	5'- CTAGAAGCTTTAGAAGATGCAGTATTG	61
	M1-OR	5'- CTAAATAGTATTCTAATTCAAGTGGATCA	61
MSP-2	1	5'- ATGAAGGTAATTAACATTGTCTATTATA	55
	4	5'- ATATGGCAAAAGATAAAACAAGTGTTGCTG	55

### Nested Round:

Gene	Allelic Family	Primer Name	Sequence	Annealing Temp. (°C)	Positive Control
MSP-1	K1	M1-KF	5'- AAATGAAGAAGAAATTACTACAAAAGGTGC	61	3D7
		M1-KR	5'- GCTTGCATCAGCTGGAGGGCTTGCACCAGA	61	
	MAD20	M1-MF	5'- AAATGAAGGAACAAGTGGAACAGCTGTTAC	61	HB3
		M1-MR	5'- ATCTGAAGGATTTGTACGCTTGAATTACC	61	
	RO33	M1-RF	5'- TAAAGGATGGAGCAAATACTCAAGTTGTTG	63	RO-33
		M1-RR	5'- CATCTGAAGGATTTGCAGCACCTGGAGATC	63	
MSP-2	IC3D7	A1	5'- GCAGAAAGTAAGCCTTCTACTGGTGCT	55	3D7
		A2	5'- GATTTGTTTCGGCATTATTATGA	55	
	FC27	B1	5'- GCAAATGAAGGTTCTAATACTAATAG	55	HB3
		B2	5'- GCTTTGGGTCTTCTTCAGTTGATTC	55	

- Work in a PCR hood to prepare the master mix as described accounting for 12.5% more than the number of samples/controls being tested. Use the table below to calculate the volumes of reagents in making the master mix. After calculating the volume of reagents needed, vortex each reagent and combine in a suitable tube or sterile trough to make a master mix. Prepare the primary PCR master mix and complete the amplification, then follow with nested PCR using primer pairs for all suitable alleles in separate reactions. The PCR product of the primary PCR is used as the template in the nested PCR. The nested PCR is not multiplexed.

### Master Mix for BOTH Primary and Nested PCR Rounds

	Stock Concentration	Final Concentration	Volume (µL)	Multiple (# wells + 12.5%)	Vol. in Master Mix (µL)
H <sub>2</sub> O	--	--	16.05	X _____	
Primer 1	10 µM	0.2µM	0.5		
Primer 2	10 µM	0.2µM	0.5		
Buffer	10x	1x	2.5		
dNTP	2mM each	200µM each	2.5		
MgCl <sub>2</sub>	50 mM	1.5mM	0.75		
Taq Polymerase	5 U/µL (INV)	1 Unit	0.2		
Template	unknown	--	2.0		
<b>Final volume</b>			<b>25.0 µL total</b>		

- Add 23 µL of master mix to each well of a standard PCR plate that corresponds to the arrangement of controls and samples using a pipettor with aerosol barrier tips.
- Add 2.0 µL of samples and controls to appropriate reaction wells using a pipettor with a new aerosol barrier tip for each sample. It is optional to add 2.0µL of PCR water to the NTC well. Be careful to keep track of sample locations and avoid cross contamination. Cap the tubes and apply pressure for a tight seal.

### PCR Amplification

- Place the sealed PCR plate in the thermal cycler block.
- Program the thermal cycler by creating the programs as follows:

**MSP-2 Primary Round PCR Program: 3.5 hrs**  
Initial denaturation  
 94°C x 5m  
PCR  
 40 cycles of 94°C x 1.5m, 55°C x 45s, 72°C x 1.5 m  
Final Elongation  
 72°C x 10 m  
Hold @ 4 °C

**MSP-2 Nested Round PCR Program: 2 hrs**  
Initial denaturation  
 94°C x 2m  
PCR  
 30 cycles:94°C x 1.5m, 55°C x 45s, 72°C x1.5 m  
Final Elongation  
 72°C x 10 m  
Hold @ 4 °C

**MSP-1 Primary Round PCR Program: 2 hrs**  
Initial denaturation  
 95°C x 5m  
PCR  
 25 cycles of 94°C x 1m, 61°C x 45s, 72°C x 1.5m  
Final Elongation  
 72°C x 5m  
Hold @ 4 °C

**MSP-1 Nested Round PCR Program: 2hrs**  
Initial denaturation  
 95°C x 5m  
PCR  
 30 cycles of 94°C x 45s, allele specific°C x 30s, 72°C x 1m  
Final Elongation  
 72°C x 5m  
Hold @ 4 °C

## Detection by gel electrophoresis

1. Prepare enough 2.5% agarose gels to be used for band detection. See Appendix 2 for formulas to prepare gels and other necessary reagents. Use 20-well combs when casting gels to run 18 samples plus 2 ladders on each gel. Ready gels are placed in the gel tank filled with enough 1X TBE buffer to cover the gel by 5-10mm.
2. Add 3  $\mu\text{L}$  of loading dye to each PCR well using a pipettor (multichannel recommended) with a new aerosol barrier tip for each sample.
3. Mix each sample/dye by pipetting up and down and carefully load 15  $\mu\text{L}$  into the appropriate well on a gel. Reserve the first and last well for the DNA size standard.
4. Once all 18 wells are loaded, vortex the vial containing prepared 50bp DNA ladder and load 15  $\mu\text{L}$  into the first and last well of the gel. Work efficiently so the sample DNA does not diffuse excessively into the gel before applying electrical current.
5. Once all wells are loaded, align the gel horizontally in the gel box, cover, and apply electrical current. Be sure to connect the gel tank so that the DNA electrophoreses towards the red/positive side of the gel.
6. Set the electrical supply to 80-85 Volts and confirm that electrophoresis has begun by observing bubbles rising from the electrodes in the TBE buffer.
7. The gel has run sufficiently and may be photographed when the bromophenol blue dye has run off the bottom of the gel. Note: for the FC27 allele, run the gel for 10 minutes past this point to allow for better separation.
8. Gently remove the gel from the tank and place into the gel imaging cabinet. Using UV light, digitally photograph the gel and save in a format compatible with GelCompar II gel processing software. Print the gel image as well for placement in the lab notebook. Refer to separate instructions for the gel imaging cabinet.
9. Immediately after the images are taken, check on the validity of the results. If any bands are present in the No Template Control, the entire experiment is invalid. For each positive control a single band of the correct size should be present for each allele as described below. For the patient samples, at least 1 allele should be detected for EITHER the IC3D7 allelic family or the FC27 allelic family and EITHER the K1, MAD20, or RO33 allelic families. Repeat PCR should be performed on pairs if any sample in the pair failed. Use 5.0  $\mu\text{L}$  of template DNA, adjusting the master mix by adding 2.0  $\mu\text{L}$  less water, and use a 21  $\mu\text{L}$  master mix per well.

### Positive Control Sizes:

Gene	Allelic Family	Positive Control Band sizes (+/- 10bp)		
		3D7	HB3	RO-33
MSP-1	K1	204	Nothing	Nothing
	MAD20	Nothing	146	Nothing
	RO33	Nothing	Nothing	150
MSP-2	IC3D7	270	Nothing	n/a
	FC27	Nothing	390	n/a

## Gel image processing using GelCompar II software

1. All gels should be stored in a folder located on the hard drive. The first step in the process is to generate an external reference standard (from your DNA ladders). To do this you will need to have a gel with good ladders at each end. Therefore run a few gels until you have a nice one that can be used to make your reference standard. A good gel is one where the ladders have run straight and even. The 150bp marker should be near the bottom but clearly visible. The 600bp marker should be clearly visible at the top. This 150bp-600bp range will be used to make the external reference standard.
2. Open GelCompar II software
3. Click "New" icon
4. Name your data base
5. Click "Next"
6. Select "Yes"
7. Click "Next"
8. Select "No"
9. Click "Finish"
10. Now your database will appear by name in the lower left corner of the GelCompar II window
11. Highlight your database name by clicking on it
12. Click "Analyze"
13. Place cursor in the "Files" window and right click
14. Select "Add new fingerprint file..."
15. Find the tiff file of interest and open it. It should now appear in the "Files" menu with a read capital "N" in front of it.
16. Place cursor over tiff file and right click
17. Select "Open fingerprint file (data)..."
18. A window titled "Confirmation" will appear. Select "Yes".
19. A window titled "New fingerprint type" will appear. Type in the word "agarose" under fingerprint type name.
20. Click "Next"
21. Select "Two dimensional TIFF files" and "8-bit (256 values)"
22. Click "Next"
23. Select "Yes" under "Do your fingerprints have inverted densitometric values"
24. Click "Next"
25. Select "No" under "Do you wish to apply a background subtraction on the densitometric curves?"
26. Click "Finish"
27. The gel will now appear in a window titled "Fingerprint data of *name of your gel*". This will begin Step 1 titled "Strips"
28. Click on maximize icon in upper right corner to enlarge the gel image
29. Move green borders to surround the area of interest on the gel. The right and left borders should be placed against the outer edge of the DNA ladders. The upper and lower borders should enclose the largest and smallest bands in your gel. Typically this should span the 200-550 bp markers, however occasionally you will need the gel to be a little smaller or larger. The key is just to have all the bands of interest within the green borders with not having too much extra space. **FOR THE FIRST GEL YOU ARE USING TO MAKE THE EXTERNAL REFERENCE, MAKE SURE THAT THE GREEN BORDERS SPAN THE 150bp – 600bp MARKERS.** Often the gel does not run completely straight. If this occurs you should adjust the corners of the green border. This is done by placing the cursor over the corner of the green border, pressing down the "shift" key and moving the green border. When the shift key is pressed both lines coming off the corner will move in a diagonal way. When you are finished each of the 4 corners of the green border should be adjacent to the same sized marker on the DNA ladder.
30. Go to the top of the window and select "Lanes". Then move the cursor down and select "Define group of lanes..". Type in the number of lanes and click "OK". All of the lanes will now be bordered by blue lines.
31. Go up to the top of the window and click the right arrow icon which will take you to the next step – "2. Curves". Now the gel will have a different appearance with a narrow red line running down the first lane and narrow blue lines running down the other lanes. Skip this step by clicking on the right arrow again. Now you will be at step 3. Normalization. On the left side of the window will appear the message "No active reference system defined".

32. Place the cursor any where in the first lane (DNA Ladder), right click and select "Use as reference lane". Do the same thing for the last lane (also the DNA Ladder). Now a icon shaped like a bottle of milk will appear at the top of these lanes.
33. Go to top of the window and click on Normalization. Select "Show normalized view".
34. Go to the 350bp marker on lane one and right click. Select "add external reference position". A new window will appear asking you to enter band name. Type in "350". Do the same for all the bands below (300, 250, 200, 150) and above (400, 450, 500, 550, 600) spanning 150bp to 600bp. Now these names should appear just to the left of the appropriate size bands on the first lane.
35. Now you have to assign each of the bands in both ladders. Do this by first clicking one of the labels to the left of the first lane (I always start with 350), press down the control key, drag the cursor over to the 350bp marker and click again. This will assign the marker. Do this for all the bands in BOTH ladders.
36. Press the right arrow in the upper window to go to the next step – 4. Bands. You will skip this step this time around and close the window by clicking on X icon in the very right upper corner. Another window will appear with the question "Configuration has been changed. Do you want to save the changes?". Click "Yes". Click "Yes on the next question as well.
37. Now you should be back to the original window. Go to the "Fingerprint types" window and place cursor over the word "agarose" and right click. Select "Edit experiment type..".
38. A new window will appear titled fingerprint type "agarose". Place the cursor anywhere under the "Reference systems" sub-window and right click. Select "set as active reference system".
39. Again place the cursor anywhere under the "Reference systems" sub-window and right click. Select "edit active reference system".
40. Click on "Metrics" from the menu at the top of the window. Select "Copy markers from the reference system". Select "Yes". Close the windows except the original one.
41. Now you are ready to read a gel and quantify band sizes.
42. Go to your same first gel and right click. Select "Open fingerprint file (data)". Skip to step 2. Strips by clicking on right arrow (you have already made your border and defined your lanes).
43. Click on "Edit" from the upper menu and select "Edit settings". Under "Curve extraction" set average thickness to 11 points and number of nodes to 2. Under filtering select "Median filter". Click OK.
44. Click on Raw data. Under image strip extraction set thickness to 25 points and nodes to 3. Click so that there is a check under "Background subtraction" and set at 6 points. Click so that there is a check under "Spot removal" and set at 50 points. You do not need to adjust anything in the "Normalization" or "Bands" windows.
45. Close this window by selecting OK.
46. Click on "Edit" from the upper menu and select "Edit tone curve..". Click "Linear" and then close window by selecting OK.
47. Click on "Curves" from the upper menu and select "Spectral analysis..". Move this window over to the right keeping the values for the Weiner cutoff scale and background scale visible.
48. Click on "Edit" from the upper menu and select "Edit settings". Move this over to the left so that you can still see the values for Weiner cutoff scale and background scale in the other open window. Click on "Apply" under the "Background subtraction" sub-window. Set disk size according to the background scale in the spectral analysis window (round up or down to the nearest whole percentage point). Click on "Apply least square filtering" and enter the value for the "cut off below" box according to the Weiner cutoff scale in the spectral analysis window (to the 3<sup>rd</sup> decimal point).
49. Select OK and then close the spectral analysis window.
50. Go to the next step by clicking the right arrow.
51. You have already assigned the reference positions for this gel. Click on Normalization and select "Show normalized view".
52. Go to the next step by clicking on the right arrow
53. Go to "Bands" and click on "Auto search bands". Make the following settings:
  - Minimum profiling = 5%
  - Gray zone = 0%
  - Rel. to max. val should be checked
  - Minimum area = 0%
  - Shoulder sensitivity = 2
54. Click OK.
55. Now all of the selected bands should have a green line through them. This is where the technique becomes a bit of an art. Basically you should have only bands selected that you think are real. This can be determined two ways: one is just to look at the original gel. The second is to go to the "Edit"

command and select "Edit tone curve". Then you click "enhance weak bands" several times to make the bands as dark as possible. The you use the cursor to scroll around so that you can visually determine which bands are real and which are not. Once you have a good idea from these two methods which bands you think are real, you should go back to the and make adjustments so that the correct bands are selected (if you enhance weak bands remember to click on "linear" to reset this window before closing it).

56. Now you have a good idea what bands you think should be select. You can make changes with the following settings to either get rid of bands you think are not real or pick up bands you think are real.

- 1) Under "Bands" "Auto search bands" you can increase or decrease the minimum profiling
- 2) Under "Edit" "Edit settings" "Raw data" you can increase or decrease the background subtraction and the spot removal.

I would not make any other changes to the settings. If you really think a band should not be selected you can just put the cursor over that band, right click and select "Delete selected band(s)". Likewise if you think a band should be added, you can put the cursor over that band, right click, and select "add new band". The most common problem is when you have a dense band with a "laddering" phenomenon below that band. We tend to think this laddering phenomenon is not real and make adjustments so that these bands are not selected.

57. Once you are satisfied that all the correct bands have been selected you go to "File" and select "Export" report. This will give you the molecular weights of all the bands that were select as a .txt file. (under the column "Metrics" are the weights in bp). I print this out and label which lane is which. Then I immediately enter the data into my SPSS database.

58. Close the Result.txt – Notepad.

59. Close the Fingerprint data window. You will get a window that asks you if you what to save the changes. You should always select "Yes". The next window asks you if you want the current settings as the new defaults. For the first gel you should select "Yes".

Now you have completed making your external reference standard (which only needs to be done once) and read your first gel. Now you can do the following steps to read the remaining gels.

60. Open GelCompar II software (you can skip the next few steps if it is already open)

61. Click on your database name in the lower left corner and then select "Analyze"

62. Go to "Files" menu and right click. Select "Add new fingerprint file"

63. Open the tiff file of interest

64. Right clinic on this file under the "Files" menu. Select "Open fingerprint file (data)"

65. Click "OK"

66. Follow steps 27-30 to create the green border around your gel and define your lanes.

67. Go to Step 2. Curves

68. Click on "Edit" icon and select "Edit tone curve". Click on Linear. Click OK

69. Click on "Edit" icon and select "Edit settings..". You should never have to change the averaging thickness or the number of nodes. Click "Median filter". Uncheck background subtraction and apply least squares. Click OK

70. Follow steps 47-50 to add spectral analysis settings to the background subtraction and apply least squares (to do this you must have previously unchecked these as instructed in step 69).

71. You should now be in Step 3. Normalization. First assign all the bands in the two DNA ladders. Do this by following step 35.

72. Click on "Normalization" and select "Show normalized view". Next, click on "Normalization" and select "Show distortion bars". Vertical colored bars will appear along each lane of the gel. Yellow to blue is good. Black to red is bad. If you have a lot of black to red you probably made an error in assigning the markers in the DNA ladder. If this is the case you should re-assign these markers and try again.

73. Click on the right arrow to go to step 4. Bands.

74. Click on "Bands" and select "auto search bands". Select OK without changing any of your defaults settings. Now you will see which bands are selected using your defaults settings. You may need to make adjustments as described in steps 55-56. Once you are satisfied with the bands that have been selected complete steps 57-59. When you exit the window each time you should select "Yes" to "Configuration has been changed. Do you want to save the changes". After my first gel, I like to select "No" to the next question "Settings have been changed. Do you want to use the current settings as the new defaults". I like to keep the defaults the same each time.

## **Interpretation of genotyping patterns**

Enter all results into a standard database (*eg.* SPSS). This will involve four string variables for IC3D7 (day 0 and day of failure) and FC27 (day 0 and day of failure), and six string variables for K1 (day 0 and day of failure), MAD20 (day 0 and day of failure), and RO33 (day 0 and day of failure). For a result to be considered valid you must have at least one band for either the allele. To be able to classify an outcome you need to have valid results for both day 0 and the day of failure.

Next code how many unique bands (both alleles combined) were detected for day 0 and the day of failure. Then compare the bands in the day of failure to day 0 to calculate a fraction (numerator = the number of bands present on the day of failure also present on day 0, denominator = total number of day of failure bands)

Bands less than 500bp in size are consider the same if they are  $\leq 10$  bp in size

# Appendix 1

## Chelex DNA Extraction

Materials: Dried, blood-blotted filter papers; 10% saponin in water (stored at  $-20^{\circ}\text{C}$ ); 1XPBS (Calcium and Magnesium Free pH7.4); 20% Chelex (stored at room temperature); a heat block at  $95^{\circ}\text{C}$

1. Cut the filter paper to appropriate size using a scissors or hole puncher. The same blade or hole puncher can be used after wiping with an alcohol swab.
2. Combine the dried, blood blotted filter papers with 1ml of PBS and 50ul of 10% saponin in a 1.5 ml Microfuge tube, invert several times, and store over night at  $4^{\circ}\text{C}$ .
3. Microfuge tubes for 5 seconds and aspirate the now reddish PBS/saponin from the tubes with a clean non-barrier tip attached to a Pasteur pipette at the end of a vacuum assembly using a new tip for each tube.
4. Add 1 ml of PBS/tube (no saponin), invert several times and incubate at  $4^{\circ}\text{C}$  for 15-30min. Turn on the heat block at this time to allow time for  $95^{\circ}\text{C}$  to be reached.
5. Microfuge and aspirate (as above) as much fluid as possible and afterwards use the tip to press the filter paper down into the lower third of the tube without packing it excessively.
6. Transfer 1ml of vortexed chelex stock solution to a 1.5 ml microfuge tube and using a tip with tapered end cut off. Dispense 50 ul to each sample, vortexing or inverting the tube every two or three transfers (to be sure you are not just transferring water with all the chelex settled to the bottom of the dispensing tube).
7. Add 100ul of sterile water to each tube.
8. Extract the parasite DNA by incubating the tubes for 10 min in a  $95^{\circ}\text{C}$  heat-block, vigorously vortexing each sample every 2 minutes or so throughout the incubation. It is best to briefly uncap each tube every two minutes or so in the block to release pressure, or else the tubes will "pop"
9. After incubation, microfuge the tubes for 5 minutes at high speed. Meanwhile, label two sets of 0.6mL microfuge tubes for transfer, the second set for final storage of the extracted DNA samples.
10. Transfer as much solution as possible from the spun tubes to the first set of microtubes with a 200uL barrier-tip, not worrying if chelex is carried over as well.
11. Spin tubes for 10 minutes at high speed and then transfer the final, white-to-yellowish supernatant (avoiding the pelleted chelex) to the final set of labeled tubes. Store at  $-20\text{C}$ .

## Appendix 2

### Reagent Formulas

#### 2.5% Agarose Gels

Makes 2 gels

5.0g agarose

200 mL 1x TBE

15 uL ethidium bromide

Microwave 4 ½ minutes for 2 gels, 6 minutes for 4 gels, then cool to 60° C and add ethidium bromide. Mix gently to distribute ethidium bromide. Pour gel into casting tray with 20 well combs and allow to set (approximately 20 min). **Note: ethidium bromide is mutagenic and should be handled carefully. Gels containing ethidium bromide should be disposed of accordingly**

#### 5X TBE Buffer Stock

Per liter of stock solution

54g tris base

27.5g boric acid

20 mL 0.5M EDTA pH 8

#### 6X Loading Dye

Makes 10mL

25 mg (0.25%) bromophenol blue

3 mL (30%) glycerol

7 mL H<sub>2</sub>O

#### DNA Size Marker With Dye

50 uL of 50bp ladder (Invitrogen)

170 uL 6X bromphenol blue loading dye

780 uL H<sub>2</sub>O to make 1 mL

#### 2mM dNTP

1 mL 100 mM dNTP from stock

11.5 mL H<sub>2</sub>O

Aliquot into 1 mL volumes