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Abstract of thesis entitled

“Identification of Novel Chloroquine Resistance Genes by Using

Yeast Complementation System”

Submitted by LAU, Chi-Keung

for the Degree of Master of Philosophy

at the Hong Kong Polytechnic University in October, 2001.
Abstract

Malaria is one of the fatal diseases in the world and the situation has become more worst due to the presence of multidrug resistance. Several phenotypic changes are associated with chloroquine resistance in *Plasmodium falciparum*, including reduced chloroquine accumulation and the changes in the binding of chloroquine to ferrirhodopsin IX. In the past, several candidate genes have been proposed, including *pfmdrl*, NHE, *cg2*, and other putative mechanisms mediating the changes in ferrirhodopsin IX binding to chloroquine. The main reason why the molecular biology of this parasite is not the same as that of bacteria is the transfection of gene to *P. falciparum* is not well established. Therefore, the role of many candidate genes in resistance mechanism remains to be searched. We have used a yeast functional complementation approach to identify novel chloroquine resistance genes. A 3D7 plasmodial cDNA library, constructed in a yeast expression vector, was used to transfect a chloroquine-sensitive yeast strain and plated on chloroquine-containing plates at a concentration which can kill the chloroquine-sensitive yeast strain. After primary and secondary
screening, about 30 colonies, supposed to contain plasmodial drug resistance genes, were picked and analyzed. By using yeast drug assay, clones that confer resistance were chosen and sequenced. Finally, about eight clones were further characterized. Since the *P. falciparum* genome project is still in progress, the function of many genes remains to be searched. By searching the sequence of this eight genes from Genbank database, three of them were found from plasmodial expressed sequence tags database, four of them can only be found from unfinished sequencing data of plasmodial database and one of them was a *Plasmodium falciparum* knob-associated histidine-rich protein. The resistance phenotype of those resistant clones were confirmed by drug assays and northern analysis shown that they would express their mRNA. By using yeast complementation system, putative chloroquine resistance genes can be rapidly found out.
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<tr>
<td>A</td>
<td>adenine</td>
</tr>
<tr>
<td>Amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulfate</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>C</td>
<td>cytosine</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CQ</td>
<td>Chloroquine</td>
</tr>
<tr>
<td>CQR</td>
<td>Chloroquine resistance</td>
</tr>
<tr>
<td>C-terminal</td>
<td>carboxyl-terminal</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>deoxy(G, A, T and C) triphosphates</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diaminetetraacetic acid</td>
</tr>
<tr>
<td>EtBr</td>
<td>ethidium bromide</td>
</tr>
<tr>
<td>EtOH</td>
<td>ethanol</td>
</tr>
<tr>
<td>G</td>
<td>guanine</td>
</tr>
<tr>
<td>kbp</td>
<td>kilo base pair</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>N-terminal</td>
<td>amino-terminal</td>
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<tr>
<td>NaAc</td>
<td>sodium acetate</td>
</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride</td>
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<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
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<tr>
<td>nt</td>
<td>nucleotide</td>
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<tr>
<td>OD</td>
<td>optical density</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>RT</td>
<td>reverse transcription</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNase A</td>
<td>ribonuclease A</td>
</tr>
<tr>
<td>SC-URA</td>
<td>Synthetic complete medium minus uracil medium</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecylsulfate</td>
</tr>
<tr>
<td>SSC</td>
<td>saline sodium citrate</td>
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<tr>
<td>T</td>
<td>thymine</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris [hydroxymethyl] aminomethane</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactoside</td>
</tr>
<tr>
<td>YPD</td>
<td>Yeast peptone dextrose (YPD) medium</td>
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1. Introduction

According to the World Health Organization (WHO), approximately 300 million people worldwide are affected by malaria and between 1 and 1.5 million people die from it every year. Most cases occur in developing countries, such as Africa, Asia and Latin America. More than eighty percent of the cases occur in tropical Africa, where malaria accounts for 10% to 30% of all hospital admissions and is responsible for 15% to 25% of all deaths of children under the age of five. Around 800,000 children under the age of five die from malaria every year, making this disease one of the major causes of infant and juvenile mortality. Pregnant women are also at risk since the disease is responsible for a substantial number of miscarriages and low birth weight babies, especially in remote rural areas with poor access to health services.

The problems of controlling malaria in these countries are aggravated by inadequate health structures and poor socioeconomic conditions. The situation has become even more complex over the last few years with the increase in resistance to the drugs normally used to combat the parasite that causes the disease.
1.1 What Is malaria

Human malaria is caused by a protozoan parasite called *Plasmodia*. There are four different species of *plasmodium* that can infect humans; namely, *Plasmodium falciparum*, *Plasmodium ovale*, *Plasmodium vivax* and *Plasmodium malariae*. *P. falciparum* is the most widespread and dangerous of the four: untreated case can lead to fatal cerebral malaria. The female *Anopheline* mosquito transmits malaria parasites from one person to another. The males do not transmit the disease, as they feed only on plant juices. There are over 380 species of *Anopheline* mosquito, but only 60 or so are able to transmit the parasite.
1.2 Life Cycle

Figure 1.1 shows the life cycle of *Plasmodium falciparum*. The life cycle started at sporozoites form in mosquito mouthpart; after asexual blood stage, some of them proceeded sexual stage and returned to mosquito for restarting the life cycle. (The picture comes from Expert Reviews in Molecular Medicine.)
(a) The life cycle of *P. falciparum* starts when an infected *Anopheles* sp. mosquito injects their mouthparts for blood bleeding. The infective sporozoites would be injected from mosquito’s salivary gland into the peripheral circulation of human.

(b) Within minutes, these sporozoites invade hepatocytes in the liver and they replicate to produce tens of thousands of merozoite forms of the parasite after one week.

(c) Then, the infected hepatocytes rupture and merozoites are released into the peripheral circulation.

(d) The merozoites start to invade red blood cells (rbcs).

(e) The merozoites then replicate inside rbc to produce about 16-20 merozoites per rbc within 48 - 72 hours and digest the hemoglobin. Finally, rbc would burst and the merozoites release.

(f) The released merozoites invade other neighboring rbcs and carry on the cycle.
(g) Some invading merozoites do not divide and proceed sexual reproduction. It differentiates into male (microgametocyte) and female (macrogametocyte) sexual forms.

(h) These sexual forms are taken from the bloodstream by a feeding *Anopheles sp.* mosquito.

(i) Gametocytes fertilize in the mosquito midgut to form zygotes. It differentiates into motile forms and migrates through the mosquito gut wall. Eventually, it divides to form thousands of sporozoites.

(j) The infective sporozoites are released in the salivary gland of mosquito where they wait for injection into another human host and restart the life cycle.
1.3 Treatment

Since the life cycle of *P. falciparum* have known, many treatments have been discovered to target on different stage of *P. falciparum*'s life cycle. Generally, vaccination and chemotherapy are the main defense of all diseases appeared in the world.

1.3.1 Vaccine

The situation has gradually worsened in recent years because of increasing resistance of the anopheline mosquitoes that transmit malaria to insecticides, and of the parasites themselves to antimalarial chemotherapy. Thus, the development of an effective malaria vaccine represents a high priority intervention strategy to control both the transmission of infection and the impact of disease (Taylor-Robinson, 1998).

In recent years, molecular tools were used to identify, produce and study malarial antigens and it has encouraged researchers to attempt to construct subunit vaccines containing multiple targets at each stage of parasite development. However, both natural and
vaccine-induced immunity are hampered by the capacity of blood stage parasites to vary critical antigenic determinants on the surface of the parasitized erythrocyte. An additional complication to vaccine development is the genetic heterogeneity of human populations. In order to overcome the difficulty of poor immunogenicity and parasite diversity, much experimental work is now focused on complex antigenic constructs delivered as synthetic peptides, such as SPF66 (Rodriguez et al., 1990; Ruebush et al., 1990; von Sonnenburg, 1995), recombinant proteins, such as merozoite surface protein-1 (MSP-1) (Daly and Long, 1995; Egan et al., 2000; Rotman et al., 1999; Urquiza et al., 1996), 'naked' (plasmid based) DNA, such as pf332 (Fricker, 1996; Martin et al., 1999; Sedegah et al., 2000). Although there were many candidate vaccines discovered, Only one candidate vaccine, SPF66, based on antigens from both merozoite and sporozoite stages, has undergone extensive field trials. Efficacy was reported in several early clinical trials in South America, and one in Africa, but results from subsequent trials in Africa and Southeast Asia were not as promising (Facer and Tanner, 1997; Miller and Hoffman, 1998).

The most recent strategy of vaccination, genetic immunization, utilizes the DNA encoding the immunogen. These polynucleotides are
directly introduced or transfected into tissues of the host individual which then express the requisite gene product, thereby obviating the necessity for production design and formulation processes tailored to each immunogen (Ulmer J B, 1996). Although almost all vaccination studies to date have used pure plasmid DNA, naked mRNA theoretically offers an attractive alternative as it does not present a potential risk of integration into the genome. However, because of its instability and the insufficient time it allows for antigen synthesis in vivo, this technique is still being developed.

Today, there is still no effective vaccine for malaria; therefore, the main line of defense now available against the malaria is chemotherapy.

1.3.2 Chemotherapy

Antimalarials can be categorized by the stage of the parasite that they affect and the corresponding clinical objective.
1.3.2.1 Tissue Schizontocides Used for Causal Prophylaxis.

These agents act on primary tissue forms of *Plasmodia* within the liver that are destined within a month or less to initiate the erythrocytic stage of infection. Invasion of erythrocytes and further transmission of infection are thereby prevented. Chloroguanide (proguanil) exemplifies an antimalarial used primarily for causal prophylaxis of falciparum malaria (Warhurst, 1987; Watkins et al., 1987; Wernsdorfer, 1990). Although primaquine also has such activity against *P. falciparum*, this potentially toxic drug is reserved for other clinical applications (Tosone et al., 1990).

1.3.2.2 Tissue Schizontocides Used to Prevent Relapse.

Primaquine is the prototypical drug used to prevent relapse (Patterson et al., 1987; Sinha et al., 1989). These compounds act on latent tissue forms of *P. vivax* and *P. ovale* remaining after the primary hepatic forms have been released into the circulation. Eventually, such latent tissue forms mature, invade the circulation, and produce malarial attacks called relapsing malaria. Schizontocides that are active against latent tissue forms are used for terminal
prophylaxis and for radical cure of relapsing malarial infections. For terminal prophylaxis, regimens with such a drug are initiated shortly before or after a person leaves an endemic area. To achieve radical cure, this type of drug is taken either during the long-term latent period of infection or during an acute attack. In the latter case, the agent is given together with an appropriate blood schizontocide, usually chloroquine, to eradicate erythrocytic stages of *P. vivax* and *P. ovale*.

1.3.2.3 Schizontocides Used for Clinical and Suppressive Cure.

Blood schizontocides act on asexual erythrocytic stages of malarial parasites to interrupt erythrocytic schizogony and thereby terminate clinical attacks. Such drugs also may produce suppressive cure, which refers to complete elimination of parasites from the body by continued therapy. Inadequate therapy with blood schizontocides may result in recrudescence of infection due to erythrocytic schizogony. With the notable exception of primaquine, virtually all antimalarial drugs used clinically were developed primarily for their blood schizonicidal activity. These agents can be divided into two
classes; the rapid acting blood schizontocides includes classical antimalarial alkaloids like chloroquine, quinine, and their related derivatives quinidine, mefloquine, and halofantrine. The antimalarial endoperoxides, for example, qinghaosu, also belong to this category. Slowing acting, less effective blood schizontocides are exemplified by the antimalarial antifolate and antibiotic compounds. These drugs are most commonly used in conjunction with their more rapidly acting counterparts (Dutta et al., 1989; Espinal et al., 1985; Peters and Robinson, 1992; Peters and Robinson, 1997; Warhurst, 1987).

1.3.2.4 Gametocytocides

These agents act against sexual erythrocytic forms of Plasmodia, thereby preventing transmission of malaria to mosquitoes. Chloroquine and quinine have gametocytocidal activity against *P. vivax* and *P. ovale* and *P. malariae*; on the other hand, primaquine displays especially potent activity against *P. falciparum*. However, antimalarials are rarely used clinically just for their gametocytocidal action (Warhurst, 1987).
Although there are a number of drugs used for treatment of malaria, only a few of them can be used clinically because of their toxicity and side effect to human. Generally, a number of drugs have been used for malaria treatment, such as quinine, chloroquine and mefloquine. All of them were quinoline antimalarial drugs.

1.4 History of Quinoline Antimalarial Drugs

The first quinoline antimalarial drugs were alkaloids extracted from the Contress of Chinchon, who according to legend was cured of malaria in 1630 by a powder made from its bark (Wallace, 1996). A crude mixture of crystalline alkaloids was extracted from cinchona bark by Gomes, in Portugal, in 1810 and the quinoline antimalarial drugs was isolated or synthesized from this crude mixture.

1.4.1 Quinine

The medicinal use of quinine dates back over 350 years. Quinine is the chief alkaloid of cinchona, the bark of the South American cinchona tree (Amabeoku, 1991). Although quinine has been synthesized, the procedure is complex; quinine and the other
alkaloids are, therefore, still obtained from natural sources. Cinchona contains a mixture of more than 20 alkaloids. The most important of these are two pairs of optical isomers, quinine and quinidine and cinchonidine and cinchonine. Quinine contains a quinoline group attached through a secondary alcohol linkage to a quinuclidine ring (figure 1.2). Quinidine has the same structure as quinine except for the steric configuration of the secondary alcohol group. Quinidine is both more potent as an antimalarial and more toxic than quinine.

Quinine acts primarily as a blood schizontocide and it has little effect on sporozoites or pre-erythrocytic forms of malarial parasites. The alkaloid is also gametocidal for *P. vivax* and *P. malariae* but not for *P. falciparum*. Because of this spectrum of antimalarial activity, quinine is not used for prophylaxis. As both a suppressive and therapeutic agent, quinine is more toxic and less effective than chloroquine against malarial parasites susceptible to both drugs (Luzzi and Peto, 1993). Generally, quinine is in a lot of time used in combination with other drugs like tetracycline in treating malaria (Alencar et al., 1982; Bourgeade and Delmont, 1998).
1.4.1.1 Mechanism of quinine action

The molecular mechanism of action of quinine is remained to be searched. As quinine is a weak base, it is highly concentrated in the acidic food vacuole of *P. falciparum*. The drug is supposed to act in these organelles by inhibiting the activity of heme polymerase, thereby allowing the accumulation of its cytotoxic substrate, heme (Slater and Cerami, 1992).
Figure 1.2 shows the structure of quinine.
1.4.2 Chloroquine

Comparing all of the drugs developed for the treatment of malaria, none of the other drugs has been found as useful as chloroquine (7-chloro-4-(-4-diethylamino-1-butylamino) quinoline), (figure 1.3). The drug was synthesized in 1934 by German chemists at Bayer AG, and it has been the most extensively used drug for the treatment of *P. falciparum* infections from the 1940s to the present day. For example, it is estimated that the US international cooperation agencies bought and distributed 300 million tablets of chloroquine between 1960 and 1962 (Coatney, 1963).
Figure 1.3 shows the structure of chloroquine
1.4.2.1 Mechanism of chloroquine action

Different theories have been proposed to explain the mode of action of chloroquine (Coatney, 1963; Ginsburg, 1992; Slater, 1993; Ward, 1988). All of them propose that chloroquine is selectively active against the developmental stages where hemoglobin is being actively degraded and malaria pigment, haemozoin, is being produced. In 1992, Slater and Cerami reported that ferriprotoporphyrin IX (FP), a toxic hemoglobin breakdown product produced during parasite feeding, was converted to a non-toxic malarial pigment called haemozoin by an enzyme-dependent polymerization of membrane-lytic FP in the acid food vacuole of the parasite. First, chloroquine was a weak base when it was in the unprotonated form so that it could cross membranes freely. When it entered the acidic food vacuole, it was protonated as a diprotic weak base that was less permeable to membranes and accumulated in there. Chloroquine inhibited polymerization in vitro at concentrations, which they claimed correlated with antimalarial activity (Slater and Cerami, 1992) (figure 1.4).
Figure 1.4 shows the action of chloroquine. *P. falciparum* would digest the haemoglobin in infected erythrocytes. FP is a by-product of this digestion and toxic to the parasite. Therefore, it would polymerize the toxic FP to non-toxic haemozoin. When chloroquine is presented, it would accumulate in parasite food vacuole and inhibit polymerization so that the toxic FP can kill the parasite.
Chloroquine is highly effective against blood stages parasites. This includes the asexual erythrocytic trophozoite stages. The drug is inactive against sporozoites and the primary and secondary exoerythrocytic stages of all strains. Chloroquine also has gametocytocidal activity against *P. vivax*, *P. ovale* and *P. malariae*, but is only active against immature gametocytes of *P. falciparum* (Anand, 1984; Bruce-Chwatt, 1986). Owing to its antimalarial activity, limited toxicity, ease of synthesis and relatively low cost, chloroquine has been very useful in malarial treatment and prophylaxis. Unfortunately, the appearance of chloroquine resistant strains of the parasites causes chloroquine to be a less-than-perfect drug.

**1.4.3 Mefloquine**

Mefloquine is a product of the Malaria Research Program established in 1963 by the Walter Reed Institute for Medical Research to develop promising new compounds to combat emerging strains of drug-resistant *P. falciparum*. Among many 4-quinoline-methanols tested based on their structural similarity to quinine, mefloquine, (figure 1.5), displayed high antimalarial activity in animal models and
emerged from clinical trials as safe and highly effective against drug resistant strains of *P. falciparum* (Trenholme et al., 1975). Mefloquine is now recommended for the prophylaxis and chemotherapy of chloroquine-resistant or multidrug-resistant falciparum malaria.
Figure 1.5 shows the structure of mefloquine.
1.4.3.1 Mechanism of mefloquine action

The exact mechanism of action of mefloquine is still not well known. As a blood schizontocide, mefloquine behaves like quinine in many respects but does not intercalate with DNA (Davidson et al., 1975). The two compounds produce similar morphological changes in early erythrocytic ring stages of *P. falciparum* and *P. vivax* (Olliaro et al., 1989). Like quinine, mefloquine competes for accumulation of chloroquine and inhibits chloroquine induced clumping of pigment in erythrocytic plasmodia (Warhurst, 1987). The major ultrastructural abnormality produced by mefloquine in *P. falciparum* is swelling of the parasitic food vacuoles (Jacobs et al., 1988). Like chloroquine, low extracellular concentrations of mefloquine raise the intravacuolar pH of plasmodia in excess of that predicted from passive distribution of a weak base (Krogstad et al., 1988). This suggests that mefloquine is concentrated in plasmodia by an unknown mechanism. Unlike chloroquine and quinine, mefloquine has been shown to inhibit heme polymerase, even though it has a high affinity for free heme (Slater and Cerami, 1992). Mefloquine may act by forming toxic complexes with free heme that damage membranes and interact with other plasmodial components (Borst and Ouellette, 1995).
As mentioned before, only few drugs can be used for malarial treatment. Besides of their toxicity and side effects, the main reason is the development of multidrug resistant strain *plasmodium*.

### 1.5 Chloroquine Resistance

The first case of chloroquine resistant strains of *P. falciparum* was reported independently in Southeast Asia and South America in the late 1950s. Over the next 30 years, chloroquine resistant strains spread to the whole of Southeast Asia and Africa (Bruce-Chwatt, 1986) (figure 1.6).
Figure 1.6. The map shows the spreading of chloroquine resistant *P. falciparum* since the late 1950s. The first case developed independently in Southeast Asia at 1957 and South America at 1959 and in the next 30 years, the resistant strains would widely spread over the world (From the website of WHO).
There are four general mechanisms of drug resistance: 1. inhibition of drug uptake; 2. drug degradation or inactivation; 3. altering the target to make it less sensitive to the drug and 4. drug extrusion.

The mechanism of chloroquine resistance involve reduced accumulation of the drug in the parasite was proposed (Krogstad et al., 1987), but, up to now, the actual resistance mechanism remains to be elucidated.

Chloroquine resistance is similar to the multi-drug resistance (MDR) phenomenon that was first observed in human cancer cells (Endicott and Ling, 1989; Gottesman and Pastan, 1993). The drug resistance mechanism in human cancer cells is an efflux-mediated mechanism involving the overexpression of a transporter protein, p-glycoprotein (Pgp) (Chen et al., 1986; Gros et al., 1986). Pgps belong to the family of adenine nucleotide binding cassette (ABC) transporters (Higgins, 1992). These are large plasma membrane glycoproteins consisting of two similar halves, each containing six putative transmembrane segments and an ATP-binding site. Drug resistance is caused by the ability of Pgps to extrude drugs against a
concentration gradient, resulting in a decrease of the intracellular drug concentration.

Resistance in cancer cell can be reversed by a number of chemicals, such as verapamil (Rogan, 1984). It has also been observed that chloroquine sensitivity can be restored in resistant cells by verapamil (Martin et al., 1987). Subsequently, several other agents that reverse MDR have been shown to reverse chloroquine resistance as well (Foote and Cowman, 1994). Observations of reduced drug accumulation in chloroquine-resistant \textit{P. falciparum} and the ability of verapamil and a number of other unrelated agents to reverse chloroquine resistance prompts comparison of chloroquine resistance in this parasite with multi-drug resistance in mammalian cancer cell lines.

\subsection*{1.5.1 Candidate drug resistance genes}

The presence of drug resistance causes many drugs useless. Therefore, it is as urgent as the discovery of new drugs to find out the molecular mechanism of drug resistance. In past decade, several well-known candidate drug resistance genes were found out.
1.5.1.1 *Pfmdr1*

Intensive investigation of *P. falciparum* has revealed a mdr homologue, *pfmdr1* (Foote, 1989). Pgh1, encoded by *pfmdr1*, is a 162kDa protein, which consist of 2 homologous halves separated by a hinge region. Each half of the protein is thought to contain six transmembrane domains and a nucleotide-binding fold. The predicted amino acid sequence has a 54% similarity to the human MDR1 (Foote, 1989). Localization of the Pgh1 by immunofluorescence and immunoelectron microscopy reveals that it is expressed throughout the erythrocytic cycle, is located in trophozoites predominantly on the vacuolar membrane (Cowman et al., 1991). This is a location consistent with its putative role as a chloroquine transporter. Therefore, it has been proposed that this is the efflux pump that causes increased efflux of chloroquine from the parasite, and that this pump is inhibited by verapamil (Foote et al., 1989).

The actual role of that gene is still not well known because different research groups have different experimental results and opinions. It has been shown by immunoblot that there is no correlation between overexpression and drug resistance (Cowman and Karcz,
1991). Subsequent work has failed to correlate the number of \textit{pfmdr1} gene copies with chloroquine resistance (Ekong et al., 1993). Moreover, a genetic cross between chloroquine-susceptible HB3 and chloroquine-resistant Dd2 clones of \textit{P. falciparum} showed that the chloroquine resistance phenotype did not co-segregate with the \textit{pfmdr1} gene (Wellems et al., 1990). The cross-generated independent offspring which exhibited the drug response characteristics of either the resistant or the sensitive parent. Restriction fragment length polymorphism analysis of the inheritance pattern reveals that the parental \textit{pfmdr1} did not segregate with the drug response of the progenies.

On the other hand, some literatures have pointed out that there has a correlation of chloroquine resistance and \textit{pfmdr1} amplification (Foote et al., 1990; Triglia et al., 1991). Moreover, it showed that \textit{pfmdr1} mutation can modulate the chloroquine resistance to antimalarials \textit{in P. falciparum} (Reed et al., 2000). The difference may be due to the fact that different field isolates were used for experiment, which were under different drug selective pressure; therefore, a derivation of genetic background was obtained.
1.5.1.2 cg2

Restriction Fragment Length Polymorphism (RFLP) analysis using markers on 14 chromosomes found a single genetic locus of around 400kb on chromosome 7 that has a perfect linkage with the chloroquine resistance phenotype (Wellems et al., 1991). Fine mapping of the locus and extensive sequencing, as well as RFLP studies, discovered the gene called cg2 (Su et al., 1997). cg2 is predicted to encode a 300kDa protein located mainly on the surface, as well as on intracellular vesicles and in haemozoin, where the target of chloroquine is expected to be located. By comparing the genotype of field isolates with that of Dd2, no typical CG2 genotype was found corresponding to the chloroquine-sensitive isolates. These results suggest a strong association between the drug-resistant and CG2 genotypes and support the hypothesis that the CG2 gene may be implicated in chloroquine resistance (Basco and Ringwald, 1999). However, recent study of cg2 gene has shown that it may not be responsible for chloroquine resistance or involvement of another nearby gene in the P. falciparum CQR mechanism (Durand et al., 1999; Fidock et al., 2000a; McCutcheon et al., 2000).
Recently, a new candidate gene was found called \textit{pfcr} which coded for a protein called \textit{Plasmodium falciparum} chloroquine resistance protein (PfCRT). It is believed to be a transporter, as are its homologues in various species. In \textit{Plasmodium} species it is localized to the digestive vacuole membrane. Mutations in this protein confer Verapamil-reversible chloroquine resistance to \textit{P. falciparum} and give rise to increased compartment acidification. PfCRT-related changes in chloroquine response may involve altered drug flux across the parasite digestive vacuole membrane, or to altered drug binding to hematin through the effect on the digestive vacuole pH since chloroquine resistance is due to diminished accumulation of the drug (Fidock et al., 2000c). The actual role of this gene is still in research.

\textbf{1.5.2 Intracellular binding to Ferriprotoporphyrin IX (FPIX)}

\textbf{Drives the Uptake of Chloroquine}

Recently, another mechanism has been proposed (Bray et al., 1999b). Ferriprotoporphyrin IX (FPIX) as described before is a toxic hemoglobin breakdown product produced during parasite feeding. It has been shown that FPIX has a high affinity binding to nitrogenous bases, such as chloroquine (Sugioka and Suzuki, 1991) and proposed
that the specific uptake of chloroquine into malaria parasite and antimalarial activity are due to such binding (Bray et al., 1999c). Recently, it has been shown that saturable chloroquine uptake by \textit{P. falciparum} is dependent on the digestion of haemoglobin and release of FPIX in the parasite (Bray et al., 1999a; Bray et al., 1999b; Bray et al., 1998). After inhibition of haemoglobin digestion, chloroquine uptake can be completely inhibited and it shows the relationship between FPIX and chloroquine. It is proposed that the mutation(s) responsible for chloroquine resistance change the local environment in which FPIX binds to chloroquine, leading to reduced apparent affinity of binding (Bray et al., 1999a).

1.6 Yeast Complementation

Since the actual molecular mechanism of drug resistance in \textit{P. falciparum} is still not well known, different molecular techniques have been used to search candidate resistance gene. Such as functional complementation. Functional complementation has been used in many systems to identify new genes. In the past, the knowledge of molecular biology of malaria has not developed as quickly as that of
cancer. One of the reasons is that the technology for transfection of genes into *Plasmodium* is not well developed. Although a transfection method was published in 1995 (Wu et al., 1995), the transfection efficiency is low and the protocol is much more complicated and time-consuming than common protocol used with other cell types. Therefore, it is technically difficult to transfect a cDNA library to a drug sensitive strain of *plasmodium* for screening of drug resistance genes.

To solve this problem, it is necessary to find another type of cell that can be transfected more easily with the cDNA library and used for drug screening. *E. coli*, yeast and mammalian cells are commonly used. However, since *E. coli* is a prokaryotic cell, it is not suitable for carrying an eukaryotic gene library. Although mammalian cells are eukaryotic, the transformation efficiency is also not high enough.

In the past decade, the molecular biology of yeast has expanded rapidly and the whole *Saccharomyces cerevisiae* genome has been sequenced. Yeast genetics have become a powerful tool in the molecular biology field. For example, antimalarial drug, quinidine,
was used to screen a yeast genomic library in *Saccharomyces cerevisiae*. Several resistance genes were found out and those genes confirmed resistance. It suggested that homologs of the identified resistance genes may perform similar functions in species other than yeast (Delling et al., 1998). Therefore, *Saccharomyces cerevisiae* can act as a tool to find out novel genes or act as a host to test the function of gene. For example, *pfmdrl* gene can convert sensitive yeast to resistant one after it was transformed (Ruetz et al., 1996). Also, *pfmdrl* gene can complement the function of mutated *ste6* gene, which encodes a mating pheromone α-factor export molecule (Volkman et al., 1995). Another plasmodial gene, *dhfr*, coded for *Plasmodium* enzyme dihydrofolate reductase (DHFR) was transformed in *Saccharomyces cerevisiae* that lacks endogenous DHFR activity. Yeast expressing constructs with *dhfr* alleles from pyrimethamine-resistant strains were resistant to both pyrimethamine and cycloguanil (Wooden et al., 1997). All these examples showed that gene from other species can function in yeast. Moreover, it has been shown that yeast can act as a model system to study drugs effective against Apicomplexan proteins (Sibley et al., 1997). Therefore, if a plasmodial cDNA library constructed from a resistant
strain was transformed in an antimalarial sensitive yeast strain, it is possible to screen out a novel drug resistance gene.

First, drug sensitive yeast should be used. Unfortunately, wild type *Saccharomyces cerevisiae* has one serious drawback: it is naturally resistant to chloroquine. Therefore, it was necessary to make a sensitive strain of the yeast before transfecting the cDNA library.

As has been described, the chloroquine resistance of *plasmodium* seems to be related to a *mdr* gene. Therefore, the basic approach to make a sensitive strain is to delete *mdr* genes in yeast. The complete genome sequence of *Saccharomyces cerevisiae* is known and it includes more than 30 ABC transporter-like molecules (Decottignies, 1995). Dr. Kuchler chose three of these to delete in a yeast strain, YPH499 (MATa ade2-101oc, his3D200, leu2-D1, lys2-801am, trp1-D1, ura3-52); namely, *pdr5*, *pdr10* and *snq2*. After “gene knockout”, the yeast strain, YHW1052 (Dpdr5::TRP1, Dsnq2::hisG, Dpdr10::hisG), was found to be chloroquine sensitive (A gift of Dr. Karl Kuchler, Austria). After YHW1052 was constructed, plasmodial cDNA library can be transformed in it and grow on drug containing agar plate for screening of novel drug resistance gene.
1.7 Objectives

1. Construction of 3D7 Plasmodial cDNA Library for screening of drug resistant clones by using yeast complementation system.

2. Identification and characterization of Chloroquine and Quinacrine Resistance Genes.
2. Experimental Design

2.1 cDNA synthesis

The *P. falciparum* strain 3D7 is used for library construction because it is being studied in a genome project. The genomic project will soon be finished, so the library constructed here may be useful for testing the functions of malarial genes. In the current protocol, the starting material for cDNA synthesis can be either total RNA or mRNA. Using total RNA is to prevent the loss of mRNA during purification but it may has some ribosomal RNA contamination. So, it is necessary to test, which is the best for cDNA synthesis.

Following SMART cDNA synthesis protocol (Clontech), a pool of different sizes of cDNA is synthesized. The reason of using this protocol is that all commonly used cDNA synthesis methods rely on the ability of reverse transcriptase (RT) to transcribe mRNA into single-stranded DNA in the first strand reaction. In some cases, RT terminates before transcribing the complete mRNA sequence. This is particularly true for long mRNAs, especially if the first strand
synthesis is primed with oligo (dT) primers only or if the mRNA contains abundant secondary structures. In addition, conventional cDNA cloning procedure use the T4 DNA polymerase to generate blunt cDNA ends after second strand synthesis. As a result, under-represented 5' ends of cDNA populations tend to be 5-30 nucleotides shorter than the original mRNA (D'Alessio and Gerard, 1988). The SMART protocols are designed to preferentially enrich for full-length cDNAs, while eliminating T4 DNA polymerase and adapter ligation. By using superscript II RT (from life technology), all the first strand cDNA contain extra oligo-dC at the end so that a specific primer with oligo-dG can prime to them and only full length cDNA can be synthesized. Incomplete transcripts or premature termination of RT would be eliminated.

After cDNA synthesis, a pool of double strand cDNA is obtained. Since primers contain SfiI restriction site, adapters or linkers ligation can be eliminated and cDNA can be ligated to plasmid after SfiI digestion. However, the shuttles vector, pYES2, does not contain SfiI restriction site and therefore, it is necessary to add this restriction site in there.
2.2 Construction of pYES2 with SfiI Restriction Site

The whole progress is shown in figure 2.1. By designing primer A, which contains EcoRI and SfiI restriction sites, and primer B, which contains XbaI and SfiI restriction sites, a known gene insert that do not contain internal sites of EcoRI, XbaI and SfiI, would be amplified by using these two primers.

Primer A:

\[
EcoRI \quad SfiI \\
5' - AA[GAATTCT]GTATCAACGAGTGGCC[ATTAT]GGCCGGG- 3'
\]

Primer B:

\[
XbaI \quad SfiI \\
5' - AT[TCCTAGAGGCGAGGCCGGCGACATG-d(T)_{30}N_{4}N- 3'
\]

After that, this gene would be cloned into pGEM T/A vector. The purpose of this step is that the restriction digestion of PCR product cannot be known whether it is successful or not. If the restriction site is too near to the ends, DNA cannot be cleaved out by restriction enzyme. After ligated to T/A vector, restriction digestion
can be surely known whether it's successful. The gene would be cleaved out by using EcoRI and XbaI restriction enzymes. On the other hand, pYES2 would also be digested by these two enzymes. After gel purification, ligation of insert DNA with vector DNA, and E. coli transformation would be performed. The successfulness of SfiI restriction site addition can be proven by using primers A and B to perform colony PCR.
Figure 2.1. The simple diagram shows the construction of pYES2 plasmid with SfiI restriction site. The insert gene with SfiI, EcoRI and XbaI restriction sites at primer sequence would be cloned in pGEM-T and then to pYES2.
2.3 cDNA Library Construction

A pool of double strand cDNA would be firstly digested by SfiI restriction enzyme. After that, size fractionation would be performed by using Sephacryl S-300 spun column (From Pharmacia) and cDNA are ready to be ligated. On the other hand, pYES2 with SfiI restriction site would be digested by SfiI restriction enzyme and gel purified for library construction. Then, self-igation would be performed to ensure that the ligation efficiency is higher enough for library construction. A pilot reaction would be firstly performed to determine insert to vector ratio for optimal ligation efficiency. After an optimal ligation efficiency is known, ligation mix would be transformed to E. coli for library construction. However, a major problem in cloning large segments of Plasmodium falciparum DNA is the instability of this very A + T-rich DNA in E. coli (Triglia and Kemp, 1991). Therefore, it is necessary to find out a suitable strain for library construction. An E. coli strain called XL1-Blue MR, was shown that it is no gene rearrangement when plasmodial DNA was transformed in it (Chakrabarti et al., 1994). Hence, XL1-Blue MR would be used for library construction.
2.4 Library Characterization

After library construction, it should be evaluated whether it is representative or not. Generally, library would be evaluated by screening for an abundant mRNA sequence, such as actin. Actin is a good choice of probes with which to evaluate the library because its sequence is highly conserved across species and probes are widely available. Table 2.1 gives a range of actin cDNAs for various tissues expressed as a percentage of total recombinant clones screened.

Also, examining the inserts from about ten randomly chosen clones can evaluate the average size range of insert.
<table>
<thead>
<tr>
<th>Tissue or cell type</th>
<th>Actin-hybridizing clones (% of clones screened)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>0.02 to 0.06</td>
</tr>
<tr>
<td>Lymphoid tissue or cells</td>
<td>0.1 to 0.5</td>
</tr>
<tr>
<td>Fibroblastoid cell line</td>
<td>1 to 4</td>
</tr>
</tbody>
</table>

Table 2.1. Frequency of Actin cDNA Clones in Various Mammalian cDNA libraries. It can be seen that a representative cDNA library should have an actin percentage lower than 1%.
2.5 Yeast Transformation

After cDNA library constructed in *E. coli*, plasmid DNA would be extracted and transformed to *Saccharomyces cerevisiae* for screening of novel chloroquine resistance gene(s). Before doing this, a sensitive yeast strain is required. First, an agar plate cytotoxicity assay will be performed to test the sensitivity of yeast to chloroquine. Then, the minimum inhibitory concentration (MIC) of chloroquine to Yeast will be found. Following Lithium acetate transformation protocol, cDNA library will be transformed to sensitive yeast strain, YHW1052 and plated on chloroquine containingYPD plate at minimum inhibitory concentration for screening of chloroquine resistance clones. All of the plasmids would be extracted from those chloroquine resistance clones and transformed to *E. coli*. Since the yield of plasmid DNA extracted from yeast is low, it is necessary to amplify plasmid DNA through *E. coli*. After *E. coli* transformation, plasmid DNA would be extracted and re-transformed to YHW1052 for secondary screening. The purpose of secondary screening is to eliminate those revertent mutants generated by drug pressure. All of the clones that can survive after secondary screening would be further characterized. Similar
approach would also be used to identify the drug resistant gene(s) with another antimalarial, called quinacrine (QNC).

2.6 Resistant Clones Characterization

2.6.1 A Luminescence-Based Measurement of Drug Resistant Level of Different Clones

By primary and secondary screening, most of the revertent mutant should be removed and the resistance phenotype should be related to the insert gene in pYES2 vector. In order to confirm the resistance phenotype of yeast transformants, yeast drug assay should be performed. Generally, the determination of resistance phenotype is based on the survival of yeast transformants in the presence of drug. There are a number of methods for this measurement. Such as, no of cells counting. However, these methods have their limitations and are not specific enough so that there is variation form batch to batch experiment. Therefore, a luminescence-based scanning biosensor for measurement of respiration of microorganism would be used. The oxygen sensing element in the biosensor is a ruthenium complex, [Ru(Ph₂phen)₃](ClO₄)₂, immobilized in silicone rubber. The principle
of this measurement is that ruthenium complex displays a strong absorption in visible region with maxim range from 440 to 480nm that can be excited by blue LED and it would emit light that wavelength is greater than 600nm. However, the emission intensity will be decreased in the presence of oxygen. The relationship between luminescence intensity and the concentration of oxygen can be correlated by applying the Stern-Volmer equation. The survivor would consume oxygen in the presence of drug. Therefore, by measurement of emission intensity, the oxygen consumption can be measured at different time periods. Then, the respiration rate of each sample could be calculated from the slope of the linear part of the dissolved oxygen concentration versus the incubation time curves and the percentage inhibition of the known concentration of drug solutions on the respiration of the yeast cell could be calculated. Hence, the drug resistance phenotype could be determined by calculating respiration inhibition concentration (IC$_{50}$) of resistance clones and comparing with that of YHW1052.
2.6.2 Agar Plate Cytotoxicity Assay

Agar plate cytotoxicity assay is another method to confirm the resistance phenotype of those resistance clones. By dropping resistance clones, YPH499 and YHW1052 cells on drug containing YPD plate, YHW1052 is expected no growth. If those resistance clones can grow on plate, it indicates that those novel resistance genes can complement the function of three-deleted mdr genes.

2.6.3 96-Wells Drug Assay

96-wells drug assay is an alternative to confirm the resistance phenotype. This assay will be used to characterize the resistance phenotype of quinacrine resistant (QNCR) clones.

2.7 Northern Hybridization

After the resistance phenotype of these clones had been confirmed, it is necessary to characterize the insert gene whether it is expressed and what is the size of mRNA. Therefore, northern
hybridization will be performed. First, RNA is separated according to size by electrophoresis through a denaturing agarose gel and is then transferred to nylon membranes. The RNA of interest is located by hybridization with radiolabeled DNA followed by phosphor-imaging.
3. Methodology

3.1 mRNA Isolation

The mRNA was isolated from total RNA by using PolyATtract® mRNA Isolation System from Promega.

3.1.1 Annealing of Probe

In a sterile, RNase-free tube, 0.1-1.0mg of total RNA was brought to a final volume of 500μl in RNase-Free Water. The tube was placed in a 65°C heating block for 10 minutes. 3μl of the Biotinylated-Oligo(dT) Probe and 13μl of 20X SSC were added to the RNA, mixed gently and incubated at room temperature until completely cooled. This should be required 10 minutes or less. While this solution was cooling, stock solutions of 0.5X and 0.1X SSC were prepared.
3.1.2 Stock Solution Preparation

1.2ml of sterile 0.5X SSC was prepared by combining 30μl of 20X SSC with 1.170ml of RNase-Free Water in a sterile, RNase-free tube. 1.4ml of sterile 0.1X SSC was prepared by combining 7μl of 20X SSC with 1.393ml of RNase-Free Water in a sterile, RNase-free tube.

3.1.3 Washing of Streptavidin-Paramagnetic Particles

The SA-PMPs were resuspended by gently flicking the bottom of the tube until they were completely dispersed and captured by placing the tube in the Magnetic Stand until the SA-PMPs had collected at the side of the tube (approximately 30 seconds). The supernatant was removed. The SA-PMPs was washed three times with 0.5X SSC (0.3ml per wash), captured by using the Magnetic Stand and then carefully removing the supernatant. The washed SA-PMPs was resuspended in 0.1ml of 0.5X SSC.
3.1.4 Capture and Washing of Annealed Oligo(dT)-mRNA Hybrids

The entire contents of the annealing reaction were added to the tube containing the washed SA-PMPs and incubated at room temperature for 10 minutes. It was gently mixed by inversion every 1-2 minutes. The SA-PMPs were captured by using the Magnetic Stand and carefully removed the supernatant without disturbing the SA-PMP pellet. The particles were washed four times with 0.1X SSC (0.3ml per wash) by gently flicking the bottom of the tube until all of the particles were resuspended. After the final wash, much of the aqueous phase was removed without disturbing the SA-PMP particles.

3.1.5 Elution of mRNA

To elute the mRNA, the final SA-PMP pellet was resuspended in 0.1ml of the RNase-Free Water and gently resuspended the particles by flicking the tube. The SA-PMPs were magnetically captured and the eluted mRNA was transferred to a sterile, RNase-free tube. The elution step was repeated by resuspending the SA-PMP pellet in 0.15ml of RNase-Free Water and the capture step was
repeated, the elute was pooled with the RNA eluted in Step 2 (0.25ml total volume). mRNA can be concentrated by using Sodium acetate/ethanol precipitation.

3.2 cDNA Synthesis

The first strand and second strand cDNA were synthesized by using SMART TM cDNA Library Construction Kit from Clontech.

3.2.1 First-Strand Synthesis

The reagents were combined in a sterile 0.5-ml microcentrifuge tube. It contains about 50ng of RNA sample, 10 μm SMART III TM Oligonucleotide, 10μm CDS III/3' PCR Primer and the final volume were adjusted to 5μl by sterile water.

The contents were mixed and centrifuged the tube briefly in a microcentrifuge. The tube was incubated at 72°C for 2 min and Cool on ice for 2 min. The tube was centrifuged briefly to collect the contents at the bottom. 2.0μl 5X First-Strand Buffer, 1.0μl DTT
(20mM), 1.0μl dNTP Mix (10mM) and 1.0μl Superscript II MMLV reverse transcriptase from Life Technology (200 units/ml) was added to each reaction tube:

The solution was mixed by gently pipetting and briefly spinning the tube. The tube was incubated at 42°C for 1 hr.

3.2.2 Second Strand cDNA Synthesis

Then, 2μl First-Strand cDNA (from Step A.7), 80μl Deionized H₂O, 10μl 10X Advantage ® 2 PCR Buffer, 2μl 50X dNTP Mix, 2μl 10μm 5' PCR Primer, 2μl 10μm CDS III/3' PCR Primer and 2μl 50X Advantage ® 2 Polymerase Mix were added in a fresh 0.5-ml tube.

The contents were mixed by gently flicking the tube and centrifuge briefly to collect the contents at the bottom of the tube. Two drops of mineral oil if necessary was added. The tube was capped, and placed in a preheated (95°C) thermal cycler. It was put in PCR machine and run at follow conditions:

95°C 20 Sec
95°C 5 Sec (25 cycles)
60°C 6 minutes

3.2.3 Proteinase K Digestion

50μl of the amplified ds cDNA (2-3μg) with 2μl of Proteinase K (20 mg/ml) were mixed in a sterile 0.5ml tube. The remaining ds cDNA at -20°C was stored in freezer. The contents were mixed and spin the tube briefly.

It would be incubated at 45°C for 20 min. The tube was centrifuged briefly. 100μl of phenol:chloroform:isoamyl alcohol were added and mixed by continuous gentle inversion for 1-2 min. The mixture was centrifuged at 14,000 rpm for 5 min to separate the phases. The top (aqueous) layer to a clean 0.5ml tube was added. The interface and lower layers were discarded. 100μl of chloroform:isoamyl alcohol was added to the aqueous layer. The tube was mixed by continuous gentle inversion for 1-2 min. The tube was centrifuged at 14,000 rpm for 5 min to separate the phases. The top (aqueous) layer was transferred to a clean 0.5ml tube. The interface and lower layers were discarded. 10μl of 3M Sodium Acetate, 1.3μl of Glycogen (20 mg/ml) and 260μl of room temperature 95% ethanol
was added. The tube was centrifuged at 14,000 rpm for 20 min at room temperature. The supernatant was discarded. The pellet was washed with 100μl of 80% ethanol. The pellet was air-dried (~10 min) to evaporate off residual ethanol. 79μl of deionized water was added to resuspend the pellet.

3.2.4 *SfiI* digestion

79 μl cDNA, 10μl 10x *SfiI* buffer, 10 μl *SfiI* enzyme and 1μl 100x BSA were combined in a fresh 0.5ml tube and incubated at 50°C for 2 hours.

3.2.5 Size-fractionation

The spun column was inverted several times to resuspend the Sephacryl S-300 gel. The top and then the bottom cap were removed and the column was allowed to drain while the gel was still suspended. The gel should not be dried. 2 ml of TEN buffer was added and the caps were replaced. Then, the column was inverted several times to resuspend the gel. After that, the caps were removed and the column was allowed to drain as before. This step was repeated twice more by
using an additional 2ml of buffer for each wash. The final wash was stopped just as the last of the buffer enters the gel. The bottom cap was replaced and then the top cap. When ready to proceed, the caps were removed from the column and the column was placed in a 15ml tube. The tube containing spun column was centrifuged for two minutes at approximately 400g in a swinging bucket rotor. The column was removed and place it upright in a rack. The cDNA sample was slowly applied to the center of the flat surface at the top of the compacted bed. 1.5ml microcentrifuge tube was placed in the bottom of a 15ml tube. The loaded column was placed inside this tube with the tip of the column inside the microcentrifuge tube. The tube was centrifuged again for two minutes at approximately 400g in a swinging bucket rotor. The column was discarded and the effluent collected in the microcentrifuge tube was used in the next step of cDNA synthesis.

3.3 Insertion of SfiI Restriction Site to pYES2 Plasmid

3.3.1 Polymerase Chain Reaction (PCR)

1µl DNA (20ng), 1µl primer A (10µM), 1µl Primer B (10µM), 1µl dNTP (5mM), 5µl 10x PCR buffer, 1µl Taq DNA polymerase (1
unit) and 40μl ddH₂O were combined in a fresh 0.2ml PCR tube. After that, tubes were taken in Thermocycler and run at following conditions.

95°C 3mins
30 cycles:
95°C 30sec
50°C 30sec
60°C 3mins
Then,
60°C 4mins

3.3.2 T/A Cloning

5 μl PCR product, 2μl pGEM-T vector, 1.5μl 10x ligation buffer, 1μl ligase and 6.5μl ddH₂O were combined in a fresh 0.5ml tube.
3.3.3 CaCl₂ *E. coli* Transformation

3μl of ligation product was added to 40 μl competent cell in 1.5ml eppendorf and incubated in ice for 30 minutes. Then, the tube was incubated at 42°C for 2 minutes and 500μl SOC medium was added and shaked at 37°C for 1 hour. After that, it would be plated on LB-Amp plate and incubated at 37°C incubator for overnight.

3.3.4 Colony PCR

Colony would be picked out and mixed in 40 μl ddH₂O. Then, 1μl primer A (10μM), 1μl primer B (10μM), 1μl dNTP (5mM), 5μl 10x PCR buffer and 1μl Taq DNA polymerase (1 unit) would be mixed in a fresh 0.2ml PCR tube containing 40μl ddH₂O with colony. After that, tubes were taken in Thermcycler and run at following conditions.
95°C  3mins
30 cycles:
95°C  30sec
50°C  30sec
60°C  3mins

Then,
60°C  4mins

The PCR products would be analyzed by gel electrophoresis.

3.3.5 EcoRI and XbaI Digestion

10μl DNA (1μg), 4μl 10x NEB buffer 2, 0.5μl EcoRI enzyme (1 unit), 0.5μl XbaI enzyme (1 unit) and 25μl ddH2O were mixed in a fresh 0.5ml eppendorf tube. The tube was incubated at 37°C for 3 hours and analyzed by gel electrophoresis. After gel electrophoresis, the DNA in gel was cut at desired size and DNA was eluted by electroelution.
3.3.6 Ligation

1μl EcoRI and XbaI cut gel-purified pYES2 (0.1μg), 1μl EcoRI and XbaI cut gel-purified insert DNA (0.1μg), 1μl 10x ligation buffer, 1μl ligase (1 unit) and 6μl ddH₂O would be mixed in a fresh 0.5ml eppendorf tube. The tube was incubated at 16°C for overnight.

The ligation product would be transformed and colonies would be analyzed by colony PCR. The desired plasmid would be digested by SfiI enzyme and gel-purified.

3.4 Library Construction

3.4.1 Ligation

The SfiI cut cDNA was ligated to SfiI cut pYES2 vector by T4 DNA ligase (BM). 1μl 100ng DNA was mixed with 1μl 100ng Vector, 2μl 10X ligation buffer, 1unit/μl ligase and 15μl ddH₂O. It would be incubated in 16°C water bath for overnight.
3.4.2 Electroporation

20 µl ligation product was proceeded ethanol precipitation and DNA pellet was resuspended in 10 µl sterile water. 2µl ligation product was mixed with 40µl competent cell and added in curette for electroporation. The condition was 2.5 kilovolt and 200 micro. After the process, 1ml SOC was added and shaked at 37°C for 1 hour. After that, cells were plated on LB-Amp plate and incubated at 37°C for overnight.

3.5 Yeast Culture

Yeast was cultivated by inoculating from frozen stock to YPD medium (yeast without pYES2 plasmid) or SC-URA medium (yeast with pYES2 plasmid) and shaked in 30°C incubator for about two days at 250 rpm.
3.6 Agar Plate Cytotoxicity Assay

A chloroquine stock solution (300mg/ml) was prepared and the YPD agar plates with different concentration of chloroquine were also prepared according to table 2.2.
Table 3.1 shows the content for preparation of agar plate with different drug concentration. The yeast culture prepared as described before was first diluted to optical density~0.5. Then one drop of culture was added to chloroquine containing plate and incubated for about two to four days.
3.7 Yeast PEG-LITE Transformation

Single colony was incubated into 5 ml of YPAD and grown at 30°C with shaking for overnight. Next day, in the early morning, 5 ml of overnight culture was incubated into 50 ml of fresh YPAD in a small flask which was enough for about 5 transformations and grown with shaking at 30°C for about 4 hours. After that, 10 μl was diluted in 90 μl YPAD and count in hemocytometer. The number of cells in culture was counted and the target is about 1 - 2 X 10^7 cells/ml. The culture was centrifuged in a 50ml Falcon tube at 3000rpm for ten minutes. While spinning, fresh 1X LITE and PEG-LITE were prepared. For 4 ml LITE solution, 3.2 ml sterile H2O, 0.4 ml sterile 10X TE at pH7.5 and sterile 0.4 ml 10X LiAc (1M, pH 7.5 in H2O, sterile) were mixed. For 4ml PEG-LITE solution, 0.4ml sterile 10X LiAc, 0.4ml sterile 10X TE at pH 7.5, and 3.2ml sterile 50% PEG 4000 in H2O were mixed. Supernatant was discarded and cell pellet was resuspended in 1ml H2O in eppendorf. The tube was centrifuged for 5 minutes at room temperature. The supernatant was discarded and cell pellet was resuspended in 1ml LITE solution. The tube was centrifuged again for 5 minutes at room temperature. The supernatant
was discarded and cell pellet was resuspended in appropriate volume
to give 2 x 10^9 cells/ml. 50μl cell suspension were aliquot into an
eppendorf. 1μl plasmid DNA (1μg/μl) was added and mixed with
10μl salmon sperm DNA (1mg/ml) and 300μl 40% PEG-LITE
solution. The eppendorf was put in a rack and grown at 30°C without
shaking for 30 minutes. After that, the tube was incubated at 42°C for
15 minutes. The tube was centrifuged for 5 seconds and PEG was
discarded as much as possible. The cell pellet was resuspended in
100μl sterile water and plated on SD-URA plate. The plate was
incubated at 30°C for overnight.

3.8 Yeast Miniprep.

5 ml cell culture was centrifuged. The supernatant was
discarded and cell pellet was resuspended in 0.2 ml yeast miniprep
buffer. 0.1 ml acid-washed glass beads and 0.2 ml phenol-chloroform
were added and vortex for three minutes. The tube was centrifuged for
5 minutes. The supernatant was discarded and ethanol precipitation
was performed. The DNA pellet was resuspended in 20 μl sterile
water.
3.9 Measurement of Dissolved Oxygen

A series of different drug solutions were prepared by diluting the stock solution with sterile water. The cell culture was diluted so that optical density was 0.01. 3.5 ml cell culture and 3.5 ml sterile water were mixed and act as a control. On the other hand, 3.5 ml cell culture and 3.5 ml drug solutions of various concentrations were mixed into sample vials. The final content of each sample vial was 7ml in volume. After that, the sample vials were sealed to prevent the oxygen in air passing through into it. The change of output voltage during incubation of yeast in the presence of drugs was measured by the biosensor. The experiment was finished when the oxygen in the control sample vial was completely used up by the yeast and the output voltage level off. The dissolved oxygen curve was obtained by plotting of the dissolved oxygen concentration (mg/L) versus the incubation time (minutes).

3.10 96-well Drug Assay

The quinacrine resistance (QNCR) cell were growth in 3ml of SC-Ura no carbon medium with 1% raffinose in 30°C shaker.
YHW1052 and YPH499 were also growth as a control. After 2 days, all cells were spin down, resuspended in 3ml of Gal-induction medium and growth for 5 hours. Within that time, 96 well microtiter plates were prepared. 100 µl of 20mg/ml QNC, which was dissolved in 1X SC-Ura medium, was added to the first row. 100ul of 1X SC-Ura was added to the last row. 50ul of SC-Ura was added to the rest of plate. 50ul of 20mg/ml QNC was pipetted out from the first row to the second row. The solution was mixed and that step was done until the six rows. The last 50ul drug solution was discarded and 20, 10, 5, 2.5, 1.25, 0.625, and 0mg/ml were produced at first seven rows. Then, 50ul of cells with optical density, O.D. = 0.05, were added to each well except the last row which used as a blank control. Therefore, the final concentration of QNC, 10, 5, 2.5, 1.25, 0.625, 0.3125 and 0mg/ml were obtained.

After induction, dilute 5 fold in 1X SC-Ura medium and measure optical density at 595nm. The cell was diluted to give final O.D. = 0.05. 50ul of diluted cells were added to row 1 to 7. Triplicate samples were done per each sample. Then, the plate was incubated at 30oC for 40 hours without shaking. Before reading the O.D., the plate was shaked gently to ensure cell clumps were well suspended. O.D.
was measured by microplate reader at O.D.=545nm. The relative percentage of growth is equal to O.D. with drug divided by O.D. without drug.

3.11 Northern Hybridization

RNA was firstly separated in formaldehyde denaturing gel for 90 minutes at 100 volts. The RNA then was transferred by capillary transfer to nylon membrane. After that, the membrane was cross-linked by UV and prehybridized in prehybridization buffer at 68°C for 60 minutes. By using Random primed DNA labeling kit (Roche), $^{32}$P labeled probes were generated. After prehybridization, about 100ng probes was added to prehybridization buffer and incubated with shaking at 68°C for overnight. Next day, the hybridization buffer was discarded and washed with washing buffer containing 2X SSC and 0.1% SDS at room temperature for 15 minutes. This step was repeated for two times. Then the membrane was further washed with washing buffer containing 0.1X SSC and 0.1% SDS at 68°C for 15 minutes. Also, this step was repeated for two times. After washing, the membrane was wrapped and exposed on Kodak storage Phosphor
screen for two hours. Then, the screen was put in phosphorimager and
the image was analyzed.
4. Results

4.1 Determination of the Sensitivity of Yeast to Chloroquine (CQ)

In order to use functional complementation system to isolate new chloroquine resistance gene(s), a chloroquine sensitive yeast strain is needed. Three yeast pleiotropic drug resistance genes were chosen to delete since their functions are related to cellular transport (Balzi and Goffeau, 1995; Bauer et al., 1999) YHW1052 (Δpdr5::TRP1, Δsnq2::hisG, Δpdr10::hisG) was kindly provided by Dr. Karl Kuchler and its sensitivity to chloroquine was tested by using agar plate cytotoxicity assay. This method can differentiate whether the cells can tolerate the chloroquine or not.

The result of agar plate cytotoxicity assay is shown in figure 4.1. Clearly, the YHW1052 strain is more sensitive to chloroquine than YPH499, the parental cell line. This suggested that pdr5, pdr10 and snq2 might be involved in the resistance of chloroquine, possibly by effluxing CQ. Deletion of these three genes would result in an increase in chloroquine accumulation, leading to cell death. As a
control, the introduction of a pYES2 plasmid into YHW1052 did not affect their chloroquine sensitivity. Similar tests also show that YHW1052 is more sensitive to other antimalarial drugs such as quinacrine and quinidine than YPH499 (table 4.1). This suggests that these three mdr genes might have broad substrate specificity and that YHW1052 may be used in functional complementation to isolate genes resistant to a broad range of drugs.
Figure 4.1. The growth of yeast on chloroquine-containing agar plate. 

The agar plate cytotoxicity assay was performed as described in methodology and cell density were diluted at O.D.~0.1 and O.D.~0.05. After three days of incubation at 30°C, (a) the growth of YPH499 and YHW1052 shown that yeast could normally grow on agar plate. (b) Only YPH499 could grow on 125 mg/ml chloroquine-containing plate.
<table>
<thead>
<tr>
<th>Concentration of antimalarial drugs that can kill YHW1052 but not YPH499.</th>
<th>Chloroquine (CQ)</th>
<th>Quinacrine (QNC)</th>
<th>Quinidine (QND)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>125 mg/ml</td>
<td>7 mg/ml</td>
<td>2.3 mg/ml</td>
</tr>
</tbody>
</table>

Table 4.1. Minimum inhibition concentration of antimalarials to yeast (n = 1). The Concentration of antimalarial drugs that can kill YHW1052 but not YPH499. It can be seen that antimalarial drugs are only sensitive to YHW1052.
As described in experimental design, it is necessary to find the minimum inhibitory concentration (MIC) of CQ. It was found that the MIC for YPH499 was higher than 125 mg/ml. On the other hand, the MIC of YHW1052 was about 125mg/ml (table 4.1). From these results, it could be concluded that 125mg/ml chloroquine could be used in the library screening.

4.2 mRNA Isolation

In order to make a cDNA library on the pYES2 vector, total and messenger RNA are prepared from asynchronous culture P. falciparum using method described elsewhere (Hyde and Read, 1993; Wallach, 1982). The size range of cDNA synthesized by poly A⁺ RNA was larger than that from total RNA (figure 4.2). Hence, Poly A⁺ RNA was chosen for cDNA synthesis.
Figure 4.2. The size range of cDNA synthesized by total RNA and mRNA. The cDNA was synthesized by using total RNA (lane2) or mRNA (lane3) separately and it could be seen that the cDNA synthesized using mRNA is larger in size. Lane 1 is a 1kb DNA marker.
4.3 cDNA Synthesis

Using poly A+ mRNA as a starting material, cDNA library was constructed using SMART cDNA kit (Clontech) protocol described in methodologies.

In order to amplify the amount of cDNA, RT-PCR was used. However, the PCR condition for amplification of plasmodial DNA was different from that of other regular PCR. The PCR method is as follow:

<table>
<thead>
<tr>
<th>Denaturation</th>
<th>30 cycles</th>
<th>Final Extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°C 3mins</td>
<td>95°C 30sec</td>
<td>60°C 4mins</td>
</tr>
<tr>
<td>50°C 30sec</td>
<td>60°C 3mins</td>
<td></td>
</tr>
<tr>
<td>60°C 3mins</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The extension temperature was 60°C rather than 72°C. This is because the plasmodial DNA is A-T rich. Higher extension temperature might denature the DNA during extension and long DNA could not be amplified (Su et al., 1996). Under such PCR condition, the size of PCR products is in the range of about 0.3kb to 2kb (figure 4.3).
PCR product was then treated with proteinase K and size-fractionated for library construction.

The SfiI digested cDNA was size-fractionated using spin column, containing Sephacryl S-300 gel. cDNA with sizes smaller than 0.3kb would be removed. Moreover, any impurity would be removed so that the cDNA was pure enough. cDNA with size ranged from 0.3kb to 2kb would then be used for ligation to pYES2 plasmid.
Figure 4.3. The size range of PCR product (lane 1) amplified by using CDSIII/3 PCR primer and SMART III primer (see methodology). Most of cDNA is in the range from 0.3kb to 2kb. Lane 2 is 1kb marker.
4.4 Construction of pYES2 Plasmid with SfiI Restriction Site

SfiI restriction enzyme was used to digest the cDNA and it was necessary to have a SfiI cut plasmid for library construction. Since the multiple cloning site of pYES2, the vector going to be used for library construction, did not have SfiI restriction site, addition of this restriction site is needed (figure 2.1). PCR product amplified by using primer A and B is shown in figure 4.4. After ligation of this PCR product to pGEM-T vector and transformed to E. coli, the plasmid of recombinant clones would be isolated and analyzed by gel electrophoresis. After transformation, about two hundreds colonies were obtained and 29 colonies were picked and analyzed. The clone with largest plasmid (named pGEM-T-Sfi) is picked for further analysis.

At the same time, pGEM-T-Sfi is then digested with EcoRI and XbaI restriction enzymes to release the desired insert. The insert would be gel purified and ready for cloning to EcoRI and XbaI cut pYES2 plasmid. After ligation and transformation of insert and vector DNA, about two hundreds colonies were obtained and ten recombinant clones were picked and analyzed by colony PCR to
check whether desired insert was successfully cloned or not. Nine out of ten clones have the desired insert and contained the *SfiI* restriction site. The desired plasmid (pYES2-*Sfi*) would be digested by *SfiI* restriction enzyme and gel purified for library construction.
Figure 4.4. The size range of PCR product amplified by using Primer A and B (see methodology). Also, the size is in the range from 0.3kb to 2kb (lane 2). Lane 1 is 1kb marker.
4.5 Library Construction

The SfiI site of pYES2-Sfi will be used for library construction. A test was done to determine the optimal ratio of cDNA and vector DNA in the ligation. Generally, a representative cDNA library should have 80-90% clones with insert. A ligation is performed using size-fractionation cDNA and SfiI-linearized pYES2-SfiI. A ratio of cDNA to vector DNA 3:1 is used. About five hundreds colonies are obtained. From which nine colonies are randomly picked for further analysis. Figure 4.5 showed the result of SfiI digestion after minipreparation of plasmid of recombinant clones. It could be seen that about 90% of clones contained inserts and the size of insert was in the range of 0.3kb to about 2kb. This range is similar to that of original cDNA.

Table 4.2 showed the summarized results of characterization of the *P. falciparum* cDNA library. For a good representative plasmid based cDNA library, the titer should have a $10^5$ colony forming unit (c.f.u.). The actin content should range from 0.05% to 0.1% and the percentage of recombinant clones should have minimum about 80%. Total colony forming units of plasmodial cDNA library is $2 \times 10^5$ per microgram DNA. Ten colonies were randomly picked and the insert
size ranged from 0.3kb to 2kb. By using an actin probe, there are eight colonies that are hybridization positive, out of ten thousands colonies. It could be concluded that a representative cDNA library was successfully constructed. Therefore, this plasmodial cDNA library can be used to transform into YHW1052 for library screening.
Figure 4.5. S/fI digestion of nine randomly picked colonies. Sizes are in the range from 0.3kb to 2kb and different size inserts (arrows) are observed (lane1-9). Lane10 is 1kb marker.
<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Total colony forming</td>
<td>$3 \times 10^5$</td>
</tr>
<tr>
<td>units/microgram of DNA</td>
<td></td>
</tr>
<tr>
<td>Insert sizes range</td>
<td>0.3kb - 2kb</td>
</tr>
<tr>
<td>Actin colonies percentage</td>
<td>0.08%</td>
</tr>
<tr>
<td>Percentage of clones with</td>
<td>90%</td>
</tr>
<tr>
<td>insert</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.2. Information of cDNA library characterization. Ten colonies were picked and the size is about 0.3kb to 2kb. By using an actin probe, there are eight colonies that are hybridization positive, out of ten thousands colonies. The titre of the library is $3 \times 10^5$. The size of malarial genome is about 300 Mb. Assuming the average insert size is 1k, about $3 \times 10^5$ independent clones can represent the whole library.
4.6 Library Screening of Novel Chloroquine Resistance

Gene(s)

Transformation efficiency using lithium acetate method of YHW1052 is about 150,000 per microgram DNA. About 150,000 library-transformed cells were seeded at a density of $10^5$ cells per plate containing 125mg/ml of chloroquine. Around 200 CQ-resistant colonies grew in 125mg/ml of chloroquine after 10 days of selection. In principle, the observed chloroquine-resistance phenotype of these clones could either be arisen from library-derived plasmid-borne gene expression, or from chloroquine-induced mutations leading to resistance. To differentiate between these two, plasmids were isolated from resistant clones, propagated in E. coli and re-transformed into YHW1052 cells. 200 CQ-resistant clones were re-plated onto YPD plates containing 125 mg/ml CQ for secondary screening. After primary and secondary screening, about 30 colonies were obtained which can consistently grow at 125 mg/ml CQ (table 4.3). The chloroquine resistance phenotype of these 30 colonies was indeed mediated by the plasmid, and not due to chromosomal mutations.
Plasmids of all thirty chloroquine resistant clones were isolated and analyzed by SfiI restriction digestion and sequenced (table 4.4a & b). It could be seen that 3 out of 30 resistant clones, CQR2, CQR5 and CQR 21, were the same. 28 different cDNAs were obtained after primary and secondary screening. DNA sequence of these cDNAs was determined (table 4.4a & b). Some of them are known genes whereas others are expressed sequence tags (ESTs). Among the 28 chloroquine resistance clones, those with an inserts size greater than 700bp were selected to be analyzed first (CQR8, CQR12, CQR13, CQR20, CQR21, CQR23 and CQR30), together with CQR17 which was a known gene (pfHRP).
<table>
<thead>
<tr>
<th>Description</th>
<th>Number of colonies counted/µg of plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td>YHW1052 transformed with <em>P. falciparum</em> cDNA library plated on SC-Ura plate</td>
<td>1.5x10^5</td>
</tr>
<tr>
<td>YHW1052 transformed with <em>P. falciparum</em> cDNA library plated on 125 mg/ml CQ plate</td>
<td>200</td>
</tr>
<tr>
<td>YHW1052 transformed with plasmids selected from primary screening and plated on 125 mg/ml chloroquine</td>
<td>30</td>
</tr>
<tr>
<td>YHW1052 transformed with pYES2 plasmid plated on 125 mg/ml CQ plate</td>
<td>0</td>
</tr>
<tr>
<td>YHW1052 transformed without pYES2 plasmid plated on Sc-Ura plate</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 4.3. The result of primary and secondary screening of CQR clones. cDNA plasmids were transformed in yeast and act as a control to show the transformation efficiency. About 150,000 transformants were then plated on 125 mg/ml chloroquine containing YPD plate and incubated at 30°C. About two hundred colonies were obtained after 10 days of incubation. After secondary screening, thirty colonies were obtained. YHW1052, transformed with or without the vector, act as negative controls to show that vector alone cannot produce resistant clones.
<table>
<thead>
<tr>
<th>Insert size</th>
<th>Blast result</th>
<th>E value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CQR1 235bp</td>
<td>PFHB3.1 (0364m)</td>
<td>8e-86</td>
</tr>
<tr>
<td>CQR2 ~1000bp (incomplete sequencing)</td>
<td>PFDd2.1 (PF2226C)</td>
<td>4e-40</td>
</tr>
<tr>
<td>CQR3 150bp</td>
<td>Ch.12 (AC006279)</td>
<td>3e-19</td>
</tr>
<tr>
<td>CQR4 410bp</td>
<td>Ch.12 (AC006281)</td>
<td>0.21</td>
</tr>
<tr>
<td>CQR5 ~1000bp (incomplete sequencing)</td>
<td>PFDd2.1 (PF2226C)</td>
<td>4e-40</td>
</tr>
<tr>
<td>CQR6 579bp</td>
<td>Ch.12 (AC005308)</td>
<td>0.009</td>
</tr>
<tr>
<td>CQR7 171bp</td>
<td>PFHB3.1 (PF0247C)</td>
<td>4e-35</td>
</tr>
<tr>
<td>CQR8 ~1000bp (incomplete sequencing)</td>
<td>PFDd2.1 (PF2203C)</td>
<td>6e-06</td>
</tr>
<tr>
<td>CQR9 572bp</td>
<td>Ch.4 (PFMAL4P3)</td>
<td>0.002</td>
</tr>
<tr>
<td>CQR10 270bp</td>
<td>PFDd2.1 (PF2023C)</td>
<td>1e-109</td>
</tr>
<tr>
<td>CQR11 504bp</td>
<td>Ch.12 (AC006281)</td>
<td>3e-16</td>
</tr>
<tr>
<td>CQR12 ~1000bp (incomplete sequencing)</td>
<td>Ch.2 (AE001430)</td>
<td>8e-04</td>
</tr>
<tr>
<td>CQR13 ~1000bp (incomplete sequencing)</td>
<td>PFMAL4P3 (AL035476)</td>
<td>4e-04</td>
</tr>
<tr>
<td>CQR14 189bp</td>
<td>Ch.2 (AE001380)</td>
<td>0.11</td>
</tr>
<tr>
<td>CQR15 314bp</td>
<td>PFHB3.1 0362c (5')</td>
<td>1e-21</td>
</tr>
</tbody>
</table>

Table 4.4a. The summarized results of 15 chloroquine resistant clones.

After primary and secondary screening, 30 chloroquine resistant clones were obtained. 3 out of 30 clones were the same.
<table>
<thead>
<tr>
<th>Insert size</th>
<th>Blast result</th>
<th>E value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CQR16 261bp</td>
<td>Ch.12 (AC005507)</td>
<td>0.15</td>
</tr>
<tr>
<td>CQR17 555bp</td>
<td>PF histidine-rich protein</td>
<td>7e-51</td>
</tr>
<tr>
<td>CQR18 259bp</td>
<td>PFDd2.1 (PF2181C)</td>
<td>9e-32</td>
</tr>
<tr>
<td>CQR19 452bp</td>
<td>MAL3P7 (AL034559.4)</td>
<td>4e-19</td>
</tr>
<tr>
<td>CQR20 ~800bp (incomplete sequencing)</td>
<td>Ch.12 (AC005140)</td>
<td>0.18</td>
</tr>
<tr>
<td>CQR21 ~1000bp (incomplete sequencing)</td>
<td>PFDd2.1 (PF2226C)</td>
<td>4e-40</td>
</tr>
<tr>
<td>CQR22 262bp</td>
<td>Ch.12 (AC004688)</td>
<td>3e-16</td>
</tr>
<tr>
<td>CQR23 1500bp (incomplete sequencing)</td>
<td>PFDd2.1 (PF2094C)</td>
<td>2e-10</td>
</tr>
<tr>
<td>CQR24 76bp</td>
<td>PF5.8s and LSU rRNA gene</td>
<td>8e-19</td>
</tr>
<tr>
<td>CQR25 496bp</td>
<td>PFMAL13P6 (AL049183)</td>
<td>6e-05</td>
</tr>
<tr>
<td>CQR26 532bp</td>
<td>PFMAL3P3 (Z98547.1)</td>
<td>0.0</td>
</tr>
<tr>
<td>CQR27 214bp</td>
<td>PFDd2.1 (PF1151C)</td>
<td>1e-15</td>
</tr>
<tr>
<td>CQR29 692bp</td>
<td>PFHd3.1 (PF0029M)</td>
<td>2e-39</td>
</tr>
<tr>
<td>CQR30 ~900bp (incomplete sequencing)</td>
<td>PFMAL3P5 (AL034556.3)</td>
<td>7e-05</td>
</tr>
</tbody>
</table>

Table 4.4b. The summarized results of 15 chloroquine resistant clones.

After primary and secondary screening, 30 chloroquine resistant clones were obtained. 3 out of 30 clones were the same.
4.7 Chloroquine Resistance Phenotype and Cross Resistance Activity

The resistance phenotype of 8 chloroquine resistant clones was analyzed by agar plate cytotoxicity assay. All CQ-resistant clones and YPH499 can grow on the chloroquine-containing YPD plate, whereas YHW1052 could not (figure 4.6a & b). Since the YHW1052 was sensitive to multiple drugs, it will be of interest to test whether these CQ resistant clones are multi-drug resistant or not. Quinacrine, another antimalarial drug, with a MIC of 7mg/ml, would be tested. It can be seen that these CQ resistant clones could also survive at 7mg/ml quinacrine indicating that the inserts can indeed mediate multi-drug resistance (figure 4.7).
Figure 4.6a & b. Growth of CQ-resistant clones on YPD plate with or without 125mg/ml chloroquine. Different chloroquine resistant clones, YPH499 and YHW1052 were plated and incubated at 30°C for two days. (a) All of them could grow on YPD plate. All of them, except YHW1052, could also grow on chloroquine containing plate (b). It indicated that all resistant clones complement the function of three-deleted mdr genes and restore resistance to antimalarial as wild type, YPH499.
Figure 4.7. Growth of CQ-resistant clones on YPD plate with 7mg/ml quinacrine. Different chloroquine resistant clones, YPH499 and YHW1052 were plated and incubated at 30°C for two days. All of them, except YHW1052, could grow at this concentration. It indicates that all novel resistance genes could mediate cross-resistance phenotype.
4.8 Luminescence-Based Measurement of Yeast Resistance Phenotype

Besides of using agar plate cytotoxicity assay to determine the resistance phenotype, a luminescence-based method has also been used to confirm the resistance phenotype. This method allows the determination of inhibition concentration (IC$_{50}$). The principle of this method is based on the respiration rate at various concentration of chloroquine (Kwok, 2000). The higher the growth rate, more oxygen will be used.

Using this luminescence-based method, the IC$_{50}$ of YPH499 and YHW1052 were found to be 72mg/ml and 33.8mg/ml respectively (figure 4.8a & b), confirming the pervious observation that YHW1052 was more sensitive to CQ than YPH499.

The IC$_{50}$ of those resistant clones are also determined (figure 4.9 – figure 4.12). It could be seen that the IC$_{50}$ all those resistant clones were higher than that of YHW1052. Moreover, all of them have a higher IC$_{50}$ than that of YPH499 (table 4.5). The highest one was CQR13, which was about six-fold higher than YHW1052. Also,
CQR17 and CQR21 were about six-fold higher than YHW1052. Four out of eight, CQR8, CQR12, CQR20 and CQR30 were about five-fold higher than YHW1052 and the rest of those clones, CQR23, was about three-fold higher than YHW1052. Therefore, all these data suggested that the expression of those novel resistance genes would be related to resistance mechanism.
Figure 4.8a & b. The plot of percentage of inhibition of YPH499 and YHW1052 versus various concentration of chloroquine. The percentage of inhibition was calculated from the respiration rate, which was calculated from the slope of the linear part of the dissolved oxygen concentration versus the incubation time. The inhibition concentration (IC$_{50}$) of (a) YPH499 was 72 mg/ml and (b) YHW1052 was 33.8 mg/ml.
(a) Inhibition concentration of chloroquine to CQR8

\[ y = -0.0006x^2 + 0.4147x \]
\[ R^2 = 0.9731 \]

(b) Inhibition concentration of chloroquine to CQR12

\[ y = 0.0013x^2 + 0.0784x \]
\[ R^2 = 0.9873 \]

Figure 4.9a & b. The plot of percentage of inhibition of CQR8 and CQR12 versus various concentration of chloroquine. The percentage of inhibition was calculated from the respiration rate, which was calculated from the slope of the linear part of the dissolved oxygen concentration versus the incubation time. The inhibition concentration (IC\textsubscript{50}) of (a) CQR8 was 152mg/ml and (b) CQR12 was 168 mg/ml.
Figure 4.10a & b. The plot of percentage of inhibition of CQR13 and CQR17 versus various concentration of chloroquine. The percentage of inhibition was calculated from the respiration rate, which was calculated from the slope of the linear part of the dissolved oxygen concentration versus the incubation time. The inhibition concentration (IC$_{50}$) of (a) CQR13 was 183 mg/ml and (b) CQR17 was 181 mg/ml.
Figure 4.11a & b. The plot of percentage of inhibition of CQR20 and CQR21 versus various concentration of chloroquine. The percentage of inhibition was calculated from the respiration rate, which was calculated from the slope of the linear part of the dissolved oxygen concentration versus the incubation time. The inhibition concentration (IC$_{50}$) of (a) CQR20 was 154 mg/ml and (b) CQR21 was 180 mg/ml.
Figure 4.12a & b. The plot of percentage of inhibition of CQR23 and CQR30 versus various concentration of chloroquine. The percentage of inhibition was calculated from the respiration rate, which was calculated from the slope of the linear part of the dissolved oxygen concentration versus the incubation time. The inhibition concentration (IC₅₀) of (a) CQR23 was 87 mg/ml and (b) CQR30 was 165 mg/ml.
<table>
<thead>
<tr>
<th>Clones</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>Fold of resistance compare with YHW1052</th>
</tr>
</thead>
<tbody>
<tr>
<td>CQR8</td>
<td>152 mg/ml</td>
<td>5 X</td>
</tr>
<tr>
<td>CQR12</td>
<td>168 mg/ml</td>
<td>5 X</td>
</tr>
<tr>
<td>CQR13</td>
<td>183 mg/ml</td>
<td>6 X</td>
</tr>
<tr>
<td>CQR17</td>
<td>181 mg/ml</td>
<td>6 X</td>
</tr>
<tr>
<td>CQR20</td>
<td>154 mg/ml</td>
<td>5 X</td>
</tr>
<tr>
<td>CQR21</td>
<td>180 mg/ml</td>
<td>6 X</td>
</tr>
<tr>
<td>CQR23</td>
<td>87 mg/ml</td>
<td>3 X</td>
</tr>
<tr>
<td>CQR30</td>
<td>165 mg/ml</td>
<td>5 X</td>
</tr>
<tr>
<td>YPH499</td>
<td>72 mg/ml</td>
<td>2 X</td>
</tr>
<tr>
<td>YHW1052</td>
<td>33.8 mg/ml</td>
<td>--</td>
</tr>
</tbody>
</table>

n = 1

Table 4.5. Summarized results of IC<sub>50</sub> of CQ resistant clones based on oxygen sensor.
4.9 Subcloned in single copy plasmid, pYX113

Plasmodial cDNA library was constructed in pYES2 vector, which was a high copy number plasmid. It was interesting to test that whether the resistance was mediated by the overexpression of those chloroquine resistance genes. Therefore, those CQR genes would be subcloned in a single copy plasmid called pYX113 and tested by using 96 wells drug assay.

From the graph (figure 4.13), it would be seen that the relative percentage cell growth of all those CQR clones were higher than that of YHW1052 at a higher concentration of chloroquine, such as 100mg/ml. Therefore, it could be concluded that the resistance of those CQR clones were not mediated by the overexpression of all CQR genes. By using two different drug assays, the resistance of all those CQR could be confirmed and also, those of them could confirm the resistance even in single or high copy number plasmid. Then, those CQR genes would be analyzed at mRNA level.
Figure 4.13. The chloroquine sensitivity of CQR genes subcloned in pYX113. After subcloning those CQR genes to pYX113 and retransfected back to YHW1052, it could be seen that those CQR clones still had a higher relative percentage of growth than that of YHW1052 in the presence of high concentration of chloroquine.
4.10 Northern Analysis of Novel Chloroquine Resistance Genes

In order to determine whether the CQR cDNAs are expressed and if so, the size of mRNA, northern analysis was performed. Messenger RNA isolated from mixed stage of CQR parasites (Dd2) was hybridized with the radioactive labeled inserts (figure 4.14 – figure 4.21).

First, it could be seen that those CQR genes could express its mRNA. Also, the sizes of mRNA of most of the genes were more or less the same as that of the corresponding cDNA (table 4.6). Only one gene, CQR23, had different sizes of its mRNA (about 3kb) and cDNA (about 1.5kb). It suggested that the cDNA might not be full length. Since plasmodial DNA was high A-T content, oligo dT might mis-prime to other region of such gene during first strand cDNA synthesis and a full length cDNA could not be obtained. The partial-length cDNA can still be identified because the system was based on the functional complementation.
Figure 4.14. The northern analysis of novel resistance gene, CQR8. 100 nanogram mRNA was loaded on lane and hybridized with radiolabeled probe of CQR8 and detected by phosphor-imaging technique. A size of about 1.2kb gene of interest was detected (the arrow).
Figure 4.15. The northern analysis of novel resistance gene, CQR12. 100 nanogram mRNA was loaded on lane and hybridized with radiolabeled probe of CQR12 and detected by phosphor-imaging technique. A size of about 1kb gene of interest was detected (the arrow).
Figure 4.16. The northern analysis of novel resistance gene, CQR13. 100 nanogram mRNA was loaded on lane and hybridized with radiolabeled probe of CQR13 and detected by phosphor-imaging technique. A size of about 1.35kb gene of interest was detected (the arrow).
Figure 4.17. The northern analysis of novel resistance gene, CQR17. 100 nanogram mRNA was loaded on lane and hybridized with radiolabeled probe of CQR17 and detected by phosphor-imaging technique. A size of about 0.7kb gene of interest was detected (the arrow).
Figure 4.18. The northern analysis of novel resistance gene, CQR20. 100 nanogram mRNA was loaded on lane and hybridized with radiolabeled probe of CQR20 and detected by phosphor-imaging technique. A size of about 0.8kb gene of interest was detected (the arrow).
Figure 4.19. The northern analysis of novel resistance gene, CQR21. 100 nanogram mRNA was loaded on lane and hybridized with radiolabeled probe of CQR21 and detected by phosphor-imaging technique. A size of about 1.1kb gene of interest was detected (the arrow).
Figure 4.20. The northern analysis of novel resistance gene, CQR23. 100 nanogram mRNA was loaded on lane and hybridized with radiolabeled probe of CQR23 and detected by phosphor-imaging technique. A size of about 3kb gene of interest was detected (the arrow).
Figure 4.21. The northern analysis of novel resistance gene, CQR30. 100 nanogram mRNA was loaded on lane and hybridized with radiolabeled probe of CQR30 and detected by phosphor-imaging technique. A size of about 1kb gene of interest was detected (the arrow).
<table>
<thead>
<tr>
<th>Resistance Clone</th>
<th>Size of mRNA</th>
<th>Size of cDNA</th>
<th>Size difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CQR8</td>
<td>1.2kb</td>
<td>1kb</td>
<td>0.2kb</td>
</tr>
<tr>
<td>CQR12</td>
<td>1kb</td>
<td>1kb</td>
<td>0kb</td>
</tr>
<tr>
<td>CQR13</td>
<td>1.3kb</td>
<td>1kb</td>
<td>0.3kb</td>
</tr>
<tr>
<td>CQR17</td>
<td>0.7kb</td>
<td>0.55kb</td>
<td>0.15kb</td>
</tr>
<tr>
<td>CQR20</td>
<td>0.8kb</td>
<td>0.8kb</td>
<td>0kb</td>
</tr>
<tr>
<td>CQR21</td>
<td>1.1kb</td>
<td>1kb</td>
<td>0.1kb</td>
</tr>
<tr>
<td>CQR23</td>
<td>3kb</td>
<td>1.5kb</td>
<td>1.5kb</td>
</tr>
<tr>
<td>CQR30</td>
<td>1kb</td>
<td>0.9kb</td>
<td>0.1kb</td>
</tr>
</tbody>
</table>

Table 4.6. Summarized results of sizes of mRNA and cDNA of different resistant clones. The sizes of mRNA of most of the clones were more or less the same as that of cDNA except CQR23.
4.11 Differentiation expression of CQR genes in Dd2 (resistant) strain and 3D7 (sensitive) strain

After northern analysis, it showed that those CQR genes could express its mRNA (figure 4.14 – 4.21). Also, it was interesting to test whether those CQR genes could differentially express in Dd2 (resistant) and 3D7 (sensitive) strain since such differential expression of gene(s) might be related to drug resistance mechanism. Therefore, those CQR genes would be dotted on the nylon membrane and the total RNA would be extracted from two different plasmodium strains. Then, total RNA would be reversibly transcripted to cDNA and labeled with digoxygenin as a probe. Dot blot hybridization would be performed (figure 4.22).

By using actin as a positive control, the signal of those genes would be quantified by using lumi-imager. From the table 4.7, it would be seen that about three out of eight CQR genes had a 1.5 to 2 fold higher gene expression in Dd2. It might indicate that those genes might mediate its effect on a particular function related to drug resistance.
Figure 4.22. The results of dot-blot hybridization. By using actin as a positive control, the signals of those CQR genes were quantified by lumi-imager. It showed that three out of eight CQR genes had a 1.5 to 2 fold higher in Dd2 strain.
<table>
<thead>
<tr>
<th></th>
<th>CQR8</th>
<th>CQR12</th>
<th>CQR13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td>1.00</td>
<td>1.04</td>
<td>1.94</td>
</tr>
<tr>
<td>CQR17</td>
<td>1.16</td>
<td>1.19</td>
<td>1.42</td>
</tr>
<tr>
<td>CQR30</td>
<td>1.46</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.7. The relative gene expression of CQR genes in Dd2 compared with 3D7. It showed that out of eight CQR genes, CQR13, CQR21 and CQR30 had about 1.5 to 2 fold higher expression in Dd2 strain.
4.12 Library Screening of Novel Quinacrine Resistance Gene(s)

Since YHW1052 is sensitive to antimalarials, it can be used to screen other drug resistance gene. Quinacrine (QNC), which is another antimalarial, can kill YHW1052 at a concentration of 7 mg/ml. The screening approach would be performed by using quinacrine

YHW1052 was transformed with the *P. falciparum* cDNA library and colonies are plated on agar plate containing 7 mg/ml QNC agar plate, around 500 colonies were obtained after 10 days of selection. All resistant colonies from the primary screening were then gave a secondary screening on 7 mg/ml QNC agar plate. There were only 22 novel QNCR clones obtained (table 4.8). As controls, YHW1052 did not grow at this concentration while the 22 QNCR clones and YPH499 could (figure 4.23a &b).

Those QNCR-clones which can survive on secondary screening would be characterized their resistant level by using 96-wells drug assay (figure 4.24). Basically, it can be divided into two groups, namely, clones with resistant levels higher than that of YPH499
(figure 4.24a) and clones with resistant levels in between YPH499 and YHW1052 (figure 4.24b & c). It can be observed that six out of twenty-two QNCR-clones have a higher resistant level.
<table>
<thead>
<tr>
<th></th>
<th>Number of colonies counted/ µg of plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td>YHW1052</td>
<td></td>
</tr>
<tr>
<td><strong>transformed with</strong></td>
<td></td>
</tr>
<tr>
<td><strong>P. falciparum</strong></td>
<td></td>
</tr>
<tr>
<td>cDNA library plated on SC-URA plate</td>
<td>1.5x10⁵</td>
</tr>
<tr>
<td>YHW1052</td>
<td></td>
</tr>
<tr>
<td><strong>transformed with</strong></td>
<td></td>
</tr>
<tr>
<td><strong>P. falciparum</strong></td>
<td></td>
</tr>
<tr>
<td>cDNA library and plated on 7 mg/ ml QNC plate (Primary screening)</td>
<td>500</td>
</tr>
<tr>
<td>YHW1052</td>
<td></td>
</tr>
<tr>
<td><strong>transformed with</strong></td>
<td></td>
</tr>
<tr>
<td>plasmid selected from primary screening and plated on 7 mg/ml QNC plate (Secondary screening)</td>
<td>22</td>
</tr>
<tr>
<td>YHW1052</td>
<td></td>
</tr>
<tr>
<td><strong>transformed with</strong></td>
<td></td>
</tr>
<tr>
<td><strong>pYES2</strong></td>
<td></td>
</tr>
<tr>
<td>plasmid plated on 7 mg/ ml QNC plate</td>
<td>0</td>
</tr>
<tr>
<td>YHW1052</td>
<td></td>
</tr>
<tr>
<td><strong>transformed without</strong></td>
<td></td>
</tr>
<tr>
<td><strong>pYES2</strong></td>
<td></td>
</tr>
<tr>
<td>plated on SC-URA plate</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 4.8. The result of primary screening and secondary screening of QNCR clones. cDNA plasmids were transformed in yeast and act as a control to show the transformation efficiency. About 150,000 transformants were plated on SC-Ura agar plate containing 6.4mg/ml quinacrine and incubated at 30°C. About 500 colonies survivors were obtained after 10 days of selection. YHW1052, transformed with or without vector, act as a negative control and they do not produce any resistant colonies.
Figure 4.23a & b. Growth of QNC-resistant clones on SC-UA plate with or without 7 mg/ml quinacrine. Different quinacrine resistant clones, YPH499 and YHW1052 were plated and incubated at 30°C for two days. (a) All clones could grow on SC-URA plate. (b) Twenty-two of QNC-resistant clones and YPH499 (arrows) could grow at this concentration.
Figure 4.24a, b & c. The results of 96-well drug assay. (a) Some of the QNCR clones have a higher resistant level than that of YPH499 but (b & c) some of them have a resistant level in between that of YPH499 and YHW1052.
5. Discussion

Chloroquine resistant *Plasmodium falciparum* spread steadily from the two locations in South America and Southeast Asia where it originated 40 years ago after the massive use of chloroquine for decades. Discovery of novel drug resistance mechanism is as important as to discover new drugs or vaccines. In the past decade, the studies of resistance mechanisms in *P. falciparum* have lagged behind similar study in bacteria and mammalian cancer cells. The main reasons are that exogenous DNA cannot be transfected to this parasite and cloned DNA sequences is not stable in *E. coli* due to high A-T content of malarial DNA. Therefore, heterologous system has been used to study material gene functions (Triglia and Kemp, 1991). The budding yeast *Saccharomyces cerevisiae* is an ideal microorganism for basic biochemical, genetic, molecular and biotechnological studies (Munder and Hinnen, 1999). Previously, yeast was chosen to study the function of plasmodial genes (Ruetz et al., 1996; Volkman et al., 1995) and identify the genes from yeast genomic library that are resistant to antimalarials drug (Delling et al., 1998; Sibley et al., 1997; Wooden et al., 1997). These examples demonstrated the feasibility of using yeast system for the identification and characterization of novel
or known genes. In the present study, we have used yeast as a model organism to search for novel genes that upon overexpression confer cellular resistance to antimalarial drugs, such as chloroquine, quinacrine and quinidine. The sensitive strain of *S. cerevisiae* YHW1052 showed different sensitivity to three above mentioned antimalarial drugs. The drug mechanism of these three drugs is not been fully understood. Moreover, the difference in sensitivity to antimalarial drugs might be related to different chemical structure as well as activities of those drugs.

Chloroquine sensitive yeast mutant, YHW1052, (Δpdr5::TRP 1, Δsnq2::his G, Δpdr10:: his G) was transformed with a 3D7 plasmodial cDNA library present on a multicopy vector, pYES2 and was then used to screen for chloroquine resistance. This approach, based on a one-step chloroquine selection, enabled the rapid isolation of drug resistance genes. In this project, twenty-eight CQ resistance genes were identified and most of them were novel genes and with different sizes. Due might question whether the genes with small insert size could be translated to a functional protein. These selected genes were selected by functional complementation. They were supposed to be able to encode for functional proteins. There is an
example where small size gene could encode for a functional protein in the *Plasmodium*. For example, thioredoxin1 was found to have a size of about 300bp (Gilberger et al., 1997).

It was observed from the northern analysis (figure 4.14 – figure 4.21) that most of the resistance genes have an insert size similar to their corresponding cDNAs except CQR23. It raised the question why CQR23 could be translated to a functional protein if the 5' start region of gene was missing. As discussed before, those genes were selected by functional complementation so that CQR23 should have its functional protein. The possible reason might be another ATG start codon presented in the middle part of the gene and therefore, the CQR23 could be translated and such part of protein might be the functional domain of the whole protein. Also, it had a similar example found in the past. A candidate resistance gene, *pfmdr1*, had an insert size about 4 kb but the corresponding size of mRNAs transcript were 7.5kb and 8.5kb (Volkman et al., 1993).

Under the yeast complementation system we use, one important assumption should be made: the drug resistance mechanism must be mediated by a single gene in *P. falciparum*. For the yeast
transformation, there was only one plasmid that could be transformed per each yeast cell and therefore, we could only study the effect of one gene in each resistant clone.

The molecular genetics of *P. falciparum* has rapidly developed and the plasmodial genome project is underway. Out of 14 chromosomes, chromosome 2 and chromosome 3 have been completely sequenced (Bowman et al., 1999; Gardner et al., 1998) and chromosome 10, 11, 12 and 13 have been fully annotated. After the sequencing analysis of that chromosome is finished, all coding regions could be determined. A plasmodial expressed sequencing tag database has also been constructed. Most of the resistant clones identified in this project can be identified in the genome or EST database. For the eight clones chosen for northern analysis, they are all found to be encoding for their mRNAs (figure 4.14 – figure 4.21).

In order to map the CQ resistance genes, a genetic linkage analysis using 85 restriction fragment length polymorphism markers was used to examine inheritance of the 14 *P. falciparum* chromosomes in a laboratory cross between a chloroquine-resistant and a chloroquine-sensitive parasite. Inheritance data from 16
independent recombinant progenies showed that chloroquine-resistant phenotype was governed by a single locus within an approximately 400-kilobase region of chromosome 7 (Wellems et al., 1991). By fine mapping and extensive sequencing, cg2 (Su et al., 1997) and pfcrt (Fidock et al., 2000c) were determined to be linked to CQ resistance.

However, among the 28 chloroquine resistant clones isolated here by yeast complementation system, none of them is located in chromosome 7. They are located in 4 different chromosome, namely, chromosome 2, 3, 12 and 14 and most of them are located in chromosome 12 (table 4.4). It was reported that the increase in size of chromosome 3 was associated with the decreased drug sensitivity in the chloroquine-selected parasites (Lim and Cowman, 1996). In such study, the chloroquine-sensitive P. falciparum clone, HB3, was selected to grow in increasing amounts of chloroquine. The size of chromosome 3 expanded with increasing chloroquine selection. That data suggested that resistance phenotype might be related to chromosome 3. This suggests that the CQR loci in chromosome 7 may not be the only CQR gene in P. falciparum. On the other hand, those candidate resistance genes, such as cg2, pfmdr1 and pfcrt, would not be identified by using yeast complementation system. Since those
candidate resistance genes were found by using different methods based on different experimental approaches, those of identified genes might be different. For instance, the proposed resistance mediated by those candidate genes is based on mutational effect. The gene, \textit{pfcrt}, found in resistant strain is different from that of sensitive strain. A point mutation (K76T) associated with CQ resistance was identified in both laboratory strains and field isolates (Fidock et al., 2000c). However, the yeast functional complementation system is based on overexpression of candidate resistance genes and not by point mutation. Since the whole picture of resistance mechanism is still in search, all candidate genes, as well as those resistance genes isolated in this study should be further characterized.

A total of twenty-eight chloroquine resistance genes were identified by yeast complementation system and eight of them were selected to be further analyzed since these genes have a larger insert size. Since the yeast sensitive mutant, YHW1052, was constructed based on deletion of three mdr genes, it is expected that all resistant genes selected have to be a mdr homolog. However, the results were out of the expectation. None of them were found to be a mdr homolog. Among eight of them, CQR8, CQR21 and CQR23 are identified as
ESTs; CQR12, CQR13, CQR20 and CQR30 are unknown sequences. All of them cannot be identified by GenBank database. It indicates that they are novel genes. Only one gene, CQR17, could be identified from plasmodial database and it encodes for the *P. falciparum* histidine-rich protein (pfHRP). In the past, these were three type of histidine-rich protein identified in the *Plasmodium*; namely, *P. falciparum* histidine-rich protein (pfHRP), knob associated histidine-rich protein (KAHRP) and small histidine-rich protein (SHRP) (Ellis et al., 1987; Howard et al., 1986; Taylor et al., 1987).

In the past, KAHRP has been proposed to be related to cytoadhesion (Biggs et al., 1989a; Biggs et al., 1989b; Oh et al., 2000; Pologe and Ravetch, 1986; Sharma, 1997; Waller et al., 1999) but it has never been reported that KAHRP is related to drug resistance. The actual function of PFHRP is still unknown. It was suggested that this protein could bind heme and it might mediate the hemozoin formation (Sullivan et al., 1996). Neither KAHRP nor PFHRP were not the mdr homolog. The possible reasons were that KAHRP and PFHRP might have multiple functions.
It is interesting to see that the insert size of CQR17 is not the same as *pfhrp* or *kahrp* identified in GenBank database. The length of *kahrp* and *pfhrp* reported in GenBank database were about 2350bp (Triglia et al., 1987) and 1020bp (Sharma, 1988) respectively. However, the length of CQR17 was about 555bp. From the northern analysis, it could be seen that the size of mRNA of CQR17 and that of corresponding cDNA were more or less the same. This indicated that CQR17 is a novel gene and it might belong to the family of histidine-rich protein.

Due to the emergence of antimalarial drug resistance, it is necessary to understand the actual mechanism of drug resistance and hopefully that it can reverse such resistance phenomenon. For the antifolate antimalarial drug resistance, such as pyrimethamine and fansider, the mechanism of drug resistance has been identified. These drugs are inhibitors of the enzyme called dihydrofolate reductase which is involved in DNA synthesis. Such enzyme have a point mutation at codon108 in the pyrimethamine-resistant parasites and the effect of drug on mutated enzyme was largely reduced, causing the parasite to become resistant to the drug (Plowe et al., 1995; Wang et al., 1997). Resistance to quinoline antimalarial drug resistance
mechanism, such as chloroquine, has been studied extensively in recent years and several chloroquine resistance mechanisms were proposed by different research groups (Bray et al., 1999c; Fidock et al., 2000b; Foote et al., 1989). Although these candidate resistance genes had been shown to correlate with clinical isolates, none of them have been definitely proven to be the real resistance gene (Babiker et al., 2001; Durand et al., 2001; Price et al., 1999).

For the 8 chloroquine resistance genes identified by yeast complementation system, only CQR17 was identified as a *P. falciparum* histidine-rich protein (*pfhlp*) and 7 of them could not be identified in the GenBank database. As discussed before, those genes could functionally complement the resistance phenotype of yeast, it was supposed that those genes mediate it effect on drug resistance mechanism.

For the CQR17, a novel gene related to *pfhlp*, have been proposed to mediate of hemozoin formation (Sullivan et al., 1996). However, this protein had been shown by equilibrium dialysis experiment in this year that it does not have any significant interaction with chloroquine (Pandey et al., 2001). It might indicate that CQR17
was not the \textit{pfh}rp but a novel histidine-rich protein, which might have interaction with chloroquine and capable of mediating the drug resistance. This hypothesis had been supported by northern analysis that the size of CQR17 was not the same as the \textit{pfh}RP or \textit{KAHRP} in GenBank database. The most well known characteristic of histidine rich protein is its high affinity binding of metal ions, such as zinc. Therefore, it may indicate that the protein may have interaction with ions rather than directly binding with chloroquine and indirectly affecting the chloroquine uptake. It could also be found that the \textit{Na}/H+ exchanger (\textit{NHE}1), which is the well-known ion exchanger, has a histidine-rich conserved region. By mutation analysis, it has been shown that such region was important for maximal activity of the \textit{Na}/H+ exchanger (Dibrov et al., 2000). Although it had been shown that the cellular uptake of chloroquine was independent to the NHE activity (Bray et al., 1999a) and there is no significantly different of NHE activity between chloroquine-sensitive and chloroquine resistant parasite (Martiney et al., 1999), one could not completely eliminate the role of NHE on drug resistance mechanism. Amiloride derivatives, the inhibitors of NHE, could inhibit the chloroquine uptake (Sanchez et al., 1997). To summarize our speculation, CQR17 codes for a novel histidine-rich protein and such protein might have a high affinity
binding to metal ions. Also, such protein can act as an ion exchanger, such as NHE, and alter the pH environment of the parasite. It had been reported that chloroquine uptake was dependent on the binding to heme (CQ-Heme) and the binding of chloroquine and heme was mediated by pH (Bray et al., 1999a). Hence, the drug resistance mechanism might be indirectly mediated by CQR17, which controlled the ions exchange and pH environment where it affected the binding between chloroquine and heme as well as the uptake of the drug.

The yeast complementation system was also used to identify quinacrine resistance genes. After primary and secondary screening, 22 resistant clones were obtained and confirmed the resistance by using 96-wells drug assay. The results showed that 6 of them have a higher resistance level than that of YPH499. After restriction analysis and DNA sequencing, it was interesting to note that none of the QNCR genes were similar to CQR genes. Therefore, it might indicate that the chloroquine and quinacrine did not have the same resistance mechanism. This also could explain why chloroquine and quinacrine had different effect on YHW1052.
Using the yeast complementation system, drug resistance genes could be rapidly identified and these drug resistance gene capabilities to confirm drug resistance has been confirmed by retransflection to YHW1052. However, this system has several limitations. First, the drug resistance mechanism was assumed to be mediated by one gene. A multi-gene resistance mechanism would not be identified by this system. Second, the minimum inhibitory concentration used for identification of drug resistance gene was much higher than that used in clinical treatment. Such high concentration of chloroquine have to be used because of the low drug permeability through the yeast cell wall. Although there was another yeast mutant, YRP3, that have a lower minimum inhibitory concentration, the transformation efficiency of that strain was much lower than that of YHW1052 and therefore it could not be used for screening. Third, although these selected drug resistance genes could mediate resistance in yeast, they must be re-transfected back to *Plasmodium* for final confirmation of their roles in drug resistance.

Some of the resistance genes identified by yeast complementation system are novel genes. Their identity might be revealed after the genome project is finished. The insert could be
detected at mRNA level (figure 4.14 – figure 4.21). Messenger RNA from Dd2 strain has been used because it is a resistant strain. Those resistant genes selected from yeast complementation system should be identified from mRNA of resistant strain (Dd2) and the expected results are obtained. Moreover, Dd2 and another strain called HB3, which is a sensitive strain, have been used for genetic cross study. Therefore, a dot blot hybridization experiment was performed to test whether those drug resistance genes would differentially expressed between the resistant (Dd2) and sensitive strain (HB3). The result showed that three out of eight chloroquine resistance genes, had about 1.5 to 2 folds higher expression in resistant strain (table 4.7). Such differential gene expression might indicate that those genes mediate a particular function although it did not show whether those functions are related to drug resistance or not. Nevertheless, it is better to check the differential gene expression by northern analysis as a comparative study.

BLOCKS, a motif search engine, cannot identify any putative motifs in the CQR genes identified in this project. Several possible functions maybe predicted: the protein may directly efflux the chloroquine out or affect the environment, such as pH, so that
chloroquine will not be accumulated inside parasite. Nevertheless, it is likely to know whether the protein can bind to chloroquine or not by using radiolabeled chloroquine.

However, it is unexpected that so many resistant genes are identified. These resistance genes are not necessarily related to CQR mechanism. There may still have false positive. The best confirmation method is to transflect these CQ-resistance genes back to *P. falciparum*. Although the transfection protocol is not as well developed as that of bacteria, this technique is greatly improved in recent years (Crabb and Cowman, 1996; Crabb et al., 1997; Wu et al., 1995) and therefore, transfection of those resistant genes should be performed.

On the other hand, the plasmodial cDNA library was also used to identify the quinacrine resistance gene(s) and twenty-two clones were obtained (figure 4.23). It indicated that the resistance mechanism of quinacrine might be different from that of chloroquine. Therefore, the experimental approach of those QNCR genes should be similar to that of CQR genes. It is hoped that different drug resistance mechanism could be completely identified out and those information
could contribute to understand the molecular mechanism of drug resistance. Eventually, it could aid the antimalarial drug discovery and solve the problem of emergency of drug resistance.
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7. Appendix

7.1 General Materials and Methods

Ethanol Precipitation of DNA and RNA

Two volumes of ice-cold absolute ethanol was added to the DNA solution (2.5 volume for RNA). 3M sodium acetate (pH 5.2) was added to a final concentration of 0.3M. The ethanolic solution was kept at −70°C for 30 minutes. The precipitated DNA or RNA was collected by centrifugation at 12,000g for 15 minutes in a microcentrifuge. After centrifugation, the DNA or RNA pellet was washed with 70% ethanol and recovered by centrifugation again. The DNA or RNA pellet was air-dried and dissolved in appropriate amount of TE (DEPC-treated water for RNA).

Spectrophotometric Quantitation of DNA and RNA

The amount of nucleic acid was determined spectrophotometrically (GeneQuant, Amersham-Pharmacia). An OD$_{260}$ of 1 correspond to approximately 50µg/ml for double-stranded
DNA and 40μg/ml of RNA. The purity of nucleic acid was determined by the ratio between the absorbance at 260nm and 280nm. Pure preparations of nucleic acid have OD\textsubscript{260}/OD\textsubscript{280} value of 1.8 or above.

Minipreparation of Plasmid DNA

A single bacterial colony was inoculated into 1.5ml Luria Bertani (LB) medium containing the appropriate antibiotic in a capped sterile tube. The culture was incubated overnight at 37°C with vigorous shaking. The bacterial cells were collected by centrifugation at 12,000g for one minute. The bacterial pellet was resuspended in 100μl of Solution I. Then 200μl of Solution II was added and the mixture was kept at room temperature for 5 minutes. After 150μl of Solution III was added, the mixture was centrifuged at 12,000g for 10 minutes. The compositions of Solution I, II and III were described at the end of section. The plasmid DNA was recovered by ethanol precipitation as described and dissolved in 50μl of TE.
Preparation of Plasmid DNA using the Rapid Plasmid Isolation kit

The following procedure is based on the method recommended by the supplier. A single bacterial colony was inoculated into 1.5ml Luria Bertani (LB) medium containing the appropriate antibiotic in a capped sterile tube. The culture was incubated overnight at 37°C with vigorous shaking. The bacterial cells were collected by centrifugation at 12,000g for one minute. The pellet was then resuspended in 210µl of G1 Buffer (50mM Tris-Cl, pH 8.0 and 10mM EDTA). 210µl of G2 (200mM NaOH, 1% SDS) was then added, mixed by inverting the tube five times and incubated for five minutes at room temperature. 280µl of G3 (contains acetate and guanidine hydrochloride) was then added and mixed by inverting the tube ten times. The mixture was then centrifuged at 12,000g for 10 minutes. The supernatant was retrieved and added to the spin cartridge and centrifuged at 12,000g for one minute. The cartridge was then washed with 700µl G4 (contains NaCl, EDTA and Tris-Cl, pH 8.0) and centrifuged again at 12,000g for one minute. Plasmid DNA was eluted by adding 50µl of
TE cartridge and the eluted DNA was collected by centrifugation at 12,000g for one minute.

**Preparation of Escherichia coli Competent Cells**

A single bacterial colony (*Escherichia coli*, XL1-Blue or JM109) was inoculated with 1.5ml LB medium. The culture was incubated at 37°C overnight with shaking at 250 r.p.m. 250µl of the overnight culture was inoculated into 25 ml LB and incubated at 37°C for about 3 hours with shaking at 250 r.p.m. until log phase was reached (OD$_{600}$ ~0.3). The culture was collected into a 50ml centrifuge tube. Bacterial cells were spun down by centrifuging at 3,000 r.p.m. (1,500 g) (Hitachi rotor 20-2) for 15 min at 4°C. The supernatant was discarded. One-third volume of the original culture of RF1 buffer (100 mM RbCl, 50 mM MnCl$_2$·4H$_2$O, 30 mM potassium acetate, 10 mM CaCl$_2$·2H$_2$O, 15% w/v glycerol, pH 5.8) was used to resuspend the bacterial cell pellet. The bacterial cells were chilled on ice for 15 to 20 min and then centrifuged at 3,000 r.p.m. (Hitachi rotor 20-2) for 15 min at 4°C. The pellet was then resuspended in 2 ml RF2 buffer for every 25 ml of the original culture (10 mM MOPS, 10 mM RbCl, 75 mM CaCl$_2$·2H$_2$O, 15% w/v glycerol, pH 6.8). The bacterial cells were
chilled on ice for another 15 min and then aliquoted into 200 µl fraction into chilled microfuge tubes. For later use, the competent bacterial cells were frozen in liquid nitrogen and stored at -70°C.

Transformation of the plasmid DNA into competent Escherichia coli cells

200 µl fraction of frozen competent cells was incubated on ice until the cell suspension was just thawed. Ligation reaction mixes were added to the cells suspension. After gentle mixing, the tubes was incubated on ice for 45 min and then heat shocked at 42°C for 2 min. Then it was immediately chilled on ice for 2 min and mixed with 800 µl LBG medium (0.2 ml sterile 1M glucose/10 ml LB medium). The tube was then incubated at 37°C for 45 min with shaking at 250 r.p.m. The transformed cells (diluted to a suitable concentration) were spread onto LB agar plates containing the appropriate antibiotics. When blue-white selection was required, 50µl of X-gal (20mg/ml) and 20µl of IPTG (0.1M) were mixed and spread onto the LB plate before the cells were plated.
Agarose Gel Electrophoresis

DNA was separated by 1 to 2% TBE or TAE (when DNA recovery was planned) agarose gel, which was prepared by dissolving 1 to 2% agarose (w/v) in 1X TBE or TAE containing 0.5μg/ml ethidium bromide. The DNA sample was mixed with appropriate amount of 6X agarose gel loading buffer to give a 1X final concentration. The samples were then loaded into slots of the gel placed in the electrophoresis tank and covered by 1X TBE or TAE electrophoresis buffer. A voltage of 8V/cm was applied. After the bromophenol blue was migrated for an appropriate distance through the gel, the gel was examined under UV. Polaroid instant photograph was taken by a Polaroid MP-4 instant camera. Composition of the TAE, TBE and gel loading buffer were described at the end of section.

Restriction Digestion of DNA

Restriction digestion of DNA was carried out in a volume of 20μl to 100μl in the appropriate buffer as suggested by the supplier. The reaction was incubated at the optimal temperature of the
restriction enzyme for an hour or longer. After digestion, the reaction mixture was analyzed by agarose gel electrophoresis.

**Linearization and Dephosphorylation of Plasmid Vector**

5μg of plasmid vector was digested with 50 units of the suitable restriction enzyme(s) in a 50μl reaction mixture as described. After complete digestion, 1U of Shrimp Alkaline Phosphatase (SAP) (Amersham-Pharmacia) was added and incubated at 37°C for another 30 minutes. Linearized and dephosphorylated plasmid was recovered by ethanol precipitation and dissolved in the appropriate volume of TE buffer.
Purification of DNA from Agarose Gel using the CONCERT® Rapid Gel Extraction System (Life Technologies).

The DNA samples were electrophoresed on a 1X TAE agarose gel. The desired DNA band was excised and dissolved in 3 volumes of L1 Buffer (contains concentrated sodium perchlorate, sodium acetate and TBE-solubilizer). Then it was incubated at 55°C for 10 min with occasional shaking until the agarose gel was dissolved. The mixture was then loaded into the cartridge and centrifuged at 12,000g for one minute. The flow-through was discarded and the cartridge was washed with 700μl L2 Buffer (contains NaCl, EDTA and Tris-HCl). The cartridge was then centrifuged again at 12,000g for 1 minute and the flow through was discarded. The DNA was eluted by adding 50μl of TE to the cartridge and collected by centrifugation at 12,000g for one minute. Eluted DNA sample was quantitated by electrophoresis on a 1% TBE agarose gel with ethidium bromide.
Preparation of Ribonuclease (RNase) Free Reagents and Apparatus

General laboratory glassware was treated by baking at 180°C for eight hours or more. Other items which were not susceptible to baking were treated with DEPC (0.1% (v/v) in water). All solutions were treated with 0.1% DEPC for at least 12 hours and then autoclaved at 121°C for 15 minutes on liquid cycle. Sterile, disposable plasticwares were used for the preparation and storage of RNA without pretreatment.

Northern Hybridization of Total or Messenger RNA

Total or messenger RNA was electrophoresed in formaldehyde gel and then transferred onto Hybond N+ Nylon membrane (Amersham-Pharmacia). Agarose gel was treated in 20X SSC for 15 minutes with gentle shaking. It was then capillary blotted in 20X SSC solution overnight. After blotting, the membrane was briefly rinsed in 2X SSC, air-dried and then UV irradiated for 5 minutes to fix RNA onto the membrane. It was prehybridized and hybridized with hybridization buffer with radiolabeled probe.
Detection of Chemiluminescent Signals

Lumi-film (Roche Diagnostics) was used to detect the chemiluminescence signals as described by the manufacturer (Roche Diagnostics). The chemiluminescence substrate, CSPD, was added to the anti-DIG antibody bound membrane and sealed in a plastic bag. The membrane was then incubated at 37°C for one hour. After that, the membrane was exposed to the Lumi-film for 20 minutes or longer. After exposures, the Lumi-film was developed with Kodak X-ray developer for 5 minutes, fixed with Kodak X-ray fixer for 5 minutes and rinsed with running water for 5 minutes.

Automated Laser Fluorescent (ALF) DNA Sequencer
(Amersham-Pharmacia)

The full details and operations of the ALF DNA Sequencer are described in the manufacturer's handbook. The system is designed for automated electrophoresis and analysis of sequencing reactions by the direct detection of fluorescently labeled DNA molecules. 6% polyacrylamide gels (0.5mm thick) were prepared using the ReadyMix Gel (ALF grade, Pharmacia) and electrophoreses were
carried out under default conditions: 1500V, 38mA & 34W; running temperature of 45°C, laser power of 3mW; sampling intervals of 2 seconds, running time of 360 minutes.
**Luria-Bertani (LB) medium**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>10g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5g</td>
</tr>
<tr>
<td>NaCl</td>
<td>10g</td>
</tr>
</tbody>
</table>

Make to 1 liter with distilled water, pH 7.2, autoclaved at 121°C for 15 minutes.

**Reagents for Preparation of Plasmid DNA**

**Solution I**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-base</td>
<td>6.06g/l</td>
</tr>
<tr>
<td>Na&lt;sub&gt;2&lt;/sub&gt;EDTA</td>
<td>3.36g/l</td>
</tr>
<tr>
<td>RNAase</td>
<td>100 µl/ml of P1 buffer</td>
</tr>
</tbody>
</table>

**Solution II**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOH</td>
<td>8g/l</td>
</tr>
<tr>
<td>SDS</td>
<td>1%</td>
</tr>
</tbody>
</table>

**Solution III**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>KAc(pH 4.8)</td>
<td>250.06g/l</td>
</tr>
</tbody>
</table>
TAE (Tris-acetate), 1X

Tris-base 4.8g/l
Na2EDTA 0.34g/l
Acetic acid 20 mM

TBE (Tris-borate), 1X

Tris-base 10.9g/l
Na2EDTA 0.68g/l
Boric acid 5.56g/l

6X Agarose gel loading buffer

Sucrose 40% (w/v)
Bromophenol blue 0.25% (w/v)

10X SSC

NaCl 87.7g/l
Sodium citrate 44.1g/l

Adjust to pH 7.0 with NaOH, autoclaved at 121°C for 15 minutes.
**TE Buffer**

Tris-HCl, pH 8.0 10mM

EDTA 0.1mM

**Hybridization Buffer**

5X SSC

0.1% (w/v) N-lauroylsarcosine

0.02% (w/v) SDS

1% Blocking Reagent (Roche Diagnostics)

**Yeast peptone dextrose (YPD) medium**

5g 50g/L YPD powder

**Synthetic complete (SC) medium minus uracil (SC-URA) medium**

0.17g Yeast Nitrogen Base

0.5g Ammonium sulphate

2g Dextrose

0.76g Mix of amino acid powders (without uracil)
**SOC Medium, pH7.0**

20g bacto-tryptone
5g bacto-yeast extract
0.5g NaCl
250mM KCl
2M MgCl₂
20mM glucose

**10X LiAc solution**

1M Lithium Acetate, pH 7.5

**50% PEG solution**

50 % PEG-3350

**LITE solution**

1X TE
1X LiAc

**PEG-LITE solution**

1x LiAC
1x TE
50% PEG-3350

**Yeast Miniprep Buffer**

2% Triton X-100
1% SDS
100mM NaCl
10mM Tris
1mM EDTA

3M Sodium Acetate, pH 5.2
12g sodium acetate
7.2 Raw Data

DNA Sequence of Chloroquine Resistant Clones

**CQR1-T7**

GGTGATGGTGTGTACCTACTACATAACAATCTTTT
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CQR6-T7

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CQR7-T7

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AAA

CQR8-T7

GATTGAAAAAAAATATATGCCTATTATTAATAAAAAAAAAT
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CQR9-T7

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CQR10-T7

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CQR11-T7

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CQR12-T7

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CQR13-T7

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CQR14-T7

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CQR15-T7

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CQR17-T7

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CQR18-T7
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CQR19-T7
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CQR20-T7
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CQR21-T7

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CQR22-T7

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CQR23-T7

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CQR24-T7

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CQR25-T7

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CQR27-T7

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CQR29-T7

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CQR30-T7

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<td>0.171</td>
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<td>0.085</td>
</tr>
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<td>9.00</td>
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<td>0.166</td>
<td>0.122</td>
<td>0.080</td>
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<td>0.164</td>
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Relative percentage of growth of quinacrine-resistant clone at different concentrations of quinacrine.
<table>
<thead>
<tr>
<th>Quinacrine/mg/ml</th>
<th>ANCR32</th>
<th>ANCR33</th>
<th>4992 (P2)</th>
<th>1052 (P2)</th>
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</thead>
<tbody>
<tr>
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<td>1.056</td>
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<tr>
<td>8.5</td>
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<td>1.053</td>
<td>0.035</td>
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<tr>
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<td>1.032</td>
<td>0.018</td>
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Relative percentage of growth of quinacrine-resistant clone at different concentration of quinacrine.
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</tr>
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</tbody>
</table>

Relative percentage of growth of quinacrine-resistant clone at different concentrations of quinacrine.
<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>0.1%</th>
<th>1%</th>
<th>3%</th>
<th>6%</th>
<th>9%</th>
<th>16%</th>
<th>32%</th>
<th>64%</th>
<th>96%</th>
<th>100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1%</td>
<td>0.0959</td>
<td>0.101</td>
<td>0.103</td>
<td>0.109</td>
<td>0.115</td>
<td>0.129</td>
<td>0.134</td>
<td>0.138</td>
<td>0.142</td>
<td>0.144</td>
<td>0.145</td>
</tr>
<tr>
<td>1%</td>
<td>0.094</td>
<td>0.102</td>
<td>0.104</td>
<td>0.109</td>
<td>0.114</td>
<td>0.128</td>
<td>0.133</td>
<td>0.137</td>
<td>0.141</td>
<td>0.143</td>
<td>0.144</td>
</tr>
<tr>
<td>3%</td>
<td>0.0925</td>
<td>0.101</td>
<td>0.103</td>
<td>0.109</td>
<td>0.114</td>
<td>0.128</td>
<td>0.133</td>
<td>0.137</td>
<td>0.141</td>
<td>0.143</td>
<td>0.144</td>
</tr>
<tr>
<td>6%</td>
<td>0.093</td>
<td>0.102</td>
<td>0.104</td>
<td>0.109</td>
<td>0.114</td>
<td>0.128</td>
<td>0.133</td>
<td>0.137</td>
<td>0.141</td>
<td>0.143</td>
<td>0.144</td>
</tr>
<tr>
<td>9%</td>
<td>0.094</td>
<td>0.102</td>
<td>0.104</td>
<td>0.109</td>
<td>0.114</td>
<td>0.128</td>
<td>0.133</td>
<td>0.137</td>
<td>0.141</td>
<td>0.143</td>
<td>0.144</td>
</tr>
<tr>
<td>16%</td>
<td>0.0959</td>
<td>0.101</td>
<td>0.103</td>
<td>0.109</td>
<td>0.115</td>
<td>0.129</td>
<td>0.134</td>
<td>0.138</td>
<td>0.142</td>
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<td>0.145</td>
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<td>0.102</td>
<td>0.104</td>
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<td>0.128</td>
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<td>0.137</td>
<td>0.141</td>
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<td>0.144</td>
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<tr>
<td>64%</td>
<td>0.0925</td>
<td>0.101</td>
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<td>0.114</td>
<td>0.128</td>
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<td>0.104</td>
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<td>0.137</td>
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<td>0.143</td>
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Relative percentage of growth of quinacrine resistant clone at different concentration of quinacrine.
<table>
<thead>
<tr>
<th>Guanine m/mg</th>
<th>OCGR23</th>
<th>OCGR25</th>
<th>OCGR27</th>
<th>OCGR2S</th>
<th>OCGR2T</th>
<th>498p</th>
<th>1052p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>0.056</td>
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<td>0.068</td>
<td>0.069</td>
<td>0.070</td>
<td>0.065</td>
<td>0.060</td>
</tr>
<tr>
<td>0.3125</td>
<td>1.172</td>
<td>1.172</td>
<td>1.149</td>
<td>1.149</td>
<td>1.143</td>
<td>1.143</td>
<td>1.149</td>
</tr>
<tr>
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<td>0.987</td>
<td>0.987</td>
<td>0.987</td>
<td>0.987</td>
</tr>
<tr>
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<td>0.772</td>
<td>0.772</td>
<td>0.772</td>
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<td>0.772</td>
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</tr>
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<table>
<thead>
<tr>
<th>Guanine m/mg</th>
<th>OCGR23</th>
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<th>OCGR2S</th>
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<th>1052p</th>
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<td>0.261</td>
<td>0.261</td>
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96-well drug assay data
| O. D. | 0 | 5 | 10 | 15 | 20 | 25 | 30 | 35 | 40 | 45 | 50 | 55 | 60 | 65 | 70 | 75 | 80 | 85 | 90 | 95 | 100 |
|------|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| 0    | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 1    | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 2    | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 3    | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 4    | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 5    | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 6    | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 7    | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 8    | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 9    | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 10   | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |

**96-Well Drug Assay Data**
<table>
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<tr>
<th>Quinacrine mg/ml</th>
<th>OCNR12</th>
<th>OCNR13</th>
<th>OCNR14</th>
<th>OCNR14</th>
<th>OCNR14</th>
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<td>0.055</td>
</tr>
<tr>
<td>0.056</td>
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<td>1.143</td>
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<td>1.143</td>
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<tr>
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<td>0.009</td>
<td>0.009</td>
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</tbody>
</table>

**Relative Percentage of Growth of Quinacrine Resistant Clone at Different Concentrations of Quinacrine**

**96-Well Drug Assay Data**