CHAPTER III

RESEARCH METHODOLOGY

SNP identification in *PSEN1* on HapMap website

The tag SNP of *PSEN1* available on the HapMap (HapMap Tutorial, 2007) website was used to select tag SNPs present at a minor allele frequency (MAF) > 5 % and with pairwise linkage disequilibrium (LD) cut-off value of $r^2 > 0.6$. Coverage of the entire gene (90,901 bp) was achieved. The procedure is shown in Figures 8-16.

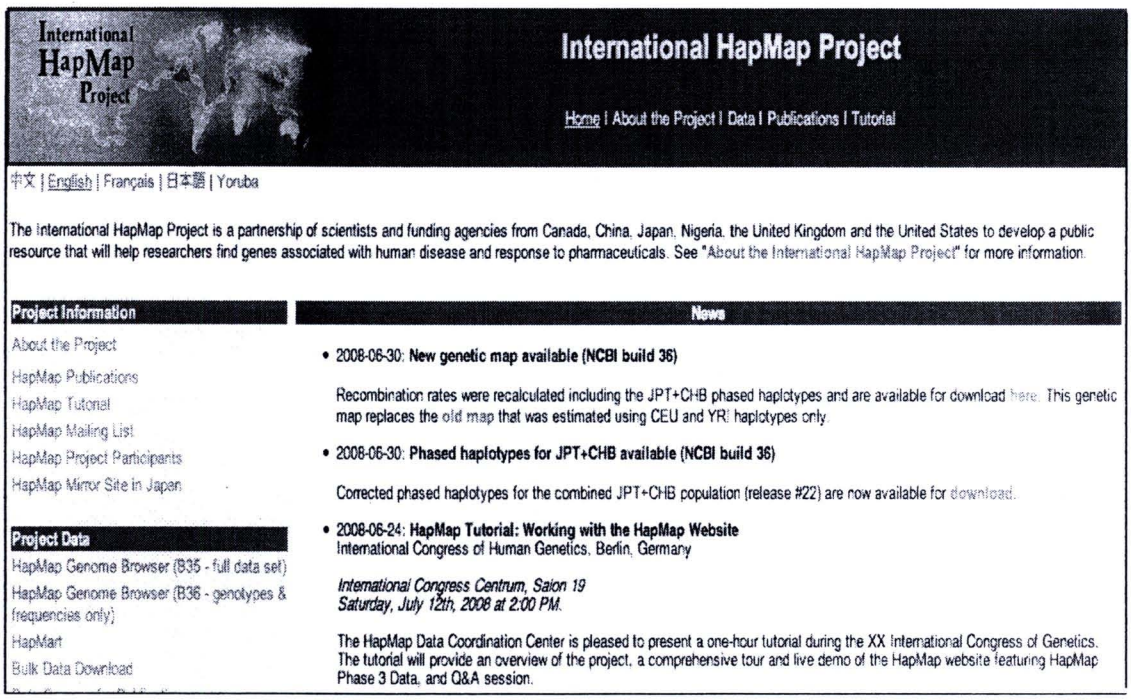

Figure 8 Surf the HapMap browser

Source: http://hapmap.ncbi.nlm.nih.gov/

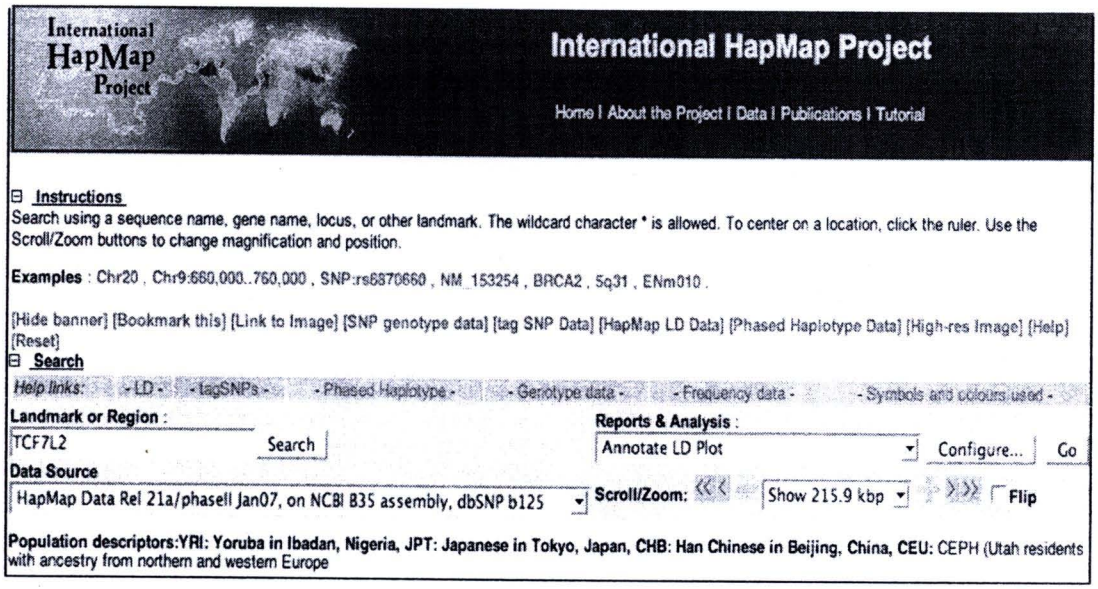

Figure 9 Searching for *PSEN 1*

Source: http://hapmap.ncbi.nlm.nih.gov/

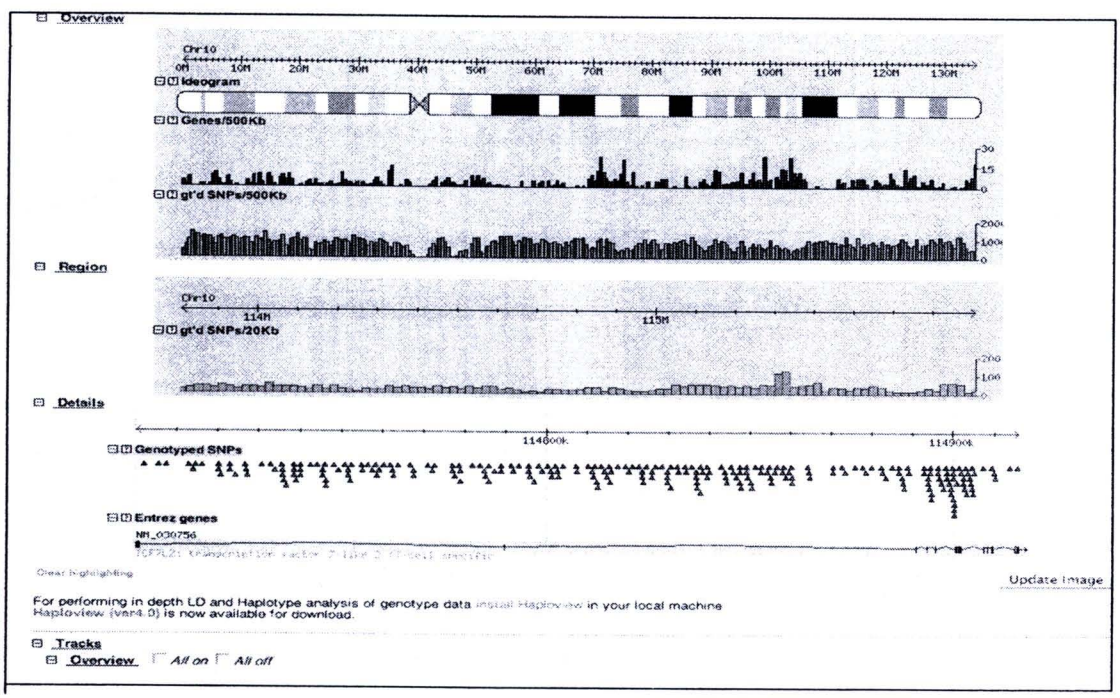

Figure 10 Examining region

Source: http://hapmap.ncbi.nlm.nih.gov/

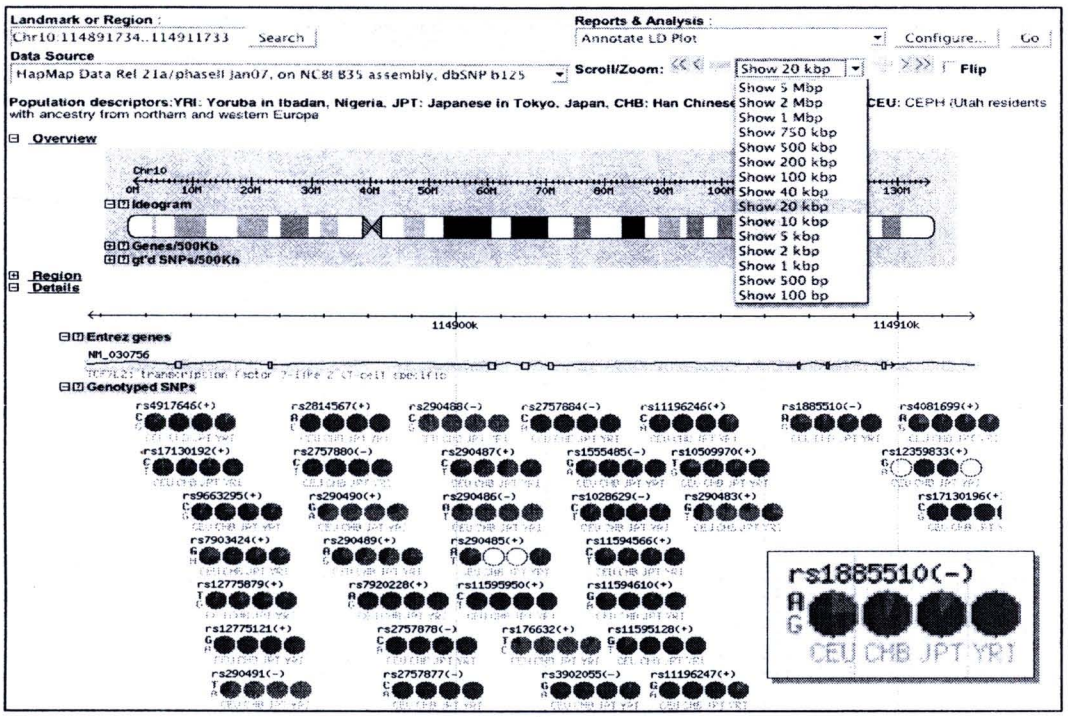

Figure 11 Turn on LD & Haplotype tracks

Source: http://hapmap.ncbi.nlm.nih.gov/

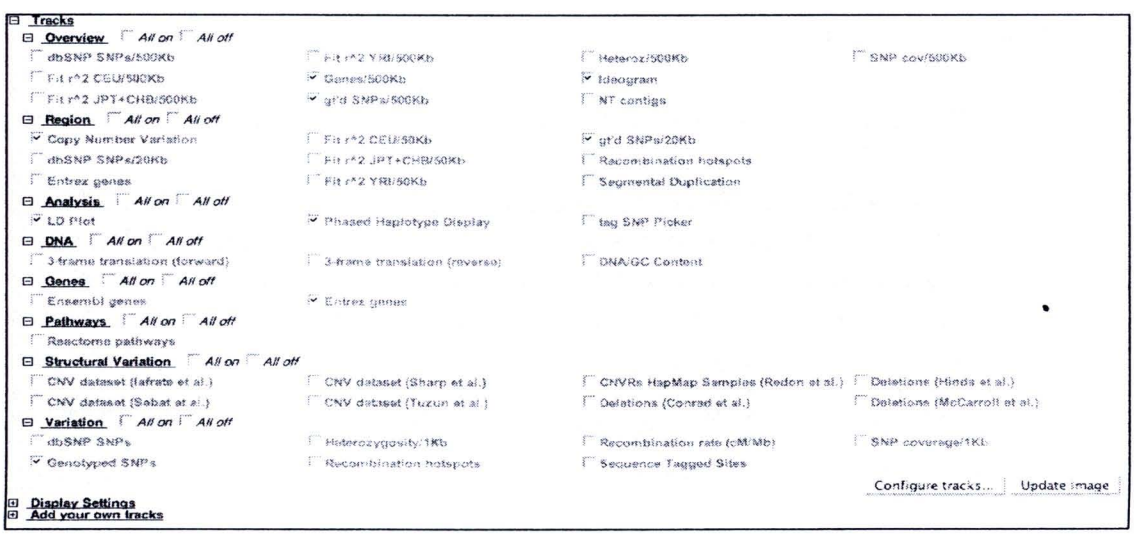

Figure 12 Viewing variation patterns

Source: http://hapmap.ncbi.nlm.nih.gov/

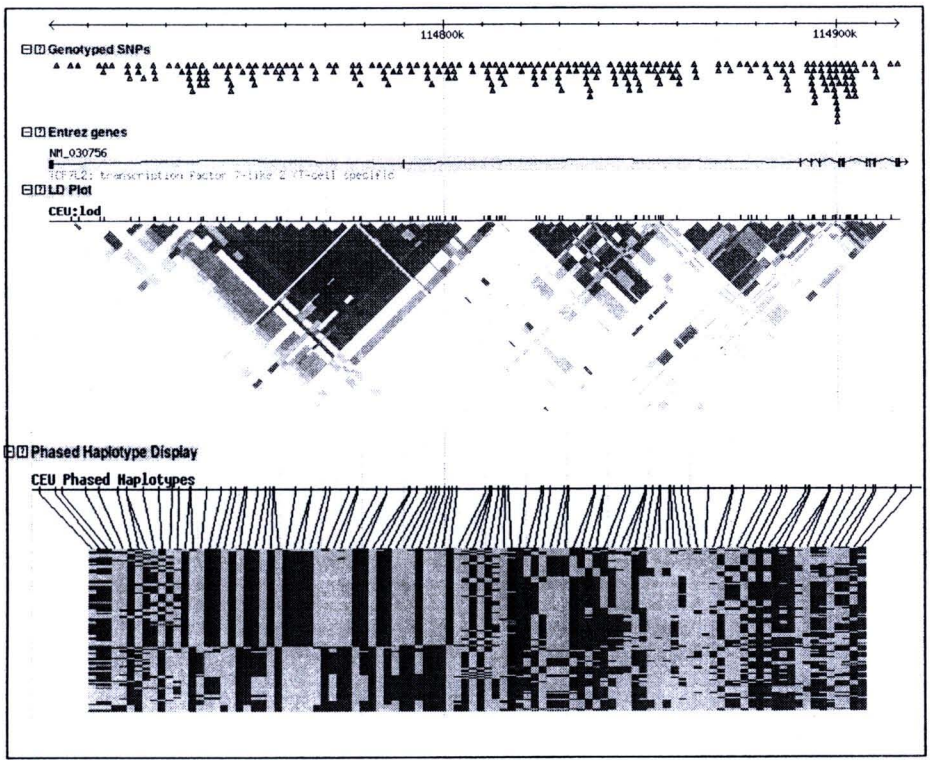

Figure 13 Adjust track settings

Source: http://hapmap.ncbi.nlm.nih.gov/

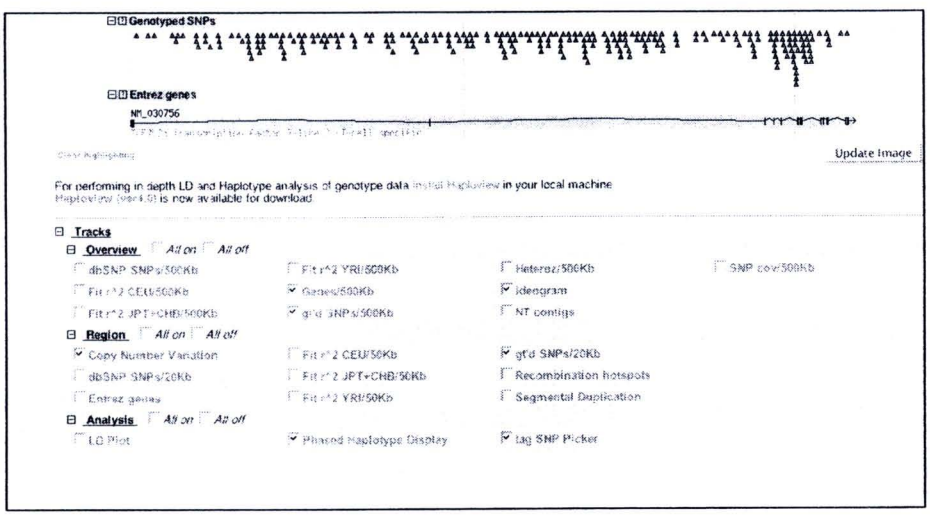

Figure 14 Turn on tag SNP track

Source: http://hapmap.ncbi.nlm.nih.gov/

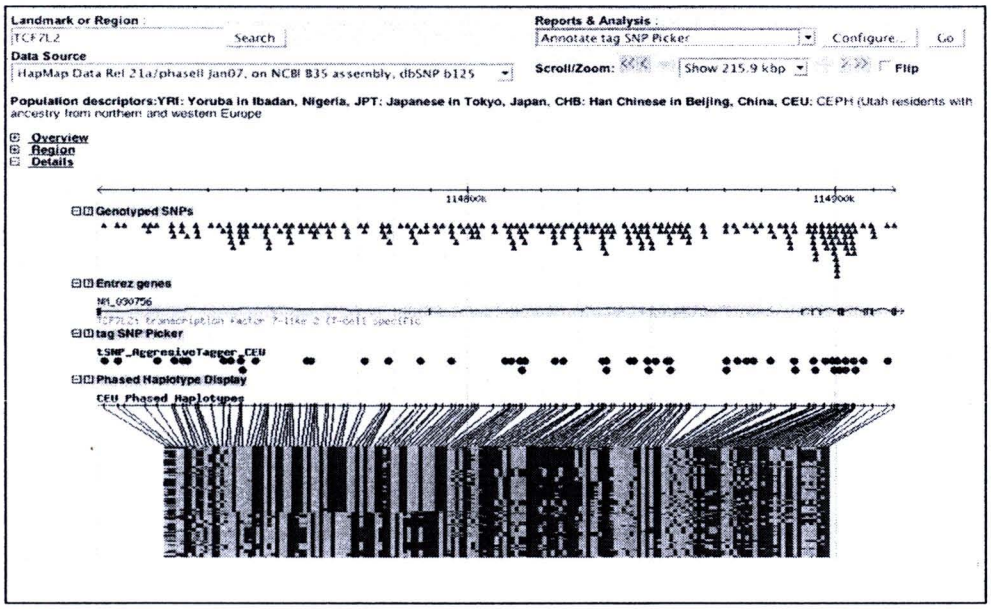

Figure 15 Adjust tag SNP picker

Source: http://hapmap.ncbi.nlm.nih.gov/

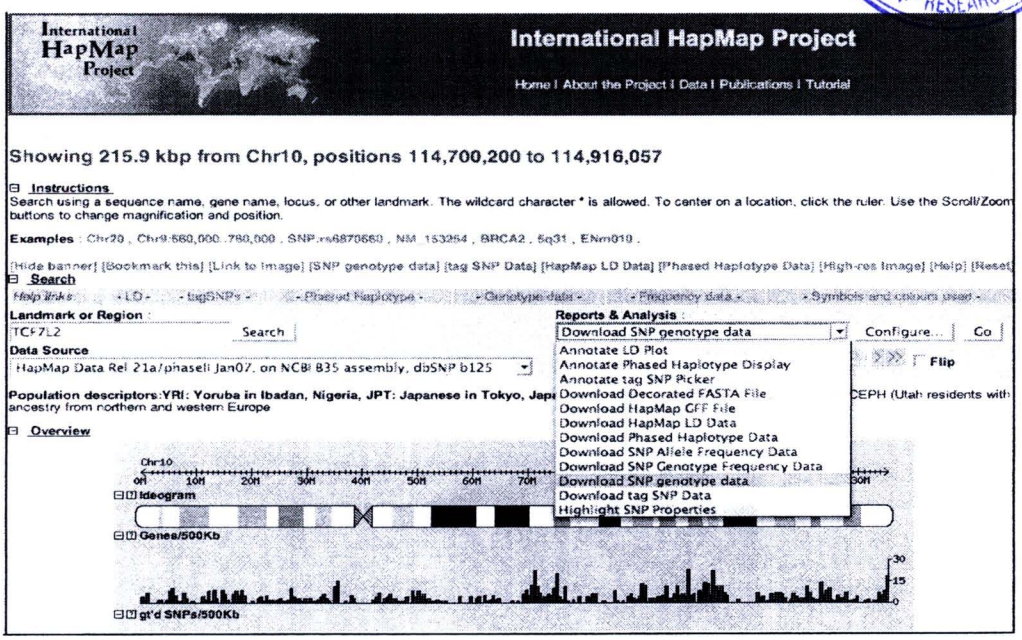

Figure 16 Generating reports

Source: http://hapmap.ncbi.nlm.nih.gov/

Sample size determination

Cases (AD patients) and controls were used with Quanto Version 1.2 developed by Jim Gauderman and John Morrison, 2007 for sample size determination. There are two alleles at the *PSEN1* locus, denoted 'Null' (allele G/A) and non-Null (allele G/G (ancestral allele)). It is thought that only those with the Null/Null(G/A) genotype are at increased risk for disease (Jim and John, 2007). The proportion of subjects in the population with the Null/Null genotype is estimated to be 38.9% (Table 1 identified rsSNPs of *PSEN1*). Assuming Hardy-Weinberg equilibrium, the prevalence of the Null allele in the population is then used to calculate frequency (prevalence) of the rs165933 in the population (Han Chinese from Beijing, China) = $38.9^{1/2}$ = 0.6237. The inheritance model is recessive, since only the heterozygous carrier is assumed to be increased risk. The relative risk for Null/Null carriers, compared to normal, is 2.0 (Rg). Desired power is 80%, at a significance level of 0.05 with a 2-sided alternate hypothesis. A matched case-control design was used. The steps for sample size determination using Quanto Version 1.2 are shown in Figures 17-24.

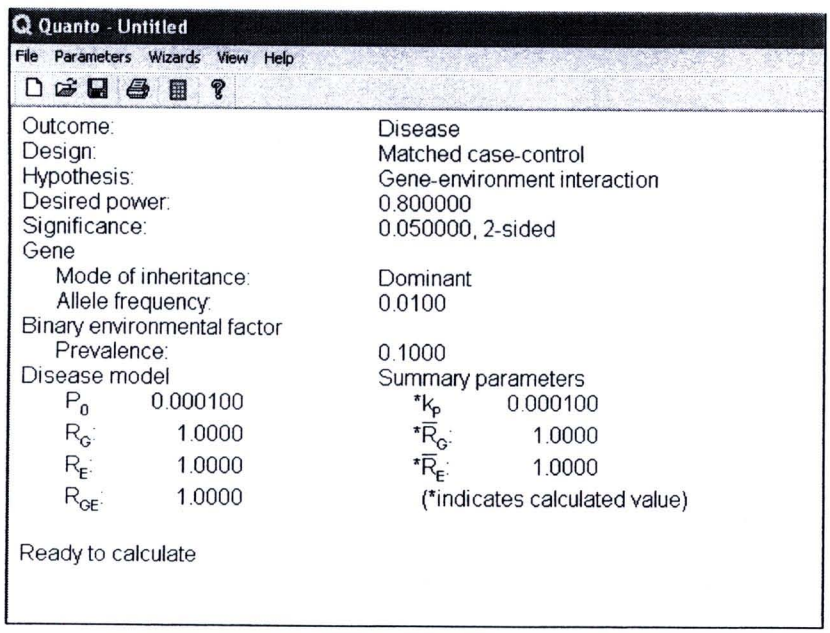

Figure 17 Quanto program

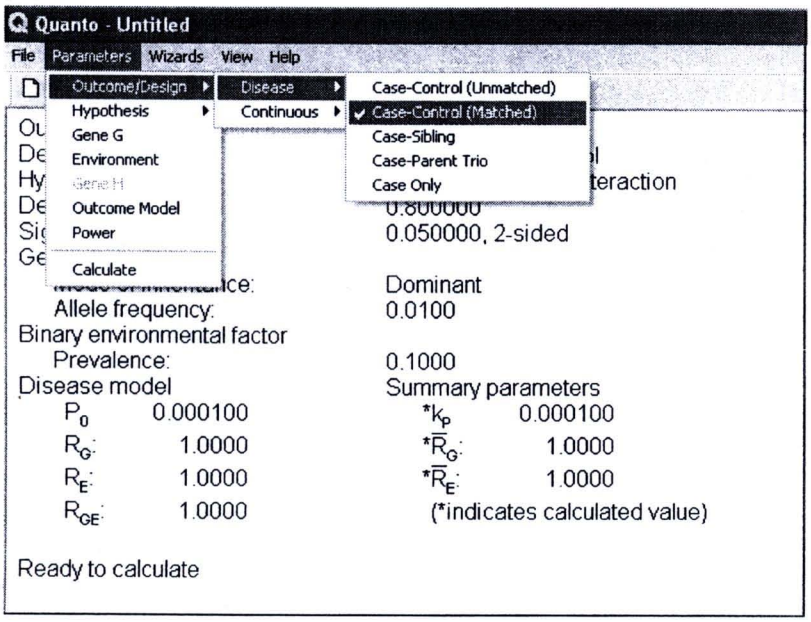

Figure 18 Selecting case-control (matched)

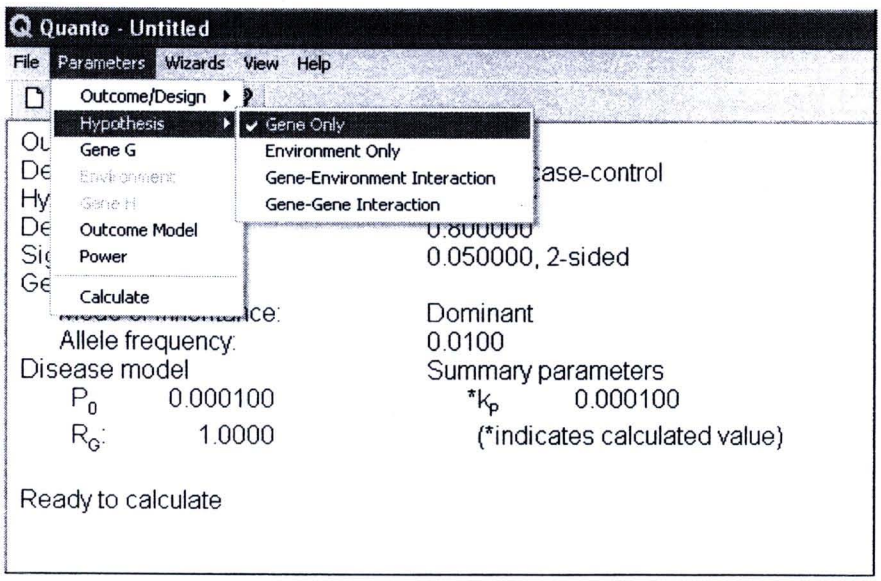

Figure 19 Selecting gene only

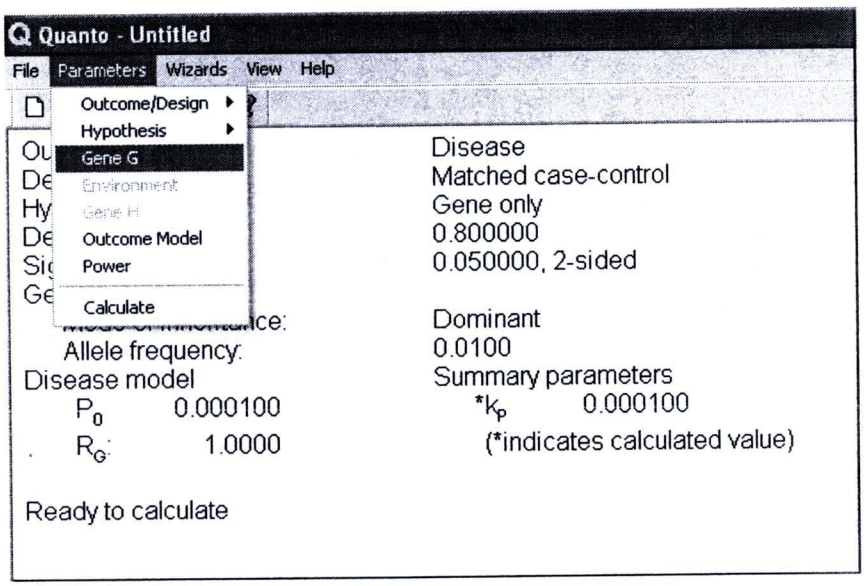

Figure 20 Selecting gene G

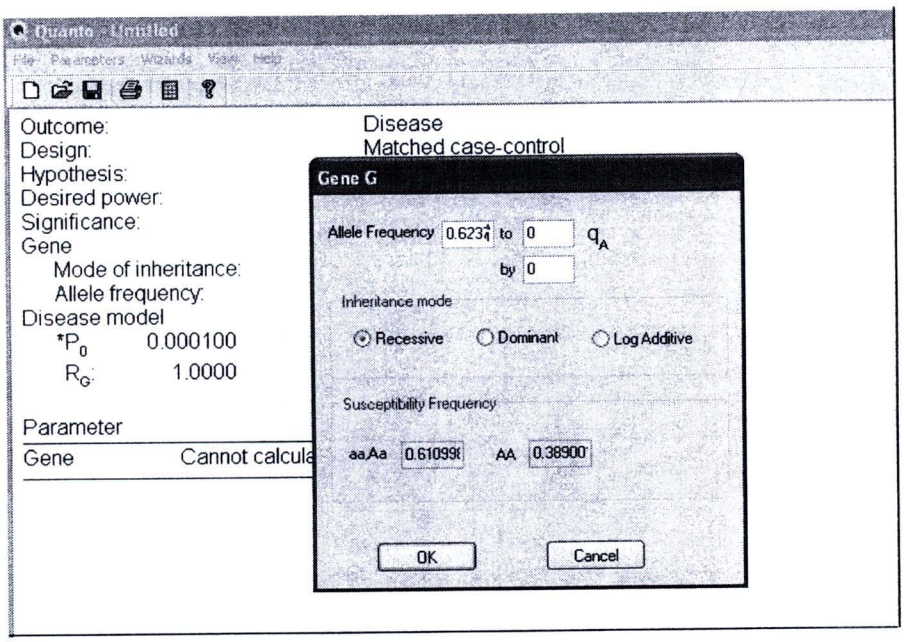

Figure 21 Put 0.6237 in allele frequency and click recessive

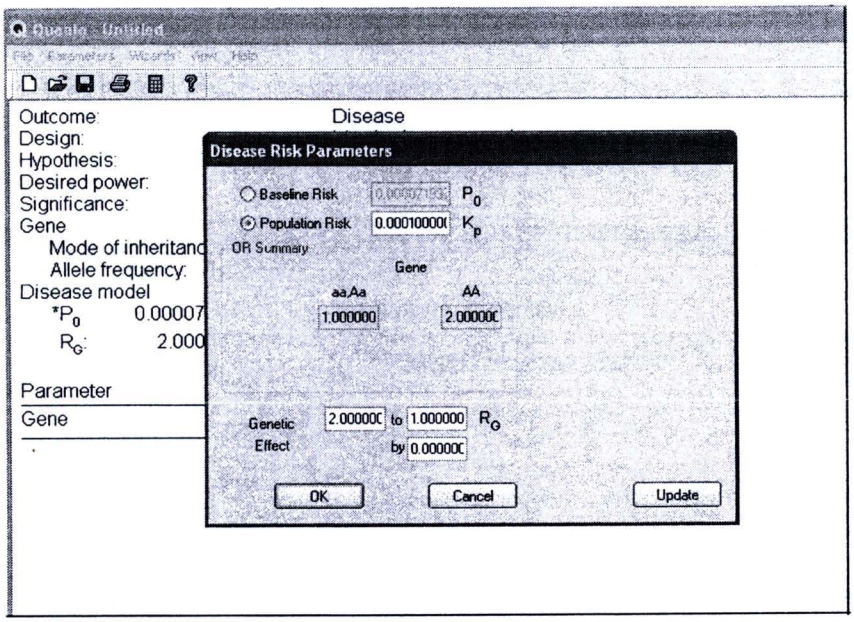

Figure 22 Click population risk and genetic is 2.00000 to 1.0000 (R_G)

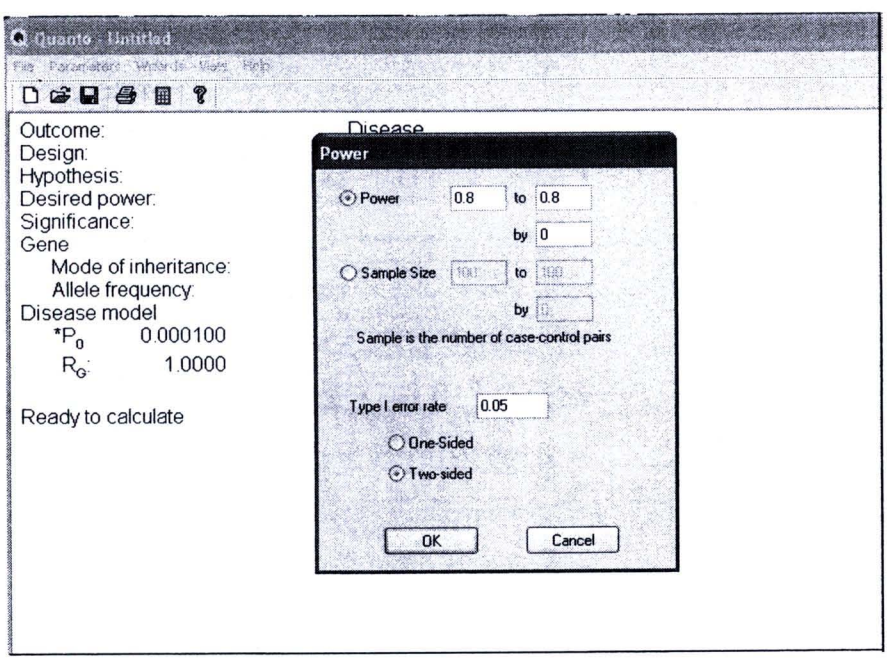

Figure 23 Put 0.8 to 0.8 in power and select two-sides

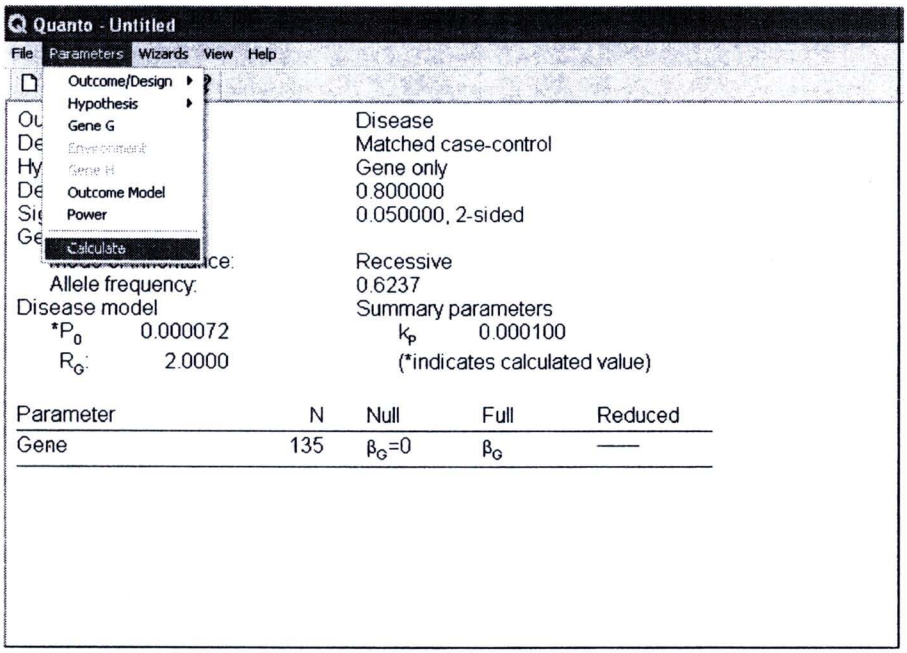

Figure 24 Select parameter and click calculate.

Note: N in figure 24 is number of sample (both AD patients and controls) suggested to be used in this project.

Alzheimer's disease patients and control subjects collection

Study sample comprised of AD patients and healthy controls. The AD patient subjects were recruited from Memorial Clinic, Chiangmai Neurological Hospital, Chiangmai, Thailand. A clinical diagnosis of probably AD established according to the criteria of the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's disease and Related Disorders Association (NINCDS-ADRDA) and Clinical Practice Guideline for Dementia (Prasat Neurological Institute, 2008). The control groups were selected from the hospital and they were confirmed healthy and neurologically normal by medical history, general examinations and Thai mini-mental state examination (TMSE). An informed consent to participate will be obtained from each subject. All patients with evidence of an autosomal dominant AD trait, or where a first degree relative had been diagnosed with familial AD, were excluded.

Primers design

The primers of DNA templates (rs362340, rs3025780, rs165932, rs10146743, rs214273 and rs165933) were designed (Figure 25) manually in the sense or antisense sequence, and check hairpins loops, self-annealing and melting temperatures (Tm) using Oligo Analyzer 1.0.3 (Teemu Kuulasma, 2001-2002), which is freely available on the internet. After that, the primers were investigated to be complemented with other sequences in the human genome by using PRIMER BLAST, which is available on the internet (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi.).

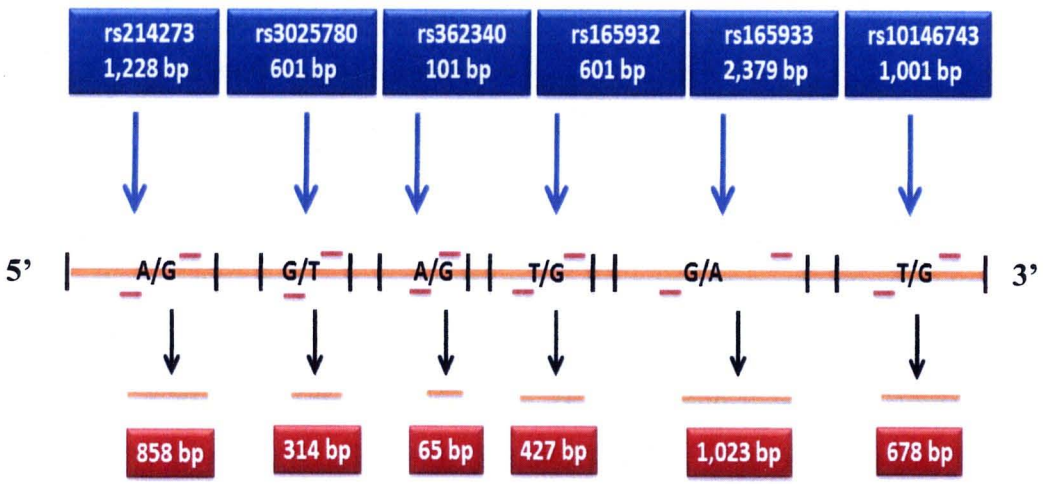

Figure 25 Primer design to specific with identified 6tagSNPs in *PSEN1*

DNA extraction

AD patient and control blood samples were extracted with Wizard® Genomic DNA Purification kit (Promega Corporation, USA). The 300 µl blood sample was added with 900µl of Cell lysis solution (Promega Corporation, USA) and inverted 5-6 times to mix. The reaction was incubated for 10 minutes at room temperature to lyse the red blood cells. After centrifugation at 14,000x g for 20 seconds at room temperature supernatant was removed and discarded as gently as possible without disturbing the visible white pellet. The 300 µl nuclei lysis solution (Promega Corporation, USA) was added to the well re-suspended and mixed by pipetting for 5-6 times. The 100 µl Protein Precipitation Solution (Promega Corporation, USA) was

added and vortexed vigorously for 10 - 20 seconds. The solution was added with 300 µl of room temperature isopropanol (SIGMA®, Germany) and gently mixed by inversion until the white thread-like strands of DNA form a visible mass. The solution was centrifuged at 14,000×g for 1 minute at room temperature. The supernatant was decanted. The 70% ethanol (Merck, Germany) was added to wash the DNA pellet. After centrifugation at 14,000×g for 1 minute at room temperature, the 70% ethanol was decanted and the DNA pellet was air-dried for 10-15 minutes. The 50 µl DNA rehydration solutions (Promega Corporation, USA) were added to the DNA pellet and the DNA was stored at 2-8 °C.

Polymerase chain reaction amplification

The PCR amplification in each identified tagSNPs in *PSEN1* was performed in a mixture containing; 5.0 µl of 10X PCR buffer minus Mg (invitrogen™, Brazil), 1.0 µl 10mM dNTP mixture (invitrogen™, Brazil), 1.5 µl of 50 mM $MgCl_2$ (invitrogen™, Brazil), 0.2 µl of 5U/µl Platinum taq DNA Polymerase (invitrogen™, Brazil), 40.3 distilled H_2O, 1.0 µl of 10 mM Forward (F_rs362340, F_rs3025780, F_rs165932, F_rs10146743, F_rs10146743, F_rs214273 and F_rs165933) and 1µl of 10 mM reverse primer (R_rs362340, R_rs3025780, R_rs165932, R_rs10146743, R_rs10146743, R_rs214273 and R_rs165933) were added each PCR reaction. Each DNA template and each forward and reverse primer were shown in Table 2.

Table 2 DNA templates, forward and reverse primers each in PCR reaction to amplify each identified tagSNPs in *PSEN1*

DNA templates	forward and reverse primer names
rs362340	F_rs362340
	R_rs362340
rs3025780	F_rs3025780
	R_rs3025780
rs165932	F_rs165932
	R_rs165932
rs10146743	F_rs10146743
	R_rs10146743
rs214273	F_rs214273
	R_rs214273
rs165933	F_rs165933
	R_rs165933

Note: F = forward, R = reverse, rs = reference SNPs

The PCR reaction was performed using PCR machine (Thermal Cycler, Model TC-25/H, BIOER Technology Co., Ltd., UK). The PCR condition program was shown in Table 3.

Table 3 PCR condition for 6 tagSNPs in *PSEN1*

Step	Temperature (°C)	Time
1 Activation of DNA polymerase	95	2 minutes
2 Denaturation	94	30 seconds
3 Annealing	55	30 seconds
4 Extension	72	1 minute
5 Go to step 2, 3 and 4 for 35 cycles		
6 Final extension	72	10 minutes
7 End of PCR	4	As long as needed

Agarose gel electrophoresis

The PCR products (rs362340, rs3025780, rs165932, rs10146743, rs214273 and rs165933) were analyzed in 2 % agarose gel electrophoresis. A 2 % agarose (GenePure LE Agarose, ICSBioEXPRESS®, USA) and 1.0XTBE were mixed and melted by heat and cooled down to approximately 60 °C before poured to a tray (A come was inserted and agarose gel was left to solidify). The gel was left to set before submerged in 1.0XTBE buffer filled in an electrophoresis chamber. The 1.0XTBE buffer was covered the agarose gel. Finally the comb was removed. Loading nucleic acid samples were mixed with one sample volume of 6X loading dye and loaded into the gel wells. Electrophoresis was carried out at 80 volts until the bromophenol blue front moved to approximately one-half of the gel. The electrophoresis gel was stained with an ethidium bromide (2.5 µg/ml) for 15 minutes and destained in distilled H_2O. Nucleic acid products were visualized under a ultraviolet transilluminator and photographed through a gel doc (Gene genius, Syngene, UK).

PCR reaction purification

The PCR products (rs362340, rs3025780, rs165932, rs10146743, rs214273 and rs165933) were purified with Wizard® SV Gel and PCR Clean-Up System (Promega Corporation, USA). An equal volume of membrane binding solution (Promega Corporation, USA) was added to the PCR products. The 700 µl Membrane Wash Solution (Promega Corporation, USA) (Ethanol added) was added followed by centrifugation at 16,000 x g for 1 minute. Flow through was discarded and mini-column (Promega Corporation, USA) was reinserted into collection tube (Promega Corporation, USA). The 500 µl Membrane Wash Solution (Ethanol added) was added and 16,000 x g for 1 minute was centrifuged. Flow through was discarded and Mini-column was reinserted into collection tube. The collection tube was empty. Mini-column was transferred to a clean 1.5 ml micro-centrifuge tube. The 50 µl Nuclease-Free Water (Promega Corporation, USA) was added to the mini-column and the reaction was incubated at room temperature for 1 minute and centrifuged at 16,000 x g for 1 minute, the mini-column was decanted and DNA was stored at 4 °C or -20 °C. The PCR products were now ready to be genotyped (DNA sequencing) with automated fluorescent DNA sequencing (Promega Corporation, USA).

Genotyping of identified tagSNPs using DNA sequencing technique

The purified PCR products (rs362340, rs3025780, rs165932, rs10146743, rs214273 and rs165933) were sequenced using 10µM forward primers (F_rs362340, F_rs3025780, F_rs165932, F_rs10146743, F_rs10146743, F_rs214273 and F_rs165933). The DNA sequencing was performed with 3730 DNA Analyzer (Applied biosystems™, USA) by Pacific Science Co., Ltd., Thailand.

Alignment of sequenced DNA with rsSNPs in *PSEN1*

All sequenced PCR products (rs362340, rs3025780, rs165932, rs10146743, rs214273 and rs165933) were aligned with rsSNPs using Bioedit sequence alignment Editor Program (Tom Hall, 1997-2011) to discover SNP in identified tagSNPs in *PSEN1*. Chromatograms were rechecked with Chromatogram Explorer version 3.1.1 (Heracle Software, 2010).

Statistical analysis

Genotype frequencies

Genotype frequencies used a percentage to calculate the percent of genotypes using Statistical Package for Social Sciences (SPSS).

Haplotype frequencies

Haplotype frequencies used a percentage to calculate the percent of haplotypes using Statistical Package for Social Sciences (SPSS).

The odds ratio

The odds ratio is a measure of effect size, describing the strength of association or non-independence between two alleles of genotyped tagSNPs in *PSEN1*. In this study, confidence intervals of odds ratio used the calculated confidence intervals of odds ratio in an unmatched case control study, for example groups of cases and controls studied, to assess a treatment or exposure to a suspected causal factor which is available on http://www.hutchon.net/ConfidOR.htm. (Martin and Douglas, 2004).