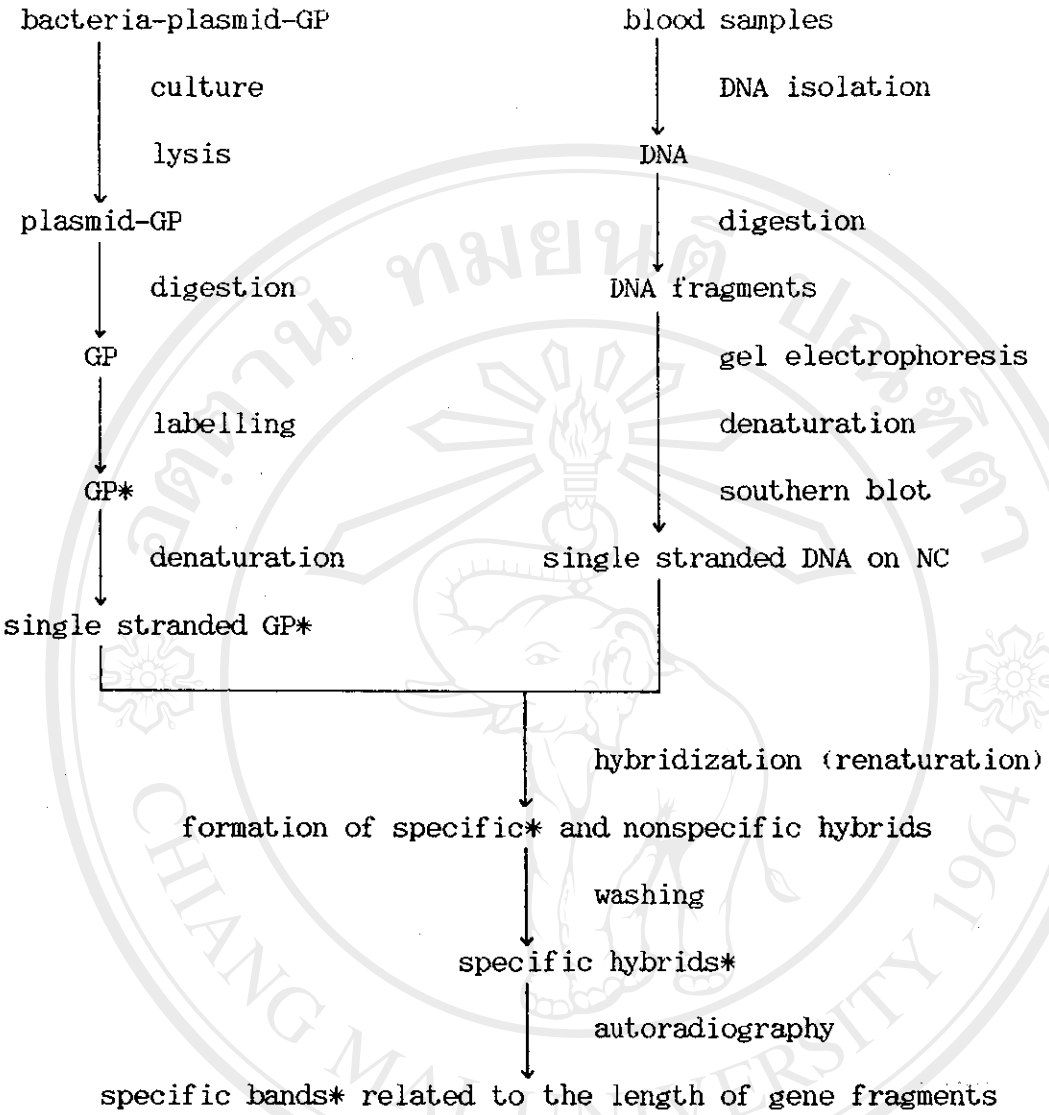
CHAPTER II

MATERIALS AND METHODS

The chemical reagents for all the procedures in this part were described in detail in the APPENDIX.

II.I. Procedure for the detection of deletional alpha-thalassemias

The restriction fragment length polymorphism (RFLP) was used as a marker in the DNA hybridization technique. DNA samples were hybridized with specific gene probes or specific DNA sequences. The whole process can be summarized as shown in the diagram on page 23



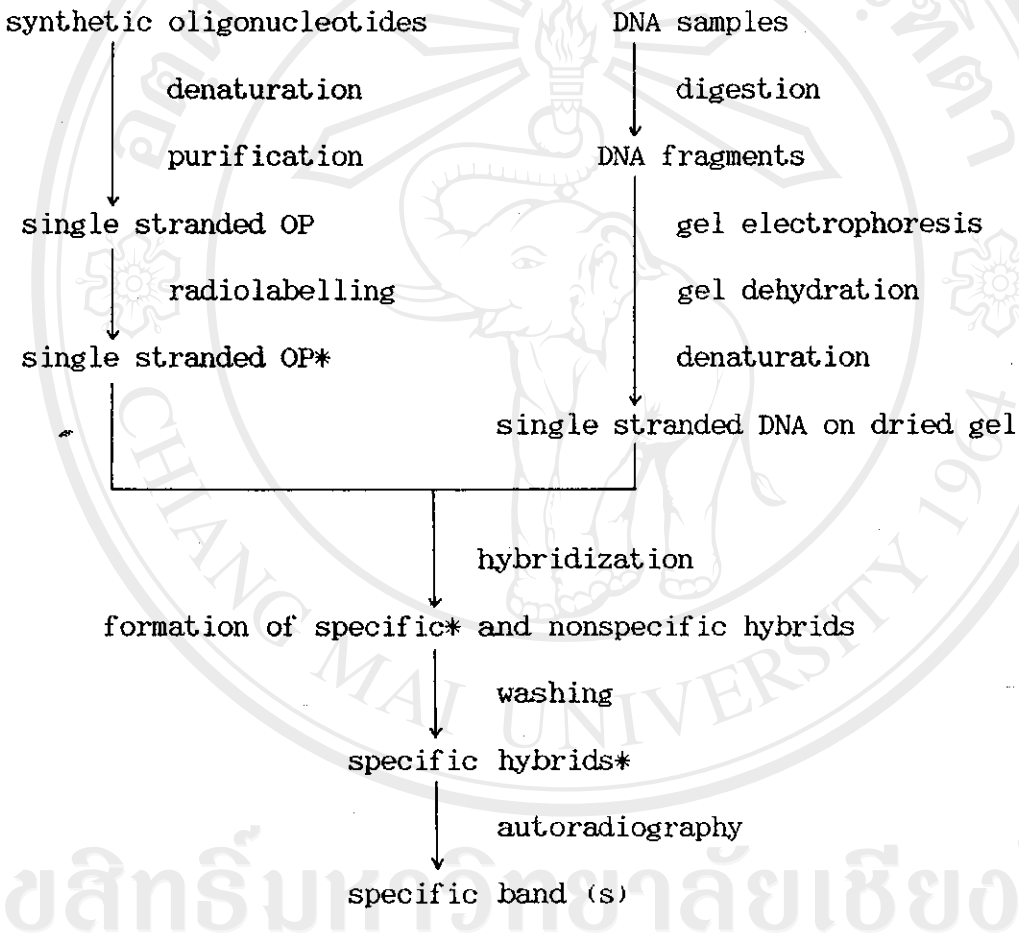
GP = gene probe or specific DNA sequence probe

NC = nitrocellulose membrane

* = radioactive labelling

II.2 Procedure for the detection of nondeletional alpha-thalassemia

The difference of duplex stability between a single base mismatch and a perfect match is the principle of the oligonucleotide hybridization technique. DNA samples were hybridized with allele specific oligonucleotide probes. The process may be summarized as follows:



OP = oligonucleotide probes

* = radioactive labelling

II.3 METHODS

II.3.1. Collection of blood samples

Venous blood samples of 3-5 ml were collected in 1 % EDTA anti-coagulant from 112 healthy males,aged 21 years, from four districts of Chiang Mai province who presented themselves for the annual military conscription examination. There were 19 samples from Jom Tong, 49 samples from Smeang, 19 samples from Mae Tang and 25 samples from Prou districts. The blood samples were transferred in the frozen state to Humangenetic laboratory of the Medizinische Hochshule Hannover,Federal Republic of Germany.

II.3.2. Isolation of DNA

The frozen blood was thawed at room temperature for 30 min, and the DNA was isolated by phenol/chloroform extraction. An aliquot of 27 ml of cold lysis buffer was added to 3.0 ml of blood and stored 10 min on ice to lyse the blood cells.The nuclei were pelleted by centrifugation with a refrigerated centrifuge (Heraeus ZETA 20)at 10,000 rpm for 10 min. The supernatant was decanted to the utmost extent. The pellet of nuclei was suspended in 9.2 ml of 1x STE with a Vortex, then 0.5 ml of 10 % SDS was added while the suspension was gently swirled. After the addition of 100 ul proteinase-K solution (20 mg/ml 1x STE, fresh preparation) the mixture was incubated overnight at 55°C. If the

the mixture was not clear after this step,a larger amount of proteinase -K and a longer incubation period were required. The mixture was extracted by adding 10 ml of 80 % phenol and chloroform and strong shaking for 6 min at room temperature. Then the aqueous layer was separated from the organic layer by centrifugation at 3,000 rpm for 6 min (Heraeus UJIIIE). The phenol-chloroform layer (the lower layer) was removed using a Pasteur pipette. The aqueous and interphase layer was then extracted twice with phenol/chloroform in the same manner,and at last the residual phenol was removed by extracting once with 10 ml of chloroform. After removing the chloroform layer, the borderline between aqueous and interphase layer was sharpened by centrifugation at 3,000 rpm for 10 min.Then the aqueous layer was transferred to a new beaker and DNA was precipitated by adding 1/10 volume of 3.0 M NaOAc and 2.5 volume of cold absolute ethanol (-70°C). The mixture was gently swirled and kept at room temperature for 30 min. A white web of DNA appeared at this stage. The isolated DNA was transferred to a new sterile Eppendorf tube and washed three times with 80 % ethanol,then once with absolute ethanol.The ethanol was evaporated at 37°C in the Thermostat 5320 (Eppendorf) for 30 min. The dried DNA was redissolved overnight in 0.1- 0.5 ml of sterile water at 4°C. The solution was adjusted with 10xTE to 1xTE buffer.After thorough mixing, 30 ul of the DNA solution was diluted with 970 ul of water.The DNA concentration in this solution was then estimated and the quality was checked by the determination of its absorbance at 260 and 280 nm. The A260/A280 ratio of the DNA solution should be 1.75-1.95. Finally, the DNA solution was diluted with 1x TE buffer to a concentration between 0.4 - 1.0 ug/ul.

II.3.3 Restriction Endonuclease Digestion

The DNA samples were digested with the restriction endonucleases Bam HI, Bgl II,Hind III,Rsa I and Apa I (Bethesda Research Laboratories, USA)

For Bam HI, Bgl II, Hind III or Rsa I enzyme, the reaction mixture contained 3.0 ug DNA, 1.6 ul of 10x reaction buffer, 10 units of enzyme and sterile water to make up the end volume of 16.0 ul.The mixture was mixed well and incubated at 37°C for 2-3 hr. When the DNA was completely digested, 4.0 ul of 5x loading buffer was added to stop the activity of the restriction enzyme.

With Apa I, a 50 ul the cleavage was performed in a sterile Eppendorf tube containing 5.0 ug DNA, 5.0 ul 10x reaction buffer, 30 units of enzyme (added twice, 15 units each time). The cleavage assay was mixed well and incubated at 30°C for 1 hr. After the second addition of enzyme, the mixture was incubated for 4 hr. Then the digested DNA was precipitated with ethanol and redissolved in 16.0 ul sterile water at 4°C for 2 days. After that 4.0 ul of loading buffer were added.

For the detection of Hb Constant Spring, 10.0 ug DNA was digested with Rsa I enzyme in a volume of 60.0 ul containing 10.0 ug DNA, 6.0 ul of 10x reaction buffer, 35 units of Rsa I enzyme (20 ul at the beginning and 15 ul after 1 hr incubation). After ethanol precipitation, the digested DNA was washed and dried at room temperature

overnight and redissolved in 10.0 ul of sterile water at 4°C for one week. An amount of 2.0 ul of 5x loading buffer was added before the gel electrophoresis.

II.3.4. Gel electrophoresis

The comb teeth used in the study were 1.0 mm thick and 4-5.0 mm wide, the mold was 15.0 cm long and 20.0 cm wide.

For the detection of deletional types of alpha-thalassemia, a 0.8 % agarose gel was prepared by boiling a suitable amount of agarose (BRL, Electrophoresis grade) in deionized water (180ml for 4.0, or 150 ml for 5.0 mm comb width, respectively). After the gel solution had cooled down to 55 - 60°C, it was poured into the mold. The comb was placed 1.0 mm over the bottom of the mold, then the solution was kept at room temperature for 30 min. The digested DNA and a Hind III lambda-DNA marker were applied to each slot in submarine fashion. The gel was electrophoresed overnight (16-18 hr) in Tris-acetate buffer in horizon tal direction at 4°C applying 200 mA. DNA was stained with ethidium bromide (1.0 mg/l Tris-acetate buffer) for 3 min and examined under a UV lamp with a wave length of 254 nm (Desaga).

For the detection of Hb Constant-Spring, the concentration of the agarose gel was 1 % instead of 0.8 %, and a 70°C gel solution was filtrated through a 0.45 um nitrocellulose filter (Sartorius) before it was poured into the mold. After electrophoresis, the gel was cut at the

corner to mark the order of samples, stained with ethidium bromide and examined under UV light. The DNA was fixed on the gel by soaking it overnight in absolute methanol with gentle shaking at room temperature. Then the gel was placed between two pieces of Whatman paper and dehydrated in a gel drying machine with a vacuum system at 30°C for 2-3 hr. This dried gel could be kept at room temperature for a year.

II.3.5. Southern blotting

For the detection of deletional types of alpha-thalassemia after gel electrophoresis, the DNA sample was transferred to a nitrocellulose membrane using the Southern blotting technique,modified from the method described by Southern (29).The gel was marked at one corner and cut 20x10 cm in size. Then the DNA on the gel was denatured by soaking the gel in a denaturation solution with gentle shaking for 40 min and application of a neutralization solution for 60 min. In the meantime,the Southern blotting apparatus was set up as shown in Figure II.1. The tray was filled with 20x SSC solution,some 20xSSC was poured on the supporter and a piece of Whatman paper (No.3) measuring 20x30 cm was placed on it with two ends being dipped into 20xSSC solution. This paper functioned as a bridge. Another piece of Whatman paper was cut to the same size as the supporter (20x20 cm) and placed on the bridge which had been moistened with 20xSSC. Air bubbles between paper and bridge were carefully removed. A piece of nitrocellulose membrane and 5 pieces of Whatman paper were cut to the same size as the

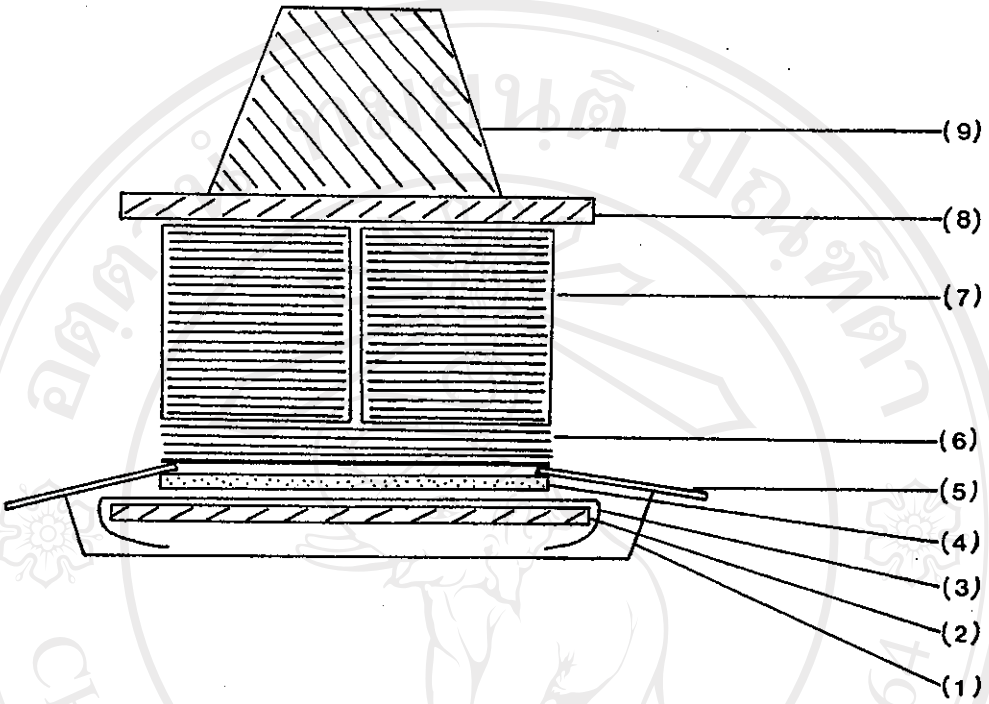


Figure II.1 Cross section of a Southern blot apparatus.

(1) tray filled with 20xSSC

(2) supporter

(3) wick = two sheets of Whatman paper

(4) gel

(5) cellophane pieces

(6) five sheets of Whatman paper and one sheet of nitrocellulose membrane

(7) a stack of toilet paper

(8) a glass plate

(9) weight = 500 gm

gel piece (20x10 cm)and soaked in 2xSSC for 10-15 min. After neutralization,the gel was soaked in 2xSSC for 5 min and placed on the supporter; all four edges of the gel were covered with cellophane. The nitrocellulose membrane was exactly positioned on the gel, followed by five pieces of Whatman paper, a stack of toilet paper, a glass plate and a weight. In every step, it was made certain that no air bubbles were trapped. The Southern blotting was carried on for 24-72 hr at room temperature with several changes of toilet paper and refillings of 20xSSC in the tray.

After reassembly of the blot, one corner of the nitrocellulose membrane was cut. The membrane was soaked in 2xSSC for 20 min and then dried at room temperature for 30 min.The DNA samples side at the corner was marked and the dried nitrocellulose membrane was placed between two pieces of Whatman paper, wrapped with aluminium foil and baked at 80°C in a vacuum oven (Heraeus) for 2 hr. This baked nitrocellulose membrane could be kept at room temperature for a year.

II.3.6. Preparation of the probe

For the detection of deletional alpha-thalassemias, three kinds of DNA specific probes were used in the study.

II.3.6.1 Alpha-globin gene probe: The alpha$_2$globin specific fragment was cloned into the Pst I cleavage site of the pEMBL vector.This vector contains the Ampicillin resistance sequence (Figure II.2). The 1.5 kb fragment of this probe could be separated from the 4.1 kb pEMBL

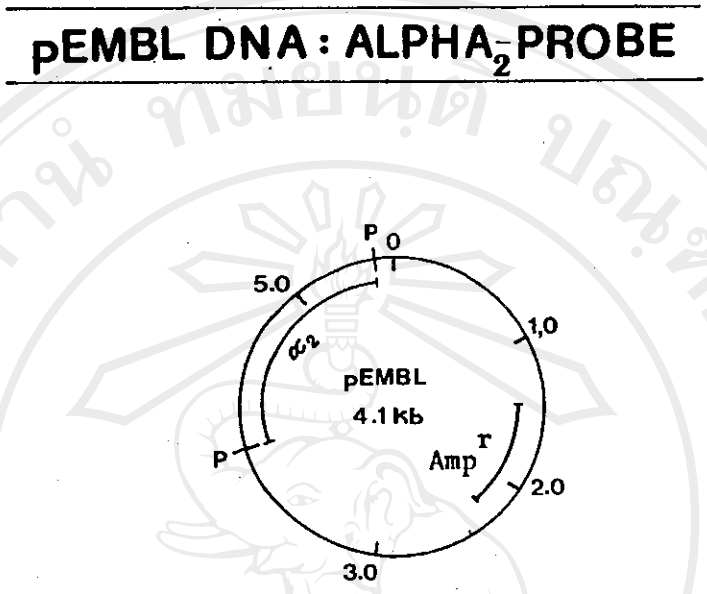


Figure II.2 The recombinant plasmid contain alpha$_2$ probe.

The alpha$_2$ globin specific fragment (1.5 kb) was cloned into the Pst I cleavage site of pEMBL vector.

P = Pst I cleavage site.

Amp^r = Ampicillin resistence sequences.

vector by cleavage with Pst I and isolated by gel electrophoresis.This DNA fragment was suitable for the detection of alpha-thal-2.

II.3.6.2 Zeta globin gene probe: The zeta globin specific fragment was cloned into the Pst I/Hind III cleavage sites of the pEMBL vector. As mentioned above, the vector contains the Ampicillin resistance sequence (Figure II.3).The 400 bp fragment of this probe was separated by cleavage with Pst I and Hind III. This DNA fragment was suitable for the detection of alpha-thal-1.

II.3.6.3 Lo probe: The 410 bp 6.8 kb Bam HI fragment upstream of the zeta gene was cloned into the Bam HI/Eco RI cleavage sites of the pUC13 vector. This vector also contains the Ampicillin resistance sequence (Figure II.4). The 410 bp of this probe were separated from the 2.73 kb pUC13 vector by cleavage with Bam HI and Eco RI. This DNA fragment was suitable for the detection of alpha-thal-1 due to a large deletion involving the entire zeta alpha globin gene complex.

The three recombinant plasmids were used to transform E. coli LK111.

II.3.6.4 For the detection of the Hb Constant Spring mutation two 19 mer oligonucleotide probes were used. One was complementary to the normal sequence (CS^{nml} = 5' CTCCAGCTTAA*CGGTATTT 3') and the other to the mutant sequence (CS^{mut} = 5'AAATACCGTC*AAGCTGGAG 3') (Figure II.5).

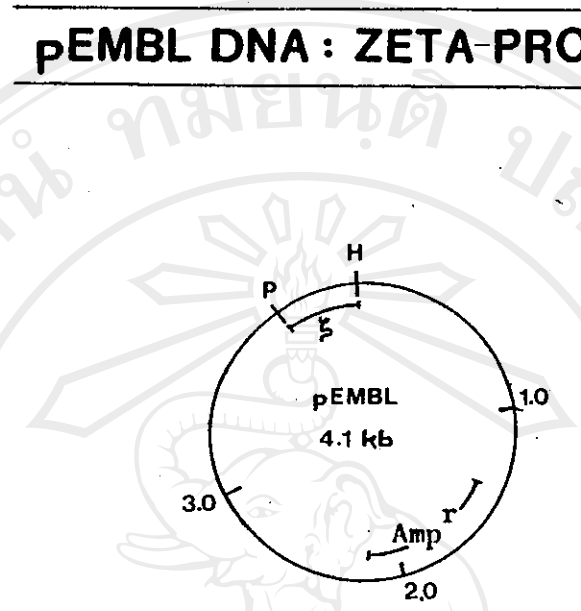


Figure II.3 The recombinant plasmid contain zeta probe.

The zeta globin specific fragment (400 bp) was cloned into the Pst I/Hind III sites of pEMBL vector.

P = Pst I cleavage site.

H = Hind III cleavage site.

Amp^r = Ampicillin resistence sequences.

pUC 13 DNA : Lo-PROBE

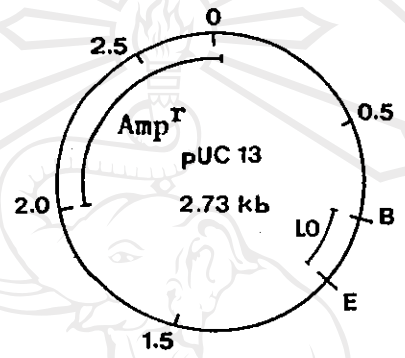


Figure II.4 The recombinant plasmid contain LO probe.

The 6.8 kb Bam HI fragment upstream of zeta globin gene (410 bp) was cloned into the Bam HI/Eco RI cleavage sites of pUC13 vector.

B = Bam HI cleavage site.

E = Eco RI cleavage site.

Amp^r = Ampicillin resistence sequences.

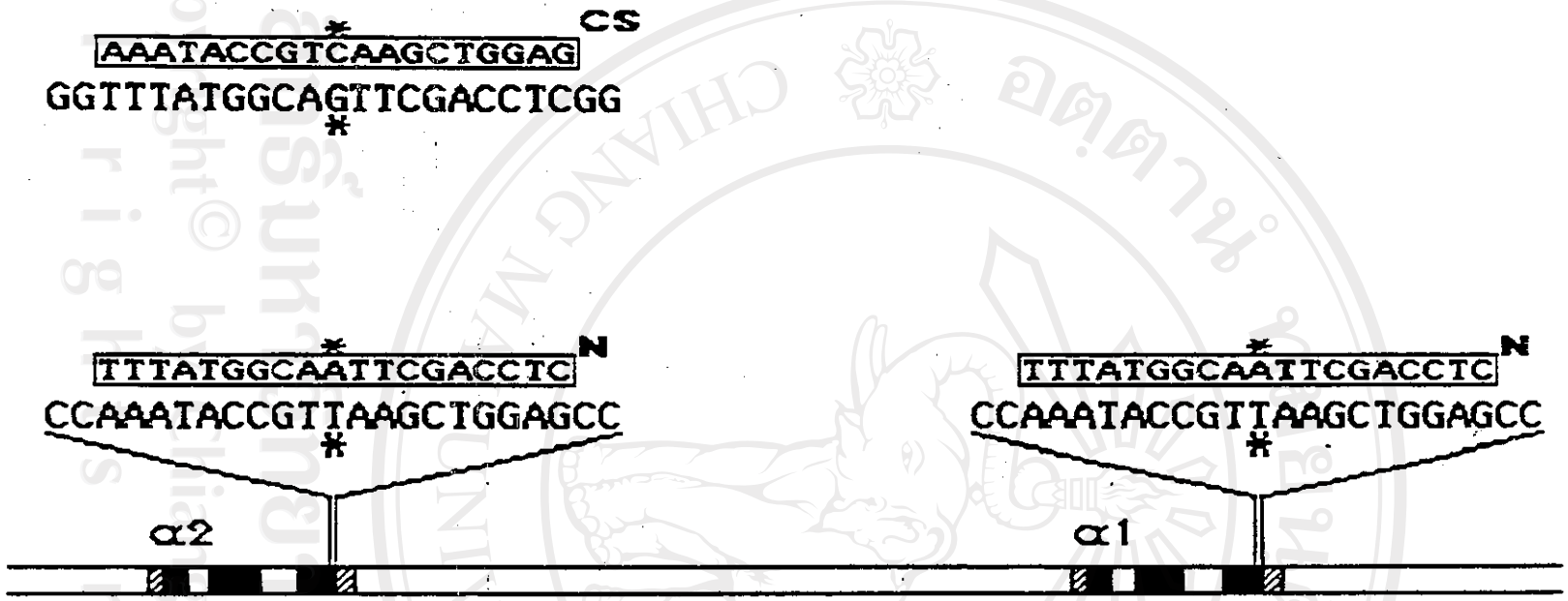


Figure II.5 Detailed comparison of alpha$_1$ and alpha$_2$ gene at the determination codon.

CS = Constant Spring sequences.

N = normal sequences.

II.3.7. Isolation and purification of probes

The procedure for isolation and purification of the probes was as follows:

II.3.7.1 Amplification of plasmid DNA: A single colony of transformed E. coli LK111 was inoculated in 25 ml of selective LB-broth, and incubated at 37°C overnight. Then 20 ml of the well grown bacteria was transferred to one litre of fresh selective LB-broth and the incubation was continued until the culture reached the late log phase (OD_{600} = 0.6-0.8). Then 200 ug/ml of Chloramphenicol was added to amplify the amount of plasmid DNA. The culture was incubated overnight at 37°C with vigorous shaking.

II.3.7.2 Isolation of plasmid DNA: The E.coli cells were harvested by centrifugation at 5,000 rpm for 15 min and washed with glucose buffer. After resuspension in 24 ml of glucose buffer, 4.0 ml of lysozyme solution (20 mg/ml glucose buffer) were added. The solution was mixed well and kept at room temperature for 10 min. After adding 55.2 ml of 1.0 % SDS solution with gentle swirling, the mixture was immediatly placed on ice for 5 min. Protein, high molecular weight DNA and RNA were precipitated by adding 28 ml of 5.0 M potassium acetate and storage for 15 min on ice. The pellets were removed by centrifugation with in a refrigerated centrifuge at 10,000 rpm for 30 min and filtrated through glass wool. The plasmid DNA in the supernatant was

precipitated by adding 0.6 volume of isopropanol (Riedel de Haen) and stored overnight at 4°C. The plasmid DNA was harvested by centrifugation at 10,000 rpm and 4°C for 30 min and washed three times with 5.0 ml of 80% ethanol.Then the pellet was spread out and dried at 37°C for 4 hr. The plasmid DNA was redissolved in 1.0-2.0 ml sterile water at room temperature overnight.

II.3.7.3 Purification of plasmid DNA: The plasmid DNA was purified by CsCl-ethidium bromide gradient centrifugation ($\underline{n}$ = 1.4080). For every 1.0 ml of plasmid DNA solution, 1.0 gm CsCl was added and gently mixed. The RNA precipitate was removed by centrifugation at 3,000 rpm for 15 min. The supernatant was transferred to a polyallomer tube, ethidium bromide (10 mg/ml 1x TE) was added (80 ul per ml supernatant) and mixed well.The tubes were balanced by filling the remaining space with paraffin oil and centrifuged in a Sorvall ultracentrifuge (DUPONT OTD 55 B) at 45,000 rpm and 20°C for 40 hr. The bands of plasmid DNA were examined under UV light (Figure II.6). The upper band consisted of linear and open circular plasmid DNA and the lower one of supercoiled plasmid DNA. RNA accumulated at the bottom of the tube. The bands of plasmid DNA were collected by suction with a 20 gauge needle and precipitated with 3.0 M sodium acetate and cold absolute ethanol. After overnight storage at -70°C the plasmid DNA was harvested by centrifugation at 10,000 rpm and 4°C for 30 min, followed by washings with 80% ethanol and drying at 37°C. The dried plasmid DNA was redissolved in sterile water overnight, and the concentration of plasmid DNA was determined at 260 nm.

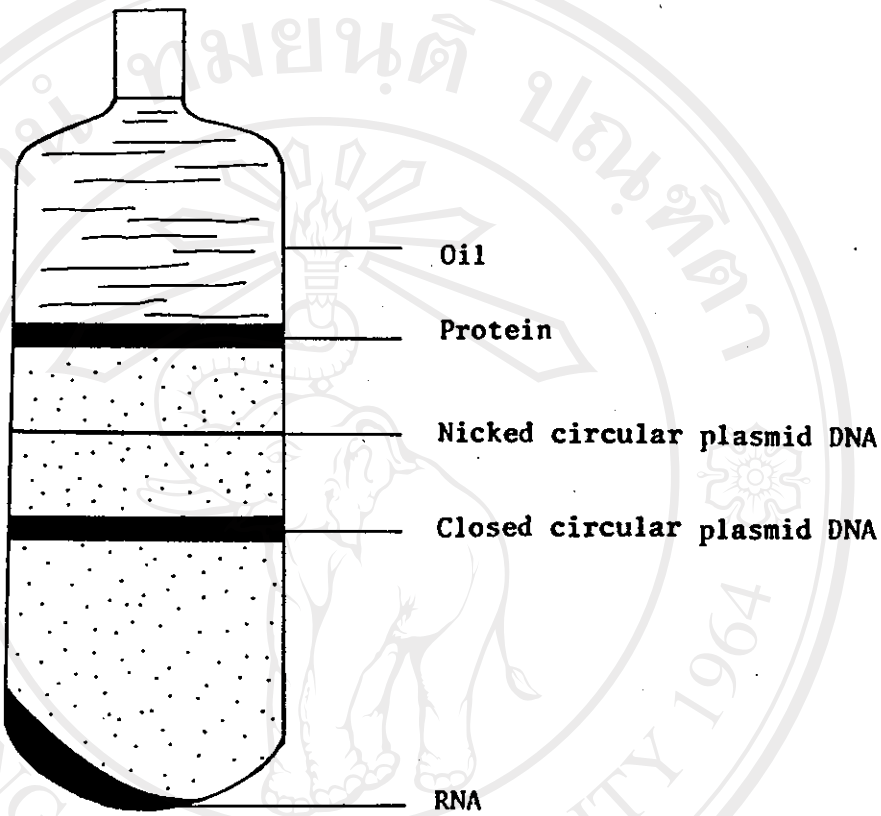


Figure II.6 Diagram showing the purification of the plasmid DNA by centrifugation to equillibrium in cesium chloride-ethidium bromide gradients.

II.3.7.4 Isolation of DNA specific probe: The DNA specific probe was isolated from the plasmid DNA by cleavage with specific restriction endonucleases and gel electrophoresis.

alpha globin gene probe : cleavage with Pst I

zeta globin gene probe : cleavage with Pst I and Hind III

LO probe : cleavage with Bam HI and Eco RI

The assay was performed in a volume of 360 ul in a sterile Eppendorf tube.The amount of 200 ug plasmid DNA,36 ul of 10x reaction buffer and 60 units of restriction enzyme were incubated at 37°C for 5 hr before adding 40 ul 5xloading buffer. A 1% agarose gel was prepared in a 10x15 cm mold with 7 mm thickness,and a 7 cm long slot for the cleavage assay and a 5 mm long slot for the Hind III lambda DNA marker was made. After loading, gel was electrophoresed at 200 mA and 4°C overnight. The bands of the specific probe were detected by ethidium bromide staining. The bands of interested were cut with a sharp scalpel and the DNA specific probe was eluted using the electroelution technique (Figure II.7).

Electroelution technique: A 9 mm thick 1.2% agarose gel was prepared in a 10x10 cm mold. In the middle, a 2x cm trough was cut with a sharp scalpel and paved with dialysis membrane. The gel slice with the DNA specific probe was placed in the trough attached to the anode. After filling the trough with Tris-acetate buffer, the gel was electrophoresed at 395 mA and 4°C until the specific probe was completely

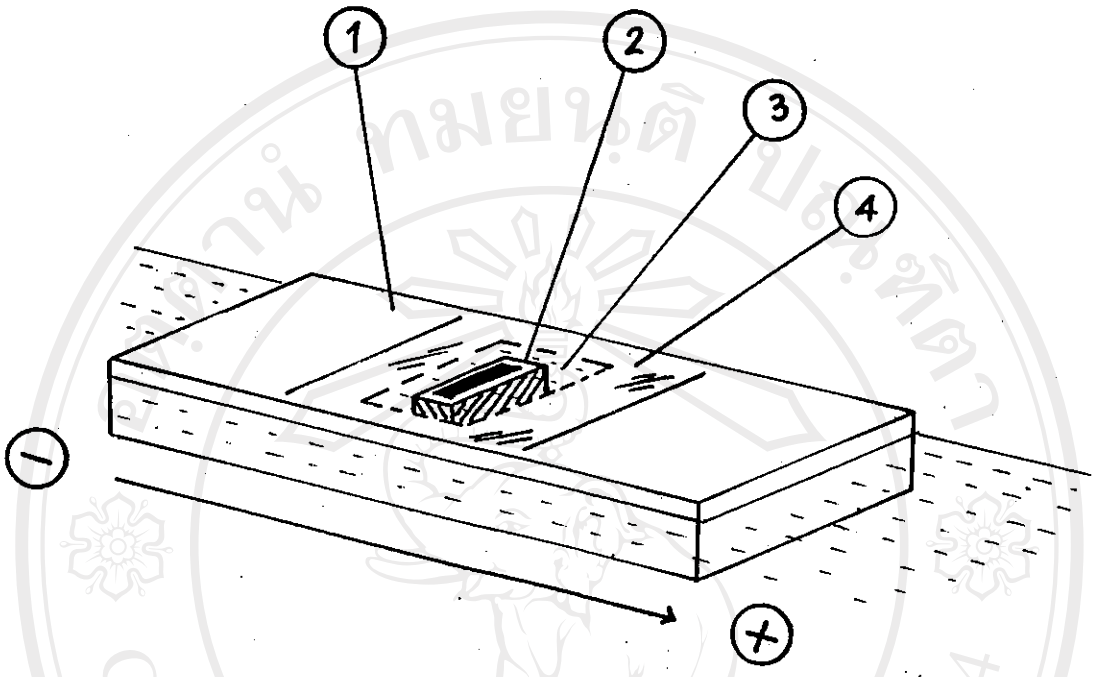


Figure II.7 The electroelution technique for the demonstration of globin specific probes preparation.

(1) 1.2% agarose gel

(2) gel slice containing the specific probe

(3) a trough paved with dialysis membrane

(4) dialysis membrane

removed from the gel slice and trapped on the dialysis membrane (examined under a UV lamp).The gel slice was removed and the polarity of the current was reversed to release the DNA specific probe from dialysis membrane. The buffer in the trough was recovered and the DNA specific probe was precipitated with 3.0 M sodium acetate and cold absolute ethanol at 70°C, overnight. The DNA specific probe was harvested by centrifugation at 10,000 rpm and 4°C for 30 min,washed with ethanol and dried at 37°C, then redissolved in sterile water to get a concentration of 0.5-1.0 ug/ul (By calculation from the ratio of DNA specific probe and recombinant plasmid size). The DNA specific probe solution could be kept at -20°C for a long time.

II.3.7.5 Purification of the oligonucleotide probe: The synthetic oligonucleotide was purified by electrophoresis in a 20 % polyacrylamide gel with 7 M urea. The gel was prepared in Tris-borate buffer and poured between two glass plates (20x20 cm) that are held apart by 1.0 mm thick spacers (Figure II.8). The gel was left at room temperature for 30 min, then the bottom spacer was removed and gel was warmed to 50°C by vertical electrophoresis at 600 V for 30 min.In the meantime,the synthetic oligonucleotide was dissolved in sterile water (6 OD/12 ul) and mixed well with 12 ul of loading buffer. After denaturation at 95°C for 5 min, the oligonucleotide solution (2 OD/8 ul) was applied to the slots (1mm x1cm); one slot was filled with the dye marker (0.05 % bromphenol blue and 0.05 % azorubin dye in Tris-borate

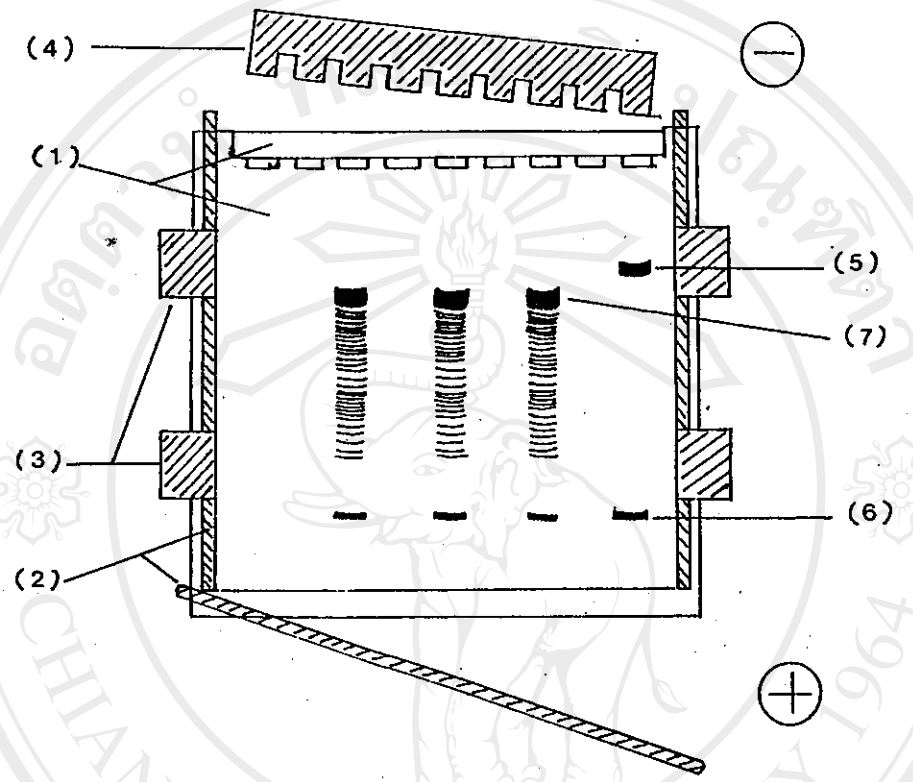


Figure II.8 Cross section of polyacrylamide gel electrophoresis for the demonstration of oligonucleotide probes preparation.

(1) two glass plates

(2) spacers

(3) clips

(4) comb

(5) dye marker = brom phenol blue

(6) dye marker = azorubin

(7) the position of 19 mer oligonucleotide probe.

buffer). The electrophoresis was carried out at 600 V until the azorubin dye was 2 cm from the bottom of the gel. The gel was removed and wrapped with Saran wrap and placed on the chromatographic screen. The band of oligonucleotide probe (19 mer) was localized under UV light and cut out with a sharp scalpel and checked under a UV lamp again to assure that the correct gel fragment had been excised. Then the gel slice was cut in small pieces and suspended in 300 ul of sterile water in a sterile siliconized Eppendorf tube. The oligonucleotide probe was eluted by incubating this suspension overnight at 37°C. Then the eluate was collected in a new siliconized Eppendorf tube. The gel pieces were then eluted twice with 300 ul sterile water at 56°C for 1 hr. The DNA concentration in the eluate was determined at 260 nm, using the blank eluate as reference (eluted from the free area on the same gel). Urea and oligonucleotide probe were denatured at 95°C for 5 min and kept at -20°C.

II.3.8. Radiolabelling

II.3.8.1 DNA specific probe labelling: the probe was labelled with alpha^{32}P-dCTP under the conditions recommended in the Multiprimer DNA Labelling Kit (Amersham). The random hexanucleotide functioned as a primer for the DNA polymerase I "Klenow fragment" (Figure II.9).

The assay was performed in a volume of 20 ul in a sterile siliconized Eppendorf tube. An amount of 0.1 ug DNA specific probe was denatured by boiling 3 min in a water bath and immediate chilling.Then

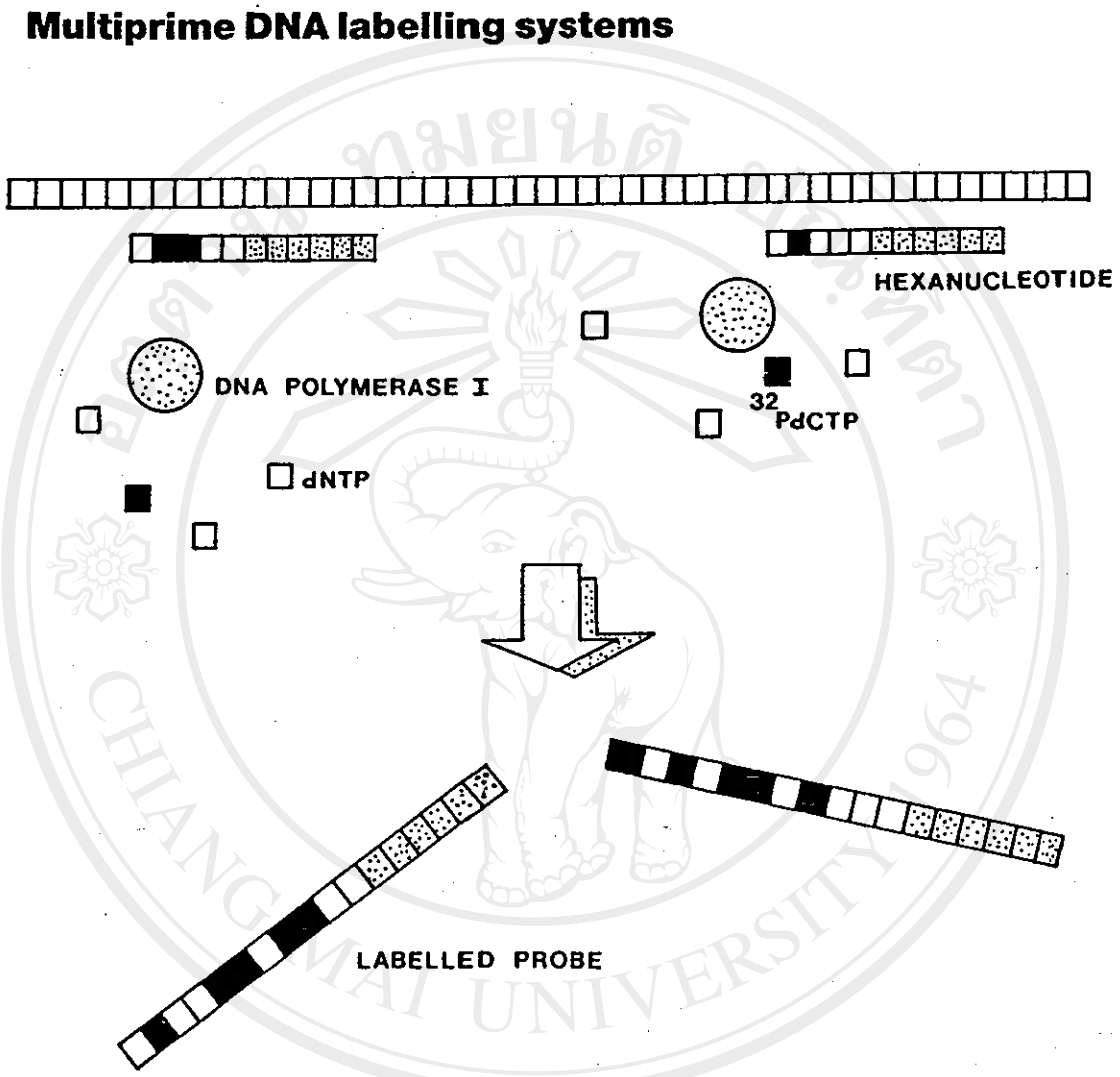


Figure II.9 The multiprimer DNA labelling systems for the demonstration of DNA specific probes labelling.

the other components were added as follows: 4.0 ul of solution 1, 2.0 ul of solution 2, 20 uCi of alpha ^{32}P-dCTP and 1.0 ul of solution 3. The reaction mixture was incubated at 25°C for 3 hr.

The specific activity was examined by separating the incorporated probe and free radioisotope by Sephadex G-75 column chromatography (1.0 ml): 1.0 ul of assay was mixed with 19.0 ul of dye marker solution (1.5 % blue dextran and 0.8 % phenol red) and eluted from a column with 1x TE.Each fraction (blue,colorless and red) was collected and measured with a Geiger counter. The blue fraction contains the labelled nucleic acids.The incorporation rate should be more than 40% of the total activity. The reaction was stopped by adding 3.0 ul of 0.25 M EDTA and 50 ug denatured salmon sperm DNA was added as competitor DNA. The labelled DNA probe was denatured by 3 min boiling and immediate chilling. The probe was kept at 4°C until hybridization .

II.3.8.2 Oligonucleotide probe: The probe was labelled with gamma ^{32}P-ATP using T4-polynucleotide kinase (BRL Laboratories USA).The phosphate group from gamma ^{32}P-ATP was transferred to the 5'end of the oligonucleotide probe.

The reaction mixture in a sterile siliconized Eppendorf tube consisted of: 10 pmole of oligonucleotide probe, 1.6 ul of 10x reaction buffer, 20 pmole of gamma ^{32}PATP and 1 unit of T4-polynucleotide kinase. The reaction was incubated at 37°C for 30 min. The specific activity was examined by separating the incorporated oligonucleotide probe and

free radioisotope by DE-cellulose 52 column chromatrography (Whatman): 1.0 ul of assay was mixed with 100 ul of 1x TE buffer and applied to the column. The free radioisotope was eluted with 2.0 ml of 0.2 M NaCl and the incorporated oligonucleotide probe was eluted with 0.5 M NaCl. The specific activity of each fraction was determined with a liquid scintillation counter (Beckman, USA). The specific activity of the labelled oligonucleotide probe should be 40-50 % of total specific activity.

II.3.9. Hybridization

The principle of this technique is that in appropriate conditions the denaturated DNA will be renaturated according to the complementary base pairing rule.

II.3.9.1 DNA specific probe hybridization :. The hybridization was performed in a sterile siliconized cylindrical tube (4x25 cm) by the following process: The tube was filled with 2xSSC solution. The nitrocellulose membrane was rolled,assuring that the immobilized DNA was on the inner surface, and transferred into the tube. Trapping of air bubbles must be avoided. About 20 ml of 2xSSC was used to warm up the nitrocellulose membrane at 42°C in a rolling incubator (Heraeus GmbH, W-Germany) for 1 hr. The 2xSSC solution is discarded and replaced with 10 ml freshly warmed prehybridization solution (50°-55°C) for 4 hr. Then the labelled DNA specific probe was added to 10 ml of freshly warmed hybridization solution (50°-55°C) and again placed in the tube.

The hybridization reaction was carried out at 42°C in a rolling incubator for 15-16 hr.

II.3.9.2 Oligonucleotide probe hybridization: The DNA sample on the dried gel was denatured in denaturation solution for 30 min with gentle shaking and neutralized for 30 min. Then this gel was placed into the hybridization tube which was filled with 2xSSC. The gel was warmed up with 2xSSC at 45°C for 30 min. After that the radiolabelled specific oligonucleotide probe ($20x10^6$ dpm) was added to 10 ml of warmed hybridization solution (60°C), mixed well and replaced into the hybridization tube. The hybridization reaction was carried out at 45°C in a rolling incubator for 15-16 hr.

II.3.10. Washing

In the hybridization step, both specific and nonspecific hybrids are formed. The nonspecific hybrids are separated and washed out by changing the stringency of the washing conditions.

II.3.10.1 DNA specific probe: The hybridized nitrocellulose membrane was transferred from the hybridization tube and washed with vigorous shaking in three solutions:

solution 1 : 10 min at room temperature (twice).

solution 2 : 20 min at 65°C (twice).

solution 3 : 30 min at 65°C

After washing, the nitrocellulose membrane was soaked in 2xSSC solution for 10 min and dried at room temperature.

II.3.10.2 Oligonucleotide probe: The hybridized gel was transferred from the hybridization tube and soaked in 2xSSC solution, then washed with vigorous shaking in three solutions:

solution 1 : 1 hr at room temperature.
solution 2 : 1 hr at 45°C
solution 3 : 1 hr at 45°C

After washing, the gel was soaked in 2xSSC solution for 10 min, then placed between two pieces of Whatman paper and dried with a gel drying machine at 30°C for 15 min (Figure II.10).

II.3.11. Autoradiography

Dried nitrocellulose and gel were wrapped with Saran wrap. In a dark room, the nitrocellulose was placed in an X-ray film holder, covered with a sheet of X-ray film (Cronex) and an intensifying screen. The dried gel was placed under the X-ray film and covered with two intensifying screens in sandwich fashion. The autoradiography was carried out at -70°C. The exposure time was between 1 and 14 days.

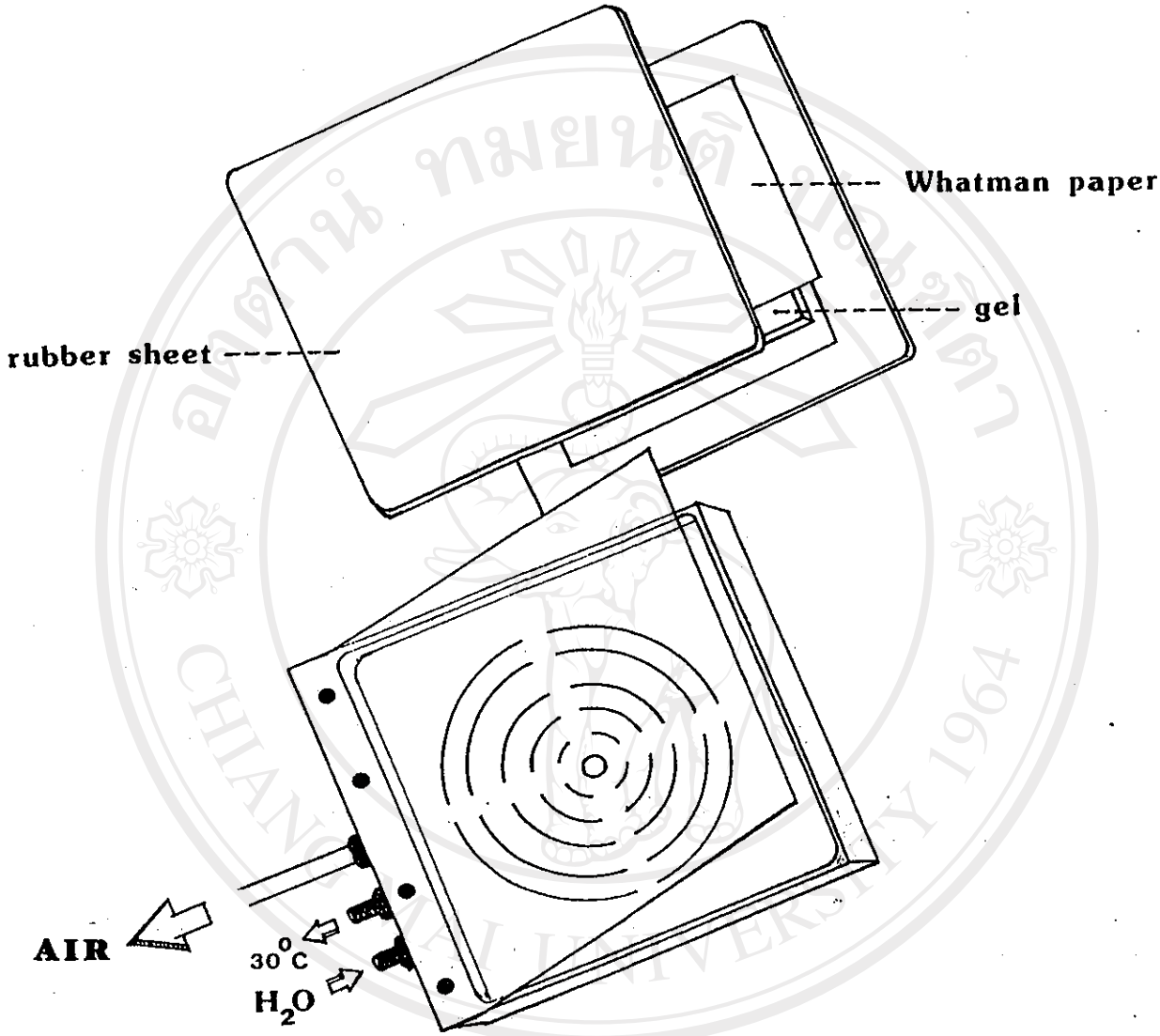


Figure II.10 Gel drying machine.