CHAPTER IV
RESULTS

1. Study subjects

1.1 Characteristics of subjects

One hundred and twenty Thai males, who attended the infertile clinic at Maharaj Nakorn Chiang Mai Hospital during 2003 to 2005, were enrolled into the study. They were divided into two groups according to their seminal profiles: forty males with azoospermia (no spermatozoon in the ejaculate even after centrifugation) and eighty males with oligospermia (sperm count less than 10^6 spermatozoa/ml). Their mean age was 34.6 years (range 22-52 years). Most of them were residences of northern Thailand. Details of the patients according to their sperm concentration were shown in Table 4.1. Clinical findings from history and physical examination were summarized in Table 4.2. Occupations of the infertile males were shown in Figure 4.1.

Fifty healthy Thai males, with normal semen analysis, were also examined for Y chromosome microdeletions. Their ages ranged from 23-42 years (mean 32.3 years). They were residences of northern Thailand. One healthy woman was included as a negative control (absence of Y chromosome) in all multiplex PCR.

Table 4.1 Classification of patients according to their sperm concentrations

Sperm concentration ($x10^6$ spermatozoa/mL)	No. of patients
0	40
0.1-2.0	25
2.1-5.0	29
5.1-10.0	26
Total	120

1.2 Hormone profiles and results of chromosome analysis of infertile males

1.2.1 Hormone profiles of azoospermic and oligospermic males

The levels of FSH and LH (mean ± SD) in azoospermic males were

significantly higher than those in the oligospermic males (FSH: 15.8±11.3 vs 7.9±5.1 mIU/mL, $P = 0.001$; LH: 9.3±6.2 vs 6.3±3.4 mIU/mL, $P = 0.001$). However, the

Table 4.2 Clinical characteristics of the patients

Clinical characteristics	No. of patients	Patients with Y chromosome microdeletions
Patient with azoospermia	40	5
Physical finding		
Cryptorchidism	4	0
Varicocele	4	0
Medical history		
Orchitis	8	0
Mump	12	2
Hernia	4	0
Trauma	5	0
Patient with oligospermia	80	1
Physical finding		
Cryptorchidism	18	0
Varicocele	6	0
Medical history		
Orchitis	19	1
Mump	22	0
Hernia	8	0
Trauma	5	0

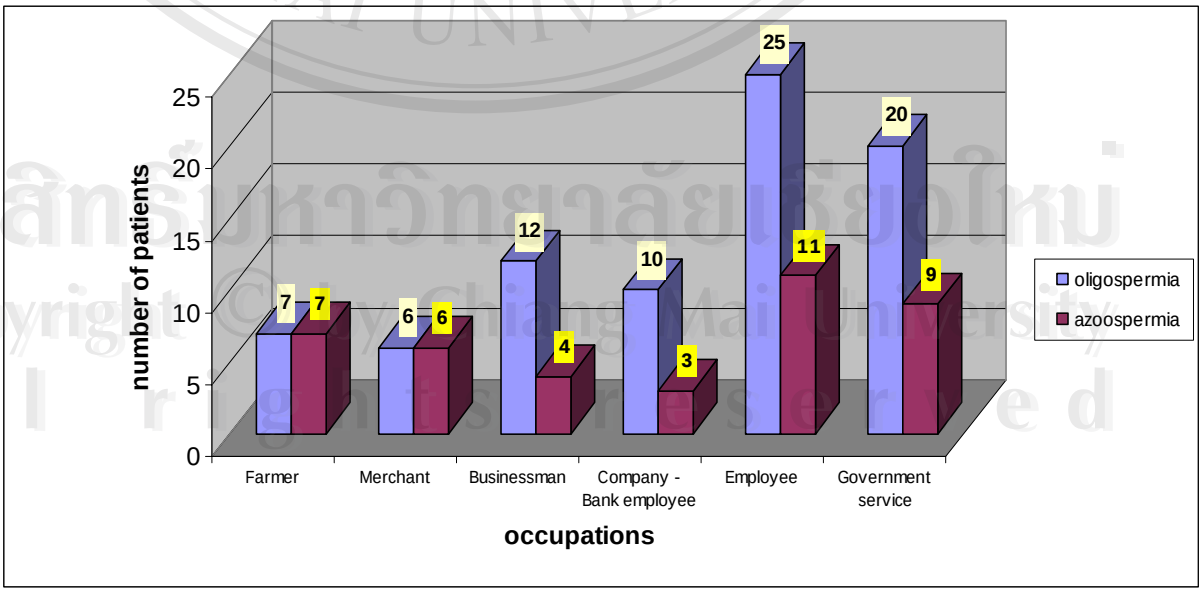

Figure 4.1 The occupations of azoospermic and oligospermic males.

levels of prolactin and testosterone (mean ± SD) in azoospermic and oligospermic males were not significantly different (prolactin: 18.9 ± 9.8 vs 18.9±11.5 ng/mL, *P* = 0.481; LH: 3.9 ± 1.7 vs 4.5± 2.6 ng/mL, *P* = 0.757). There was no significant difference in the levels of FSH, LH, prolactin and testosterone between azoospermic/oligospermic patients, with (n = 6) or without (n = 114) Y chromosome microdeletions (*P* = 0.187-0.766; Table 4.3).

Table 4.3 Hormonal profiles of azoospermic and oligospermic males with Y chromosome microdeletions. Mean hormonal levels in those with and without Y chromosome microdeletions were also shown and compared.

Patient No.	Age (years)	PRL (ng/mL)	FSH (mIU/mL)	LH (mIU/mL)	T (ng/mL)
36	38	9.1	6.8	4.7	5.6
45	31	12.5	20.4	16.5	2.6
67	33	13.8	17.6	9.5	2.5
91	28	23.0	6.6	6.7	4.2
96 †	35	16.5	12.3	7.2	4.5
116	39	18.1	13.0	10.1	5.2
Azoospermia (n=40)					
With AZF deletion (5)*	34±4.6	15.3±5.4	12.9±6.2	9.5±4.5	3.8±1.3
without AZFdeletion (35)*	34±6.0	19.4±10.3	16.2±11.9	9.4±6.6	3.9±1.8
Oligospermia (n=80)					
With AZF deletion (1)	35	16.5	12.3	7.2	4.5
without AZFdeletion (79)*	36±6.5	18.8±11.6	7.8±5.0	6.4±3.5	4.5±2.6

† oligospermic male, * values were mean±SD

1.2.2 Chromosome abnormalities in azoospermic and oligospermic males

Cytogenetic analyses were performed in 120 infertile males. Abnormalities were found in six males, corresponding to a frequency of 5.0%. None of the six cases had Y chromosome microdeletions.

Two of them had translocations, 46,XY, t(7;14) and 46,XY, t(7;16). The other four patients had 47,XXY (Klinefelter's syndrome), as shown in Table 4.4.

The frequencies of chromosome abnormalities among azoospermic and oligospermic males were 12.5% (5 out of 40 azoospermic males) and 1.25% (1 out of 80 oligospermic males), respectively.

Table 4.4 Chromosome abnormalities in azoospermic and oligospermic males

Patient number	Karyotype	Sperm count (million/mL)
14	46,XY, t(7;14)	0
16	46,XY, t(7;16)	0
25	47,XXY	0
35	47,XXY	0
52	47,XXY	0
114	47,XXY	3.2

2. Genomic DNA concentration

Genomic DNA, recovered from peripheral blood mononuclear cell extraction of 120 infertile males, 50 normal males and 1 male/1 female control samples, ranged from a concentration of 250 to 800 ng/µL. The DNA concentration of each sample was adjusted to approximately 200 ng/µL before subjected to PCR amplification.

3. Determination of each specific primer efficiency

To evaluate the efficiency of each specific primer, a single-primer PCR was performed one by one under universal PCR conditions. The sizes of the PCR products were then examined.

Single-primer PCR amplification of *RBM1* gene, under referenced PCR conditions[99] at an annealing temperature of 62ºC, did not give any product during the first attempt. The *sY14 (SRY), SMCY,* and *EIF1AY* primers gave the expected PCR products (Figure 4.2). In order to improve the amplification efficiency of *RBM1* primers, titration of annealing temperature was performed at 1-degree increment from 55-60 ºC. The optimal annealing temperature was found to be 55-57ºC (Figure 4.3). Therefore, the annealing temperature of 57 ºC was selected for subsequent studies. To achieve a higher yield of *RBM1* amplification product, various concentrations of this

primer-pair (0.2, 0.4 and 0.8 µM/reaction) were tested. However, the resulting amplification products were not markedly different (Figure 4.4). Therefore, a concentration of 0.4 µM was selected for subsequent studies. The primers for *sY14(SRY)*, *SMCY*, *EIFIAY* and *RBM1* genes were combined into a set of multiplex PCR (set # 1). The result of this multiplex PCR amplification was shown in Figure 4.5.

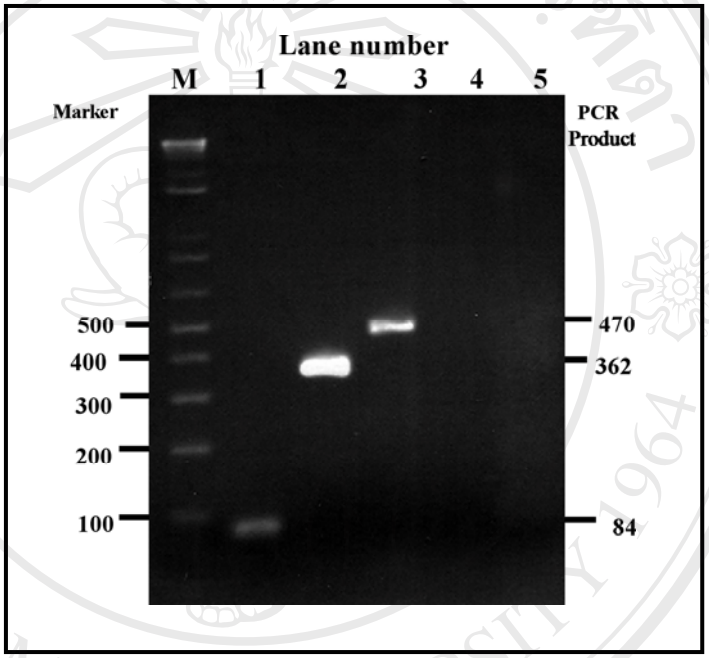

Figure 4.2 The amplification products of singleplex PCR in multiplex set # 1. Lane 1, 2 and 3 show 84 bp, 362 bp and 470 bp PCR products of *EIF1AY*, *SMCY* and *sY14(SRY)* gene amplification. Lane 4 shows no expected band (800 bp) of *RBM1* gene amplification product. Lane 5 shows negative (DW) control.

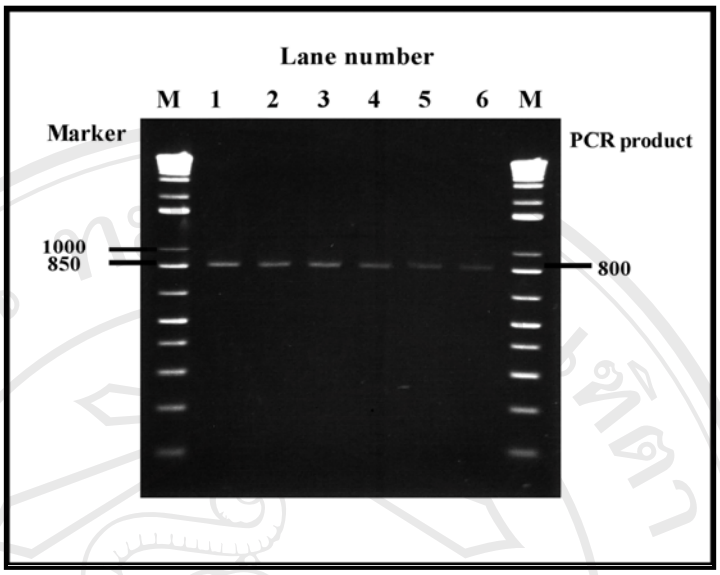

Figure 4.3 Singleplex PCR amplification product (800 bp) of *RBM1* gene at various annealing temperatures. Lane 1: 55 ºC, Lane 2: 56 ºC, Lane 3: 57 ºC, Lane 4: 58 ºC, Lane 5: 59 ºC , Lane 6: 60 ºC. Lane M showed DNA markers.

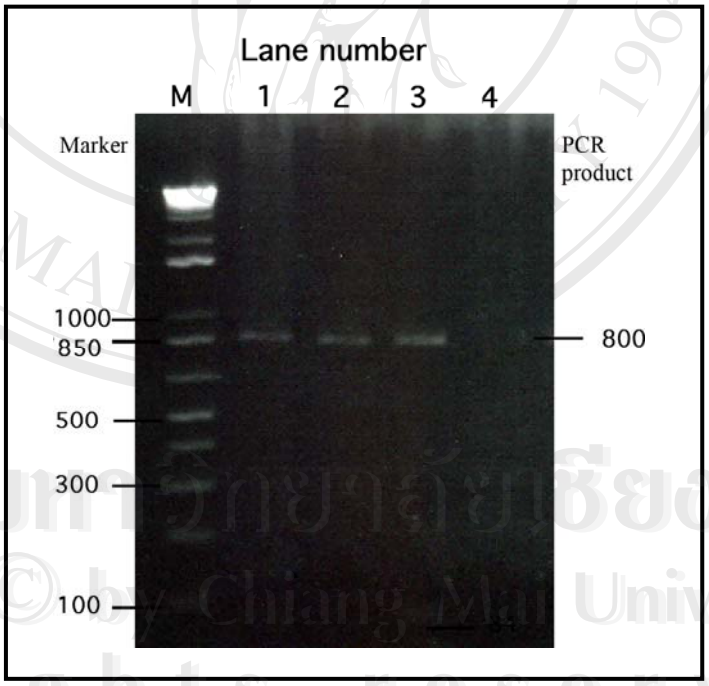

Figure 4.4 The single PCR amplifications of *RBM1* gene achieved when different primer concentrations were used. Lane 1, 2 and 3 show PCR products when 0.2, 0.4 and 0.8 µM of *RBM1* primer concentrations were used, respectively. Lane M showed DNA markers. Lane 4 showed a negative (DW) control.

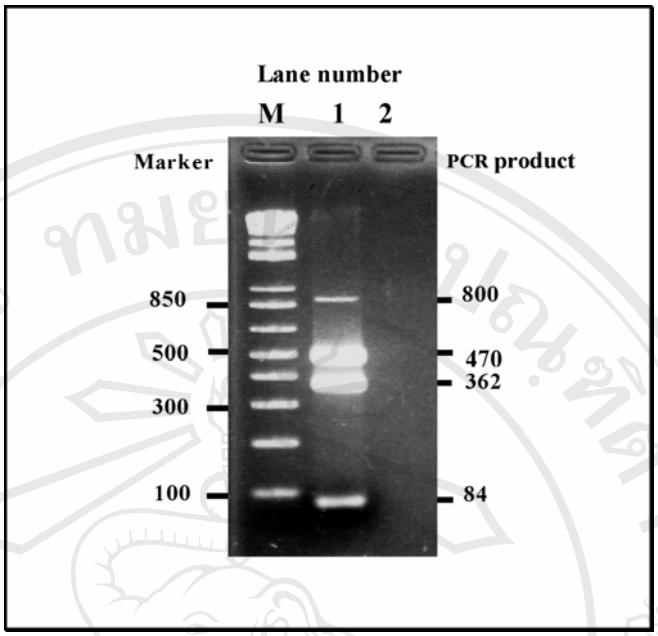

Figure 4.5 Amplification products of multiplex PCR set # 1. Lane M showed DNA markers. Lane 1 showed PCR products of *EIF1AY* (84 bp)**,** *SMCY* (362 bp)**,** *sY14(SRY)* (470 bp)**,** and *RBM1* (800 bp) genes**,** respectively. Lane 2 showed negative control (DW).

Primer pairs for *PRY*, *DAZ(sY283)* and *DBY* genes were tested individually in singleplex PCR systems. The results showed that these primers successfully amplified their DNA targets and generated PCR products of 80 bp, 498 bp and 689 bp as expected. However, when they were combined with primers for *sY14(SRY)* into multiplex PCR set, the PCR products of *DAZ(sY283)* and *sY14* (498 bp and 470 bp, respectively) could not be separated on a standard 2.5% agarose gel electrophoresis (Figure 4.6). In an attempt to separate these two PCR products, three concentrations of agarose gel, 1.5%, 2.5% and 3.0% were tested. The results, however, showed that adjusting agarose gel concentration did not help in the separation of DNA bands. As a competition between the 2 primer pairs was suspected, a new pair of primers specific to *DAZ(sY283)* was designed and tested for its efficiency and specificity (Figure 4.7).

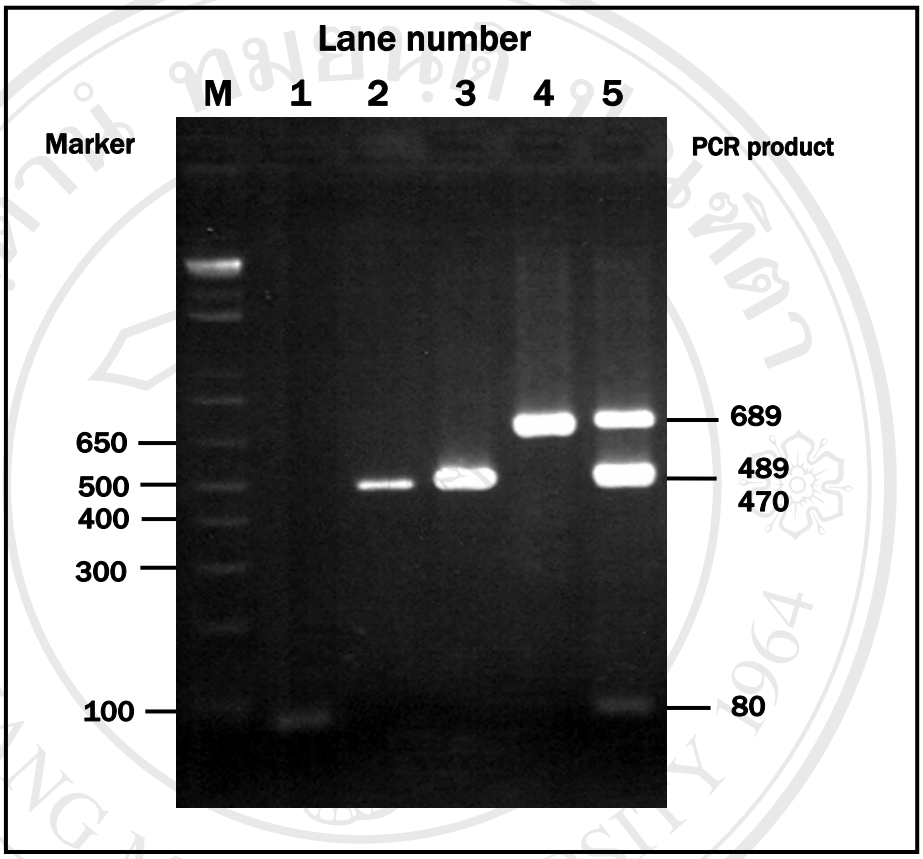

Figure 4.6 The PCR products of singleplex PCR amplification and PCR products of four primer pairs in multiplex PCR set # 2. Lane M shows the DNA marker. Lane 1, 2, 3 and 4 show 80 bp, 470 bp, 498 bp and 689 bp PCR products of *PRY, sY14(SRY), DAZ(sY283)* and *DBY* gene. Lane 5 shows the PCR products of multiplex PCR 2 with only 3 separated bands. The PCR products of sY14 and sY283 primers (470 bp and 498 bp) fail to show separated bands in this condition.

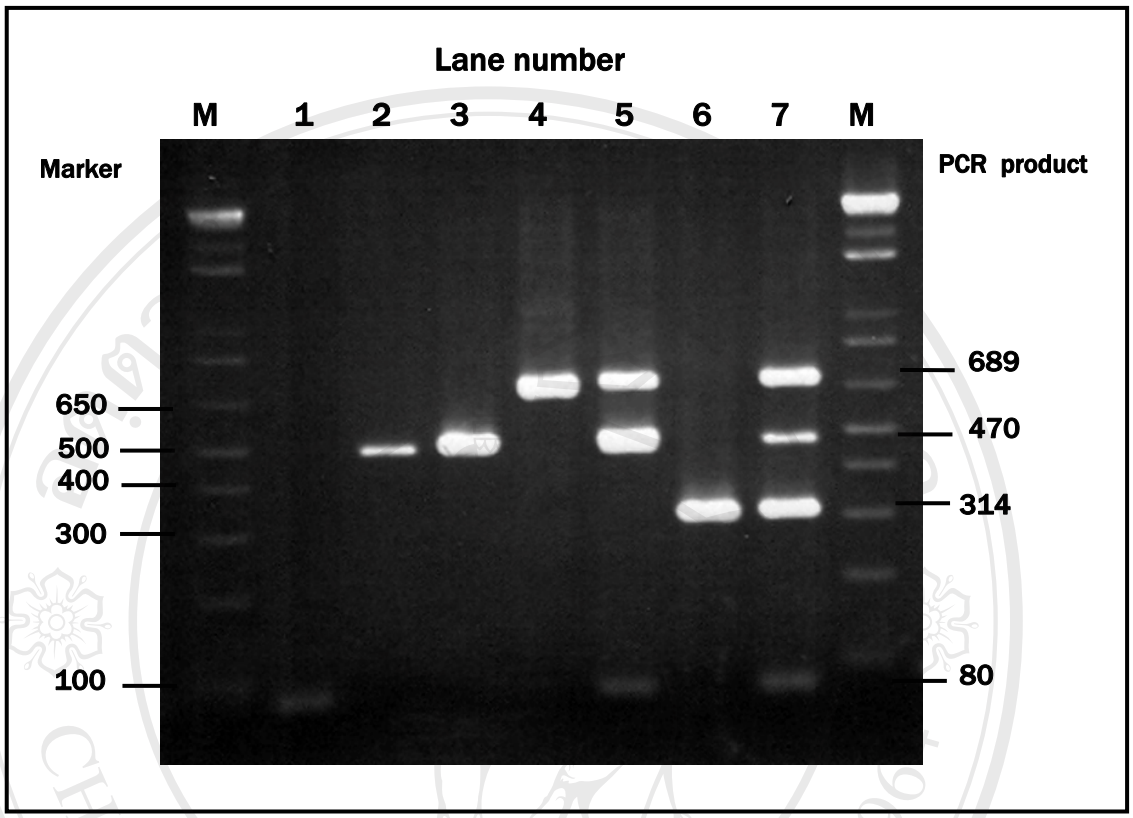

Figure 4.7 The PCR products of singleplex PCR amplifications and four PCR products of multiplex PCR set # 2. Lane M shows the DNA marker. Lane 1, 2, 3 and 4 show single PCR product after the following genes amplification; *PRY* (80 bp), *sY14(SRY)* (470 bp), *DAZ(sY283)*/ original primers (498 bp) and *DBY* (689 bp), respectively. Lane 5 shows the multiplex PCR 2 amplification when original primers specific to *DAZ(sY283)* gene were used. Lane 6 shows single PCR product (314 bp) of a new *DAZ(sY283)* primer pairs. Lane 7 shows the PCR products of multiplex PCR 2 amplification when the new primers specific to *DAZ(sY283)* were used.

In a similar manner, primers specific for other selected genes were tested individually for their efficiencies by single-primer PCR systems, before combining them into the preliminary multiplex PCR set # 3 [*sY14(SRY), CDY1, DFFRY*] and set # 4 [*sY14(SRY), TTY2, sY277, BPY*]**.** Both multiplex PCR sets were tested under standard PCR conditions. The results were shown in Figure 4.8 and Figure 4.9, respectively.

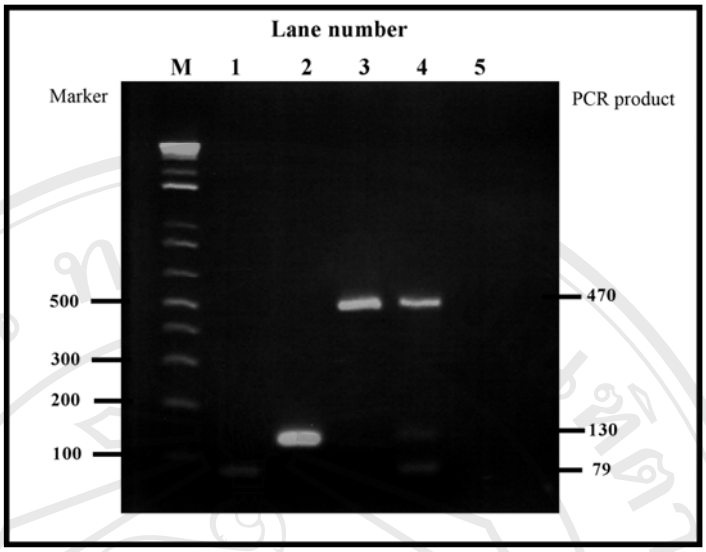

Figure 4.8 PCR products of three singleplex PCR and one multiplex PCR set #3. Lane M showed DNA markers. Lane 1, 2 and 3 showed singleplex PCR products of *CDY1* (79 bp), *DFFRY* (130 bp) and *sY14(SRY)* (470 bp) genes, respectively. Lane 4 showed multiplex PCR products when the three primer-pairs were combined. Lane 5 showed negative control (DW).

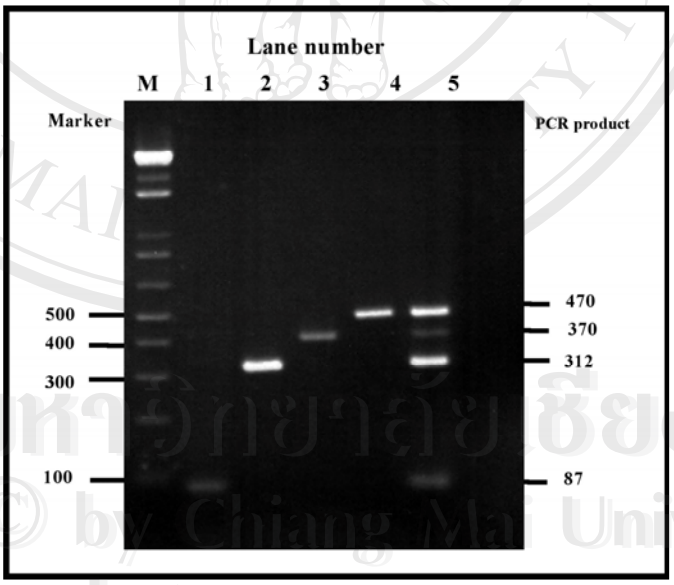

Figure 4.9 PCR products of four singleplex and one multiplex PCR set # 4. Lane M showed DNA markers. Lanes 1, 2, 3 and 4 showed singleplex PCR products of *TTY* (87 bp), *sY277* (312 bp), *BPY2* (370 bp) and *sY14(SRY)* (470 bp) genes, respectively. Lane 5 showed multiplex PCR products of all 4 genes. Lane 6 showed negative control (DW).

4. Optimization of multiplex PCR conditions

4.1 Multiplex PCR group

Primer-pairs specific to the eleven genes of interest and one primer-pair specific to *SRY* gene (control) were combined into 4 multiplex PCR sets. They were optimized in order to achieve balanced PCR products in the multiplex, as follows:

Multiplex PCR set # 1: Based on previously published report [99], the concentrations of *sY14 (SRY), SMCY, RBM1*, and *EIF1AY* primers were tested at 0.2, 0.12, 0.4 and 0.2 µM/reaction, respectively. The result showed that *EIF1AY* primers competed with *RBM1* primers and produced a markedly unbalanced yield of both PCR products. To solve this problem, three lower concentrations (0.12, 0.08, and 0.04 µM/reaction) of *EIF1AY* primers were tested (Figure 4.10). A concentration of 0.08 µM/reaction of *EIF1AY* primers appeared to give acceptable amount of both *EIF1AY* and *RBM1* amplification products in this multiplex PCR system. To get a higher yield of *RBM1* product, various concentrations of this primer-pair (0.2, 0.4 and 0.8 µM/reaction) were tested. However, the results were not different. Therefore, a concentration of 0.4 µM/reaction was selected for further study. The final optimal concentrations of the 4 primer pairs for *SRY, SMCY, RBM1*, and *EIF1AY* in multiplex PCR set # 1 were 0.2, 0.12, 0.4 and 0.08 µM/reaction, respectively (Figure 4.11).

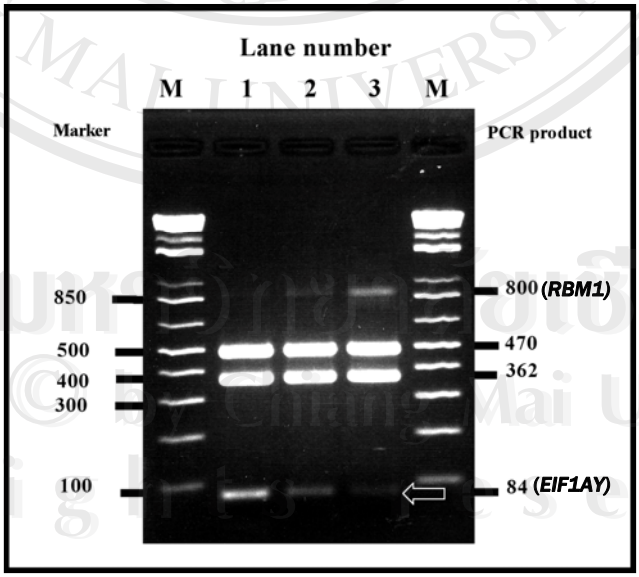

Figure 4.10 Multiplex PCR products at different concentrations of *EIF1AY* primers. Lane 1, 2, and 3 showed the amount of 84 bp PCR product of the *EIF1AY* primer, at a primer concentration of 0.12, 0.08 and 0.04 µM, respectively.

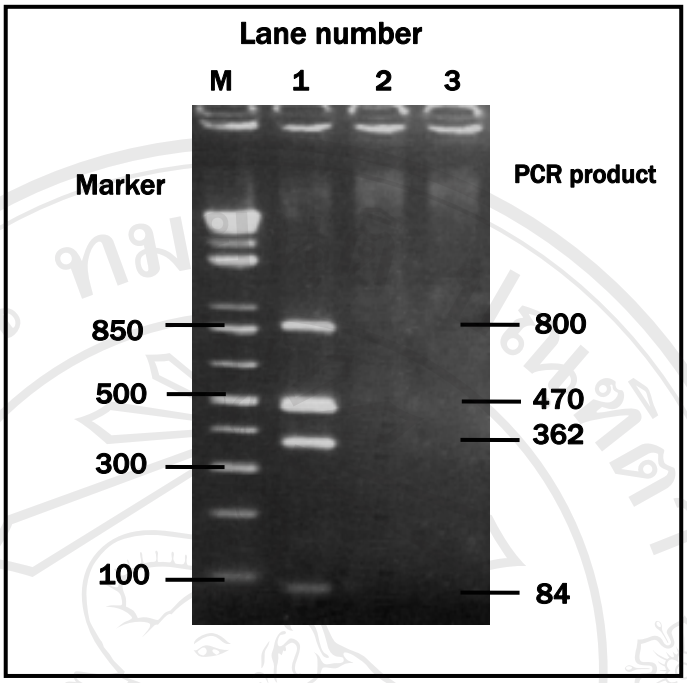

Figure 4.11 Optimized multiplex PCR set # 1, showing all amplification products. Lane 1 showed PCR product of *EIF1AY* (84 bp)**,** *SMCY* (362 bp), *sY14(SRY)* (470 bp) and *RBM1* (800 bp) genes. Lane M showed DNA markers. Lane 2 (female DNA) and Lane 3 (DW) showed no amplification products.

Multiplex PCR set # 2: Based on previous reports, the concentrations of *sY14 (SRY), DBY, DAZ (sY283)* and *PRY* primers were started at 0.2, 0.2, 0.2 and 0.2 µM/reaction, respectively. Various concentrations of the three primers [*DBY, DAZ (sY283)* and *PRY*] were then tested. For illustration purpose, only the PCR products from different concentrations of *DAZ (sY283)* primers were shown in Figure 4.12. A final concentration of 0.08 µM for *DAZ (sY283)* primers was selected for further use in subsequent multiplex PCR.

The optimal concentrations of the 4 primer pairs, for *sY14 (SRY), DBY, DAZ (sY283)* and *PRY* in the final multiplex PCR set # 2, were found to be 0.2, 0.1, 0.08 and 0.1 µM/reaction, respectively, as shown in Figure 4.23.

Multiplex PCR set # 3. Based on previous reports, the primers for *sY14 (SRY), DFFRY* and *CDY1* were initially tested at a concentration of 0.2, 0.5 and 0.2 µM/reaction, respectively. Due to gross imbalance of PCR products, the primer

concentrations were adjusted. The optimal concentrations of *sY14 (SRY), DFFRY* and *CDY1* primers were found to be 0.2, 0.4 and 0.2 µM/reaction, respectively. This PCR condition provided the acceptable balance in PCR products as shown in Figure 4.13.

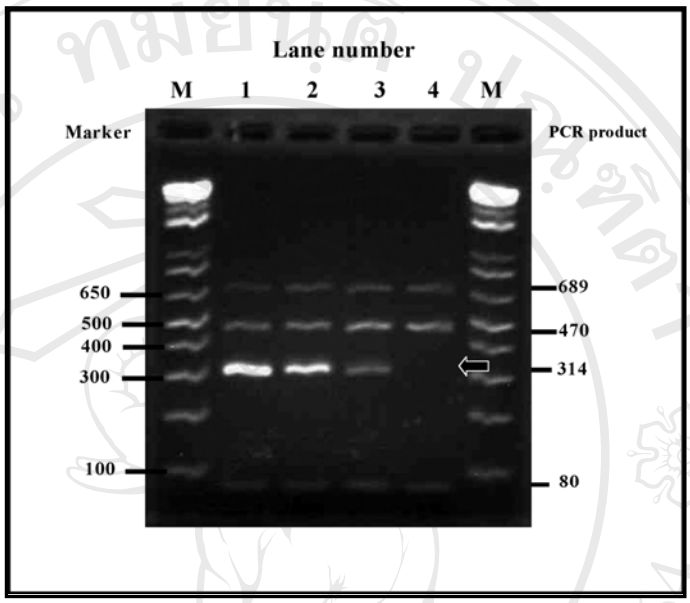

Figure 4.12 Amplification products of multiplex PCR set # 2 at different concentrations of *DAZ(sY283)* primers. Lane 1, 2, 3 and 4 showed 0.2, 0.1, 0.08 and 0.04 µM/reaction, respectively. This primer produced a 314 bp PCR product. The primer concentration of 0.08 µM was selected for further use. Lane M shows DNA markers.

Multiplex PCR set # 4: The primers for *sY14 (SRY), DAZ (sY277), BPY2, and TTY2* were initially used at a concentration of 0.2, 0.4, 0.2 and 0.4 µM/reaction, respectively, as described in previously published report [99]. The primer concentrations were titrated by increasing the concentration of primers, that gave fewer amounts of PCR products and vice versa. In this way, a 312 bp product of *DAZ(sY 277)* primers was increased to an optimal amount when a *TTY2* primer concentration of 0.3 µM/reaction was used (Figure 4.14). The optimal concentrations of *sY14 (SRY), DAZ (sY277), BPY2, and TTY2* primers in the final multiplex PCR set # 4, were found to be 0.2, 0.2, 0.2 and 0.3 µM/reaction, respectively. This condition provided the better balanced PCR products as shown in Figure 4.15.

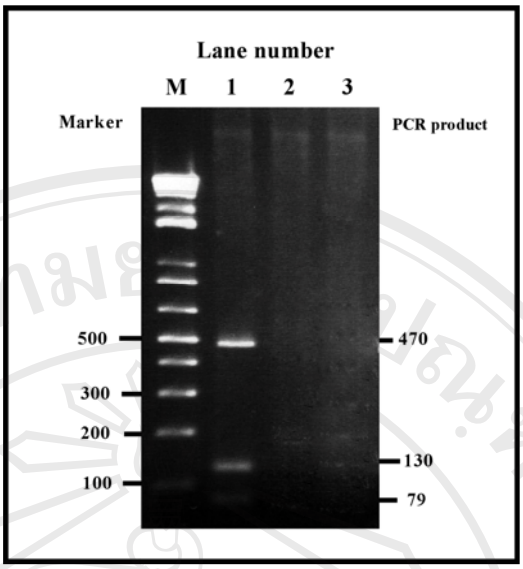

Figure 4.13 Products of multiplex PCR set # 3. Lane M showed DNA markers. Lane 1 showed amplification products of *SRY* (470 bp), *DFFRY* (130 bp) and *CDY1* (79 bp). Lane 2 (female DNA) and Lane 3 (DW) showed no PCR products.

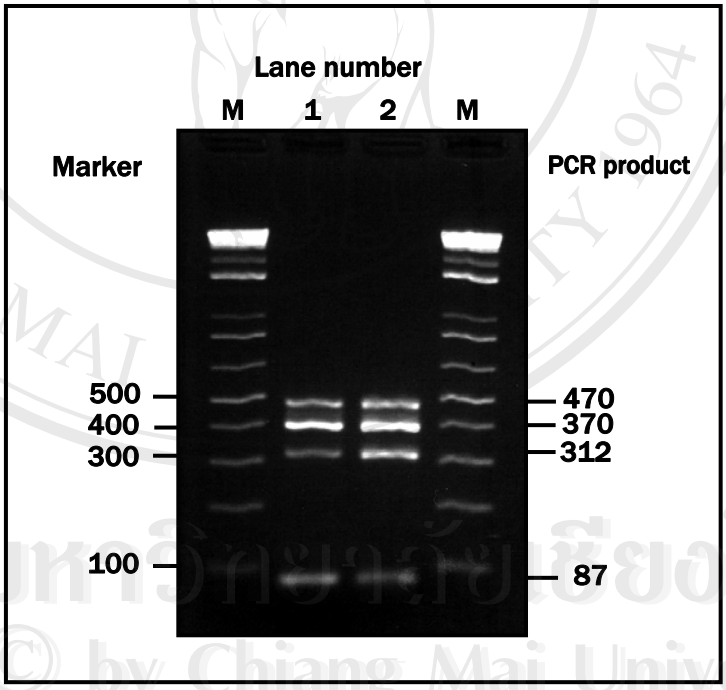

Figure 4.14 PCR products generated by the multiplex PCR set # 4, when 2 concentrations of *TTY2* primers were used. Lane 1 and 2 showed 0.4 and 0.3 µM/reaction, respecively. The amount of a 87 bp PCR product of *TTY2* primers at 0.3 µM/reaction was just slightly decreased while a 312 bp product of *DAZ(sY277)* primers increased significantly. Therefore, *TTY2* primer concentration at 0.3 µM/reaction was selected.

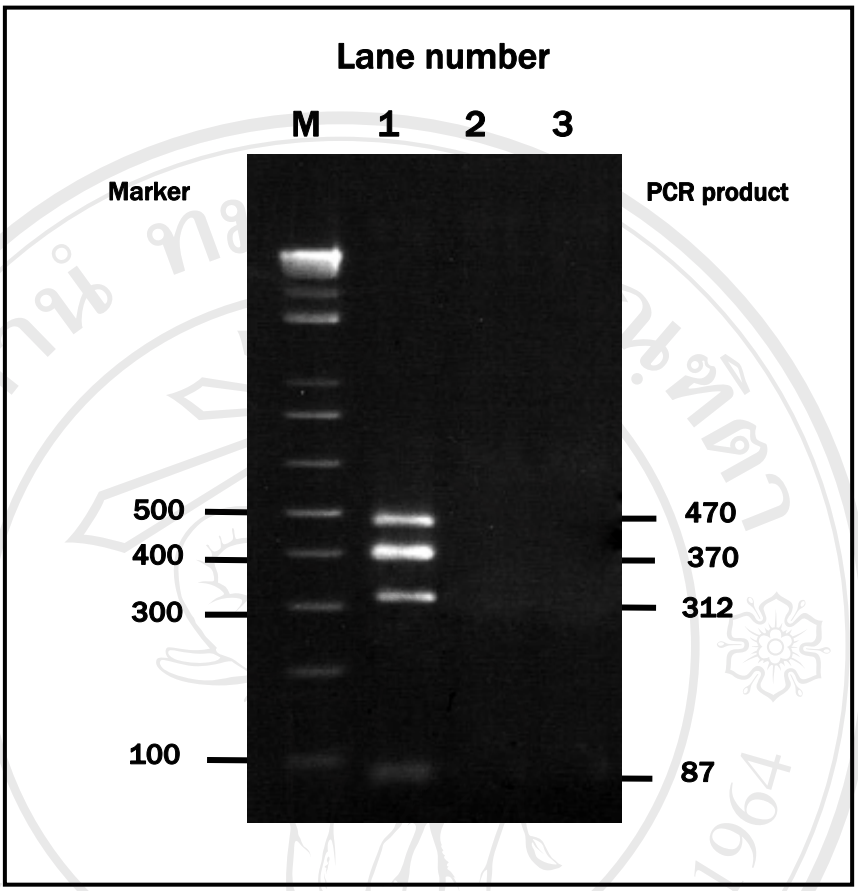

Figure 4.15 Products of multiplex PCR set # 4 after optimization. Lane M showed DNA markers. Lane 1 showed PCR products of *SRY* (470 bp), *BPY2* (370 bp), *[DAZ(sY277)*, 312 bp] and *TTY2* (87 bp). Lane 2 (female DNA) and Lane 3 (DW) showed no PCR products.

4.2 Determination of the optimal concentration of genomic DNA

Genomic DNA concentrations of 100, 200, 300 and 400 ng (for 25 µl reaction volume) were tested in the multiplex PCR sets # 2 and # 4. For multiplex PCR set # 2, there was no difference in the amount of PCR products among the various DNA concentrations tested (Figure 4.16). However, a genomic DNA concentration of 100 ng/25 µl reaction volume in multiplex PCR set # 4, gave too few amount of a 370 bp PCR product (Figure 4.17). A 200 ng/25 µl concentration of genomic DNA was, therefore, selected for use in all multiplex PCR studies.

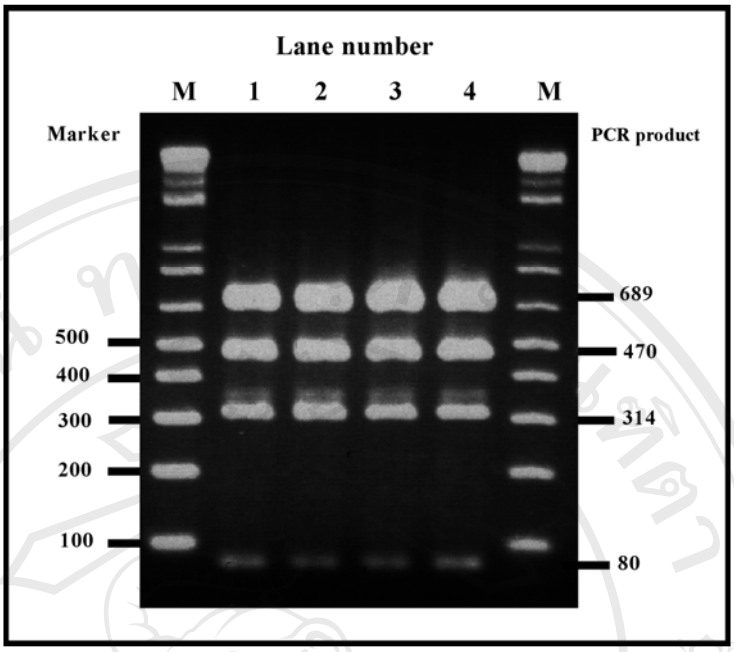

Figure 4.16 PCR products of multiplex PCR set # 2, using various concentrations of genomic DNA. DNA concentrations of 100, 200, 300 and 400 ng/25µl reactions are shown in Lanes 1, 2, 3 and 4, respectively. Lane M showed DNA markers.

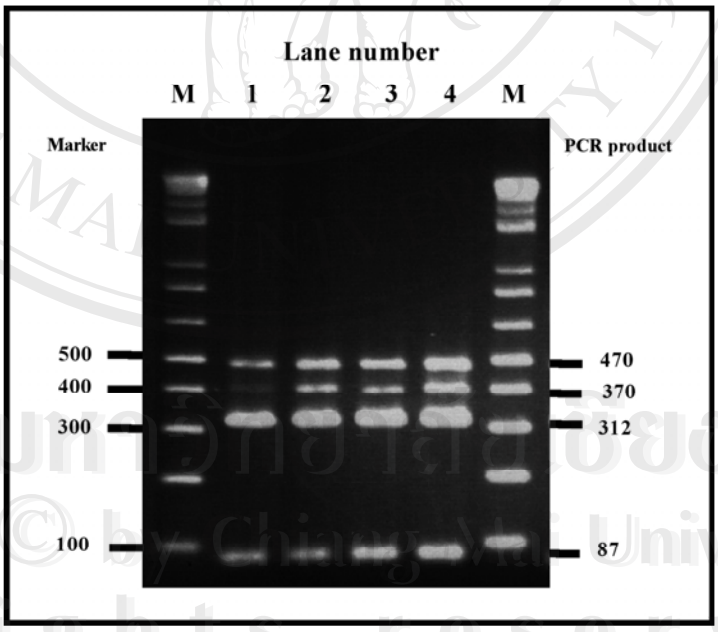

Figure 4.17 PCR products of multiplex PCR set # 4, with various concentration of genomic DNA (100, 200, 300 and 400 ng/25 µl reaction in Lanes 1, 2, 3 and 4, respectively). Lane M showed DNA markers. A DNA concentration of 200 ng/25 µl reaction was selected for all further studies.

4.3 Determination of the optimal annealing temperature

Multiplex PCR sets # 1: The annealing temperature was first set at 62 ºC, following a previously published report[99]. The result showed that PCR product from *RBM1* primers was missing (Figure 4.2). Adjustment of the annealing temperature for a single-primer PCR for *RBM1* was performed. The annealing temperature of 57ºC was found to be optimum (Figure 4.3). At this annealing temperature, the multiplex PCR set # 1 produced all expected PCR products.

Multiplex PCR sets # 2, # 3 and # 4, the annealing temperature was first set at 62 °C according to a previously published report by Lin *et al*[99]. At this annealing temperature, all multiplex PCR sets could produce satisfactory PCR products.

4.4 Optimization of the extension time

The extension times were varied from 1-1.5 min for multiplex PCR sets # 1, # 2, # 3 and # 4. The extension time of 1.5 min produced larger amount of PCR products for the multiplex PCR sets # 1, #2 (Figure 4.18) and # 3. However, the extension time of 1 min gave a better result for multiplex PCR set # 4 (Figure 4.19).

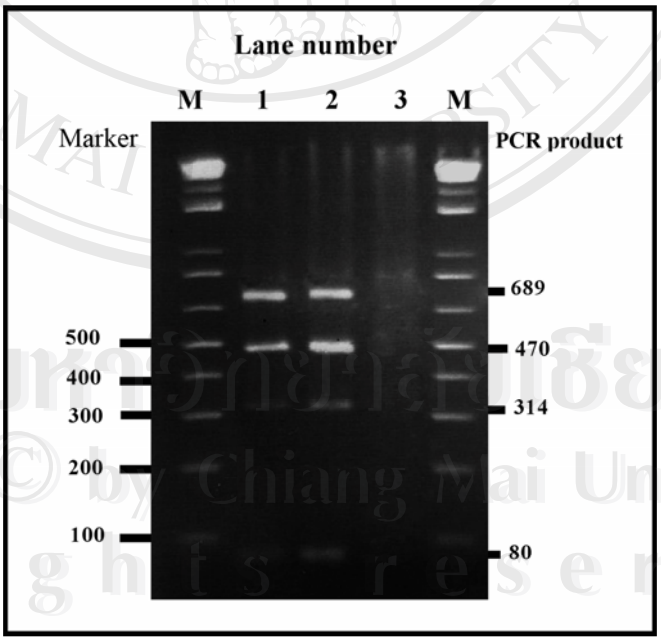

Figure 4.18 Amplification products of multiplex PCR set # 2, with an extension time of 1.0 (Lane 1) and 1.5 min (Lane 2), respectively. Lane 3 showed negative control (DW). Lane M showed DNA markers.

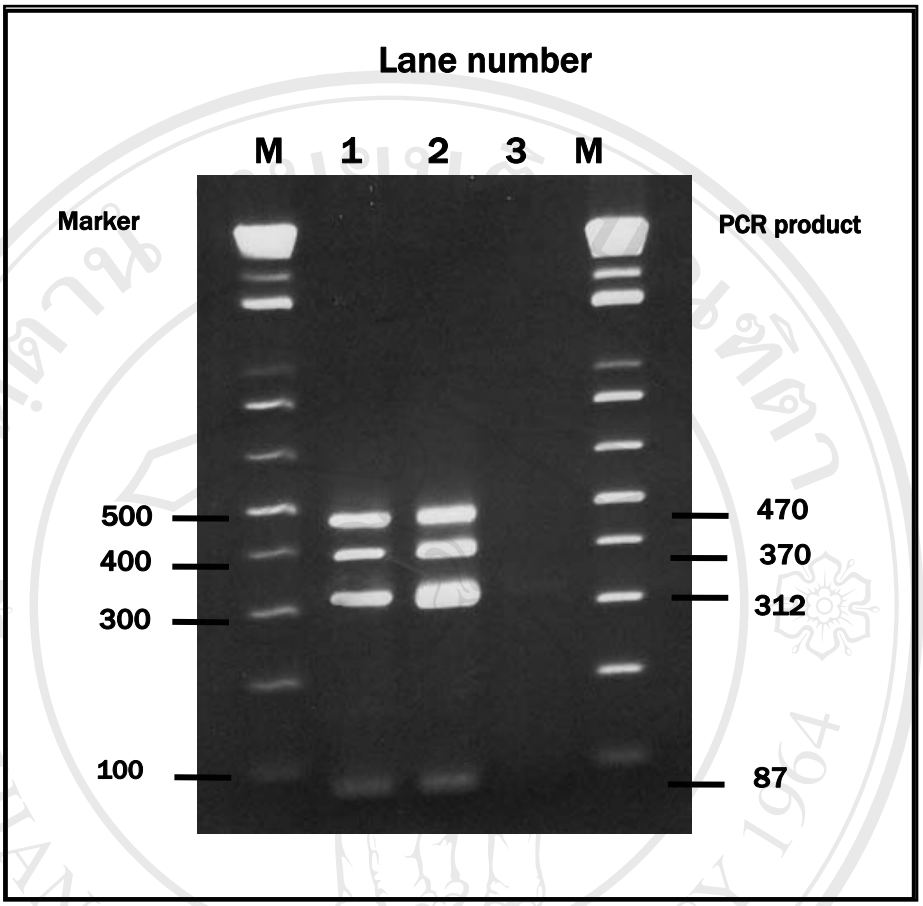

Figure 4.19 PCR products of multiplex PCR set # 4, with an extension time of 1.0 (Lane 1) and 1.5 min (Lane 2), respectively. Lane 3 showed negative control (DW). Lane M showed DNA markers.

4.5 Optimizing the number of PCR cycles

Thirty cycles of multiplex PCR were initially performed, as described by Lin *et al*[99]. However, low product yields were obtained. To optimize the PCR conditions, cycle numbers were increased to 35 and 40 cycles. Forty PCR cycles gave higher yield of products than 35 cycles for multiplex PCR set # 1 (Figure 4.20). For multiplex PCR set # 4, 35 cycles outperformed 40 cycles in three of the four PCR products (Figure 4.21). Thirty-five PCR cycles were found to be optimal for multiplex PCR sets # 2 and # 3. In order to save time by running all 4 mutiplex PCR for each patient in a single round, the PCR conditions should be as similar to each other as possible. Towards this goal, the number of PCR cycles was fixed at 35 and other PCR conditions were further

optimized. The results showed that multiplex PCR sets # 2, # 3 and # 4 could be run together for 35 cycles, by increasing the extension time to 1.5 min. Under this PCR condition, multiplex PCR set # 4 gave satisfactory amplification products as shown in Figure 4.22 However, this condition could not be applied for multiplex PCR set # 1. Therefore, two separate rounds of multiplex PCR were required for each patient.

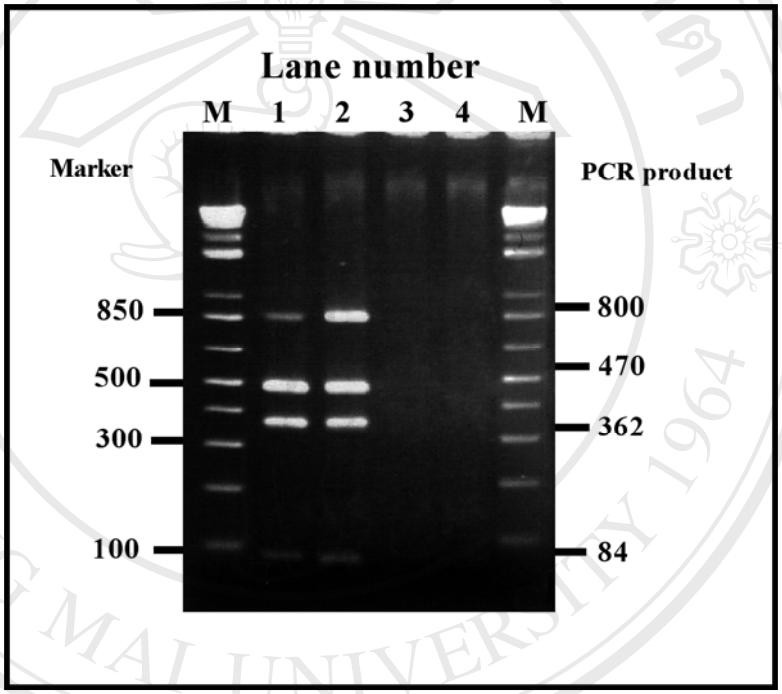

Figure 4.20 Products of multiplex PCR set # 1 with 35 and 40 PCR cycles.. Forty PCR cycles (Lane 2) gave higher yield of products than 35 PCR cycles (Lane 1). Lane 3 (female DNA) and Lane 4 (DW) showed no amplification product. Lane M showed DNA markers.

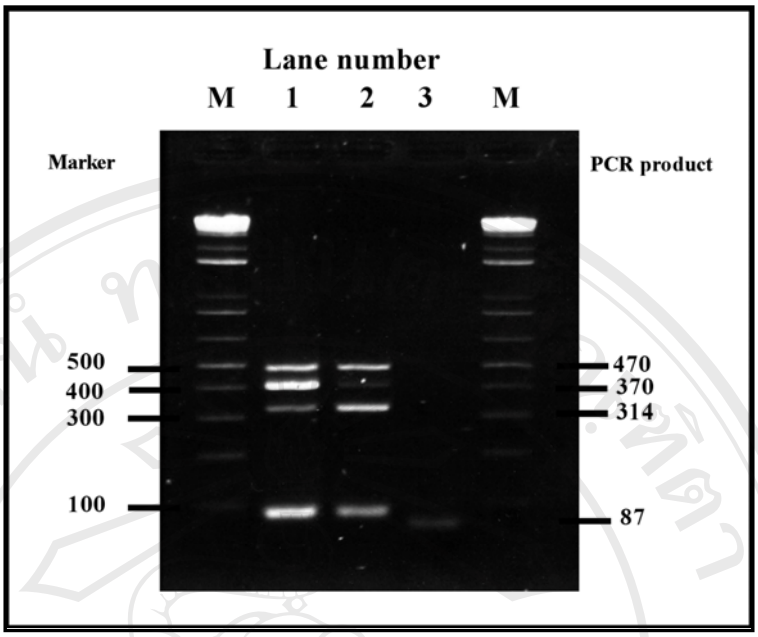

Figure 4.21 Amplification products of multiplex PCR # 4, with 35 PCR cycles in Lane 1 and 40 cycles in Lane 2. Lane 3 showed negative control (DW). Lane M showed DNA markers.

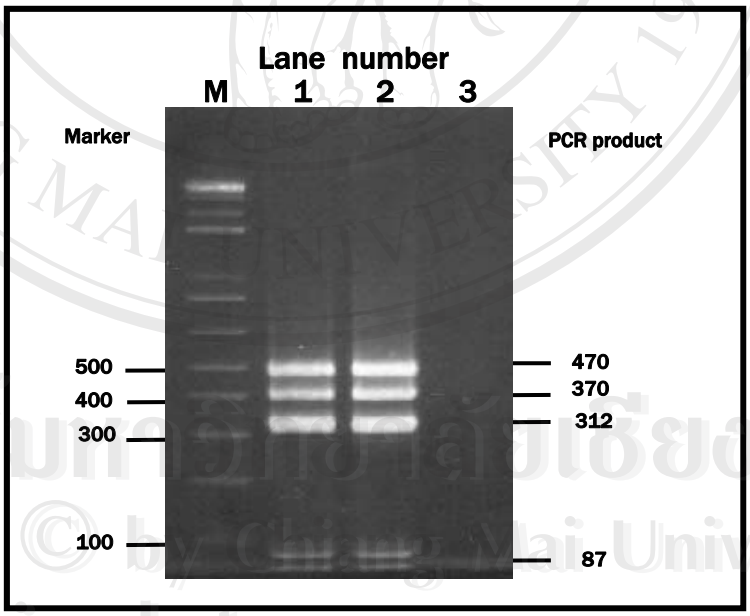

Figure 4.22 Amplification products of multiplex PCR set # 4. Lane M showed DNA markers. Lane 1 showed amplification products after 40 PCR cycles with an extension time of 1 min. Lane 2 showed products after 35 PCR cycles with an extension time of 1.5 min. Lane 3 showed negative control (DW).

4.6 Multiplex PCR conditions for the detection of Y chromosome microdeletions

Amplifications were carried out in a thermal cycler (Px2 Thermal Cycler, Thermo Electron Corporation, Bremen, Germany) using the following conditions:

4.6.1 PCR cycling conditions

Multiplex PCR set # 1 consisted of the following steps:

a) Initial enzyme activation at 95 ºC for 15 min; followed by
b) Forty cycles of PCR: Denaturation at 94 ºC for 1 min, annealing at 57 ºC for 1 min and extension at 72 ºC for 1.5 min;
c) Final extension at 72 ºC for 10 min.

Multiplex PCR sets # 2, # 3 and # 4 were performed together under the same PCR conditions as follows:

a) Initial enzyme activation at 95 ºC for 15 min; followed by
b) Thirty-five cycles of PCR: Denaturation at 94 ºC for 1 min, annealing at 62 ºC for 1 min and extension at 72 ºC for 1.5 min;
c) Final extension at 72 ºC for 10 min.

4.6.2 Multiplex PCR components

Each PCR reaction consisted of 0.12-0.5 µM of each primer (Table 4.5), 1X PCR buffer, 1X Q solution, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 200 ng of genomic DNA, and 1 unit of HotStarTaq DNA polymerase in a total volume of 25 µl.

5. Prevalence of Y chromosome microdeletions in infertile males

Genomic DNA samples from 120 infertile males were tested for Y chromosome microdeletions, using the 4 optimized multiplex PCR sets. Amplification products were shown in Figure 4.23. Samples with Y chromosome microdeletions were shown in Figure 4.24.

Table 4.5 The final concentrations of primers used in multiplex PCR

Mixture no.	Genes	Product size (bp)	Primer concentration (µM)
control	*sY14(SRY)**	470	0.2
1	*SMCY*	362	0.12
	RBM1	800	0.4
	EIF1AY	84	0.04
2	*DBY*	689	0.1
	DAZ(sY283)	314	0.08
	PRY	80	0.1
3	*DFFRY*	130	0.4
	CDY1	79	0.2
4	*DAZ(sY277)*	312	0.2
	TTY2	87	0.3
	BPY2	370	0.2

* Primers specific to *SRY* (control gene) was added to all multiplex PCR sets

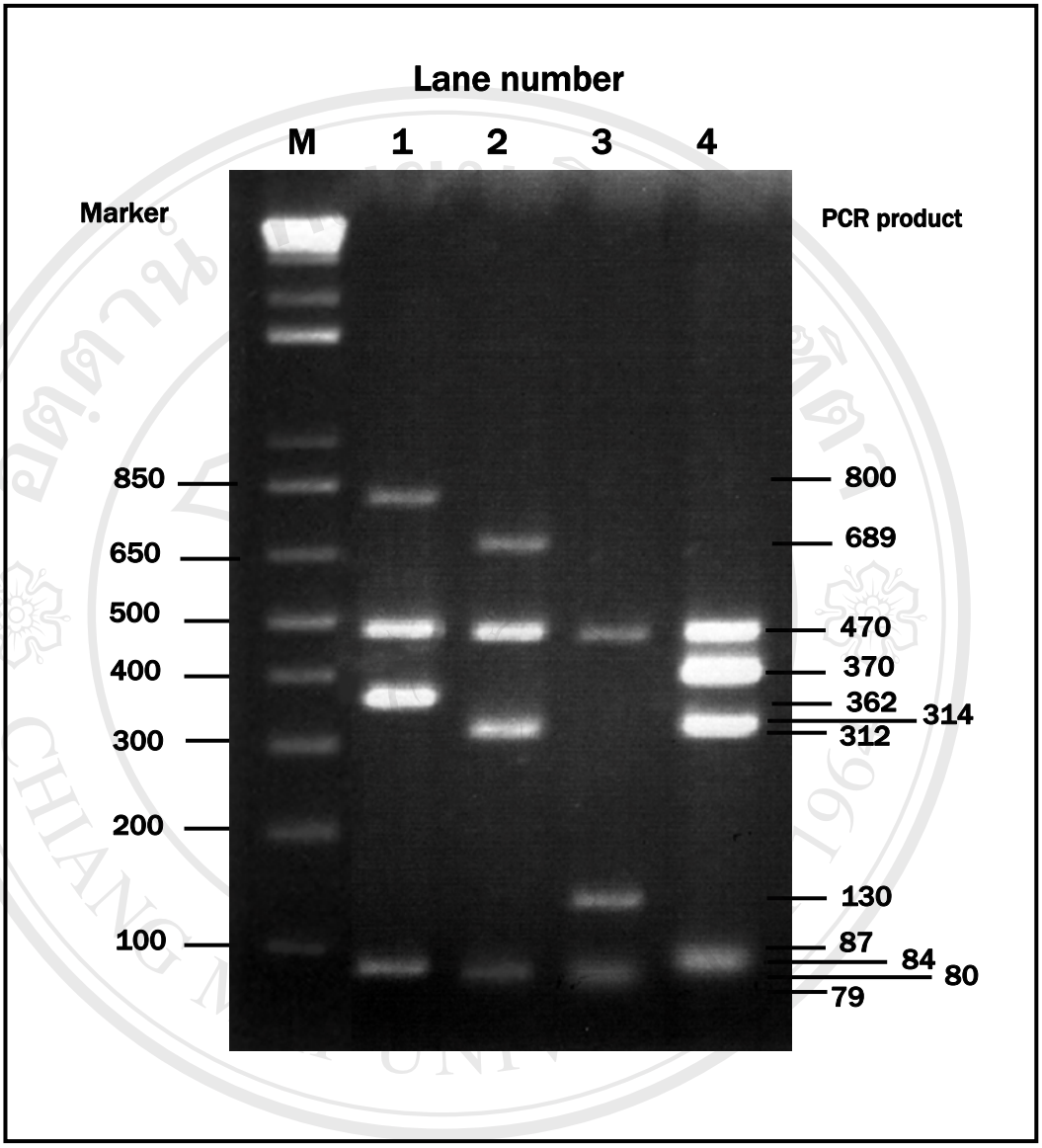

Figure 4.23 Amplification products of the 4 optimized multiplex PCR sets. Lane M showed DNA markers. Lane 1, 2, 3 and 4 showed products of multiplex PCR sets # 1, # 2, # 3 and # 4, respectively. A product of 470 bp represented amplification of *sY14(SRY)* (internal control gene) that was present in all multiplex PCR sets.

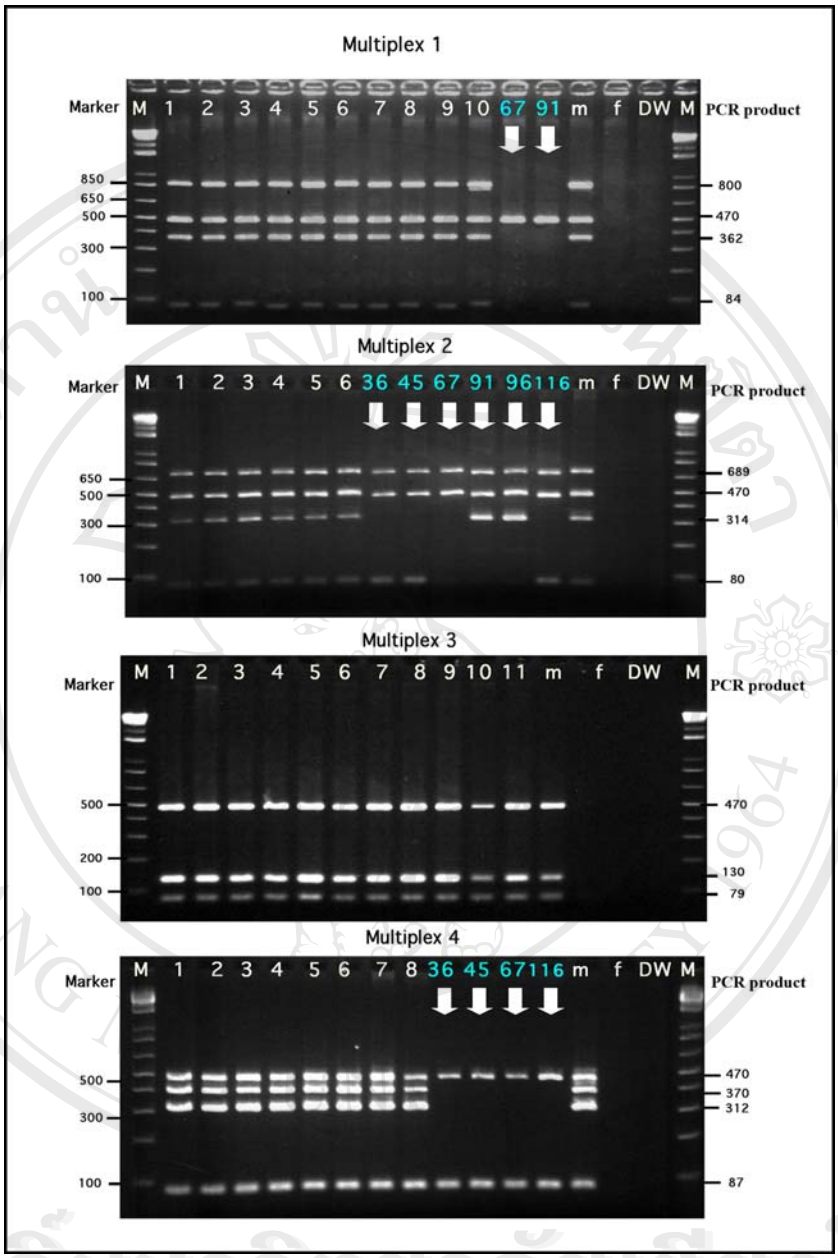

Figure 4.24 Detection of Y chromosome microdeletions using the four optimized multiplex PCRs. Samples with microdeletions were indictaed with arrows and labeled in blue letters and those without microdeletions were labeled in white letters. In multiplex PCR set # 1, patient numbers 67 and 91 had *RBM1*, *SMCY* and *EIF1AY* microdeletions. In multiplex PCR set # 2, patient numbers 36, 45 and 116 had *DAZ(sY283)* microdeletions, patient number 67 had *DAZ(sY283)* and *PRY* microdeletions, patient numbers 91 and 96 had *PRY* microdeletions. None had microdeletions in multiplex PCR set # 3. In multiplex PCR set # 4, patient numbers 36, 45, 67 and 116 had *BPY2* and *DAZ(sY277)* microdeletions. (M = DNA marker, m = normal male control, f = female control, DW = double distilled water)

Six of 120 infertile males (5.0%) had microdeletions of one or more genes on the Y chromosome. Five of them (patient numbers 36, 45, 67, 91 and 116) had azoospermia (5 in 40 azoospermic males or 12.5%) and one (patient number 96) had oligospermia (1 in 80 oligospermic males or 1.25%). Three azoospermic patients (numbers 36, 45 and 116) had microdeletions in the AZFc region. One azoospermic patient (number 91) and one oligospermic patient (number 96) had microdeletions in the AZFb region. This oligospermic patient, who presented with severe oligospermia, had deletion of a single gene (*PRY*) in the AZFb region. Another azoospermic patient (number 67) had a wide range of microdeletions involving AZFb and AZFc regions. The remaining patients had microdeletions of more than three genes. Patients numbers 36, 45 and 116 had deletion of *DAZ(sY277), DAZ(sY283)* and *BPY2* in the AZFc region. Patient number 91 had deletion of *SMCY, EIF1AY, RBM1* and *PRY* in the AZFb region. Patient number 67 had a wide deletion, involving *SMCY, EIF1AY, RBM1* and *PRY* genes in the AZFb region and *DAZ(sY277), DAZ(sY283)* and *BPY2* genes in the AZFc region. No patient had microdeletion involving AZFa region. The results were summarized in Figure 4.25 and Table 4.6.

DNA samples from 50 control males were subjected to multiplex PCR amplification under the same conditions as the infertile males. None of them showed Y chromosome microdeletions.

For each assay, three samples were incorporated as internal controls: a genomic DNA sample from a normal fertile male, a genomic DNA sample from a normal fertile woman and a PCR mixture containing water instead of DNA (blank control). Amplification failures in suspected microdeleted cases were further confirmed by at least two more amplification failures.

6. Cost comparison between the in-house multiplex PCR assay and the commercial kit (Promega®, MI, USA)

Cost calculation was based on market prices of reagents, consumables and the price of the commercial kit in the year 2005. Only direct cost was considered. The cost of instruments, other hardware, salary, labor cost, intangible costs and other indirect costs were not included. The details of the costs for the in-house multiplex PCR assay

and the commercial kit (Promega®) were shown and compared in Table 4.7. Overall, the commercial kit was five times more expensive than the in-house assay.

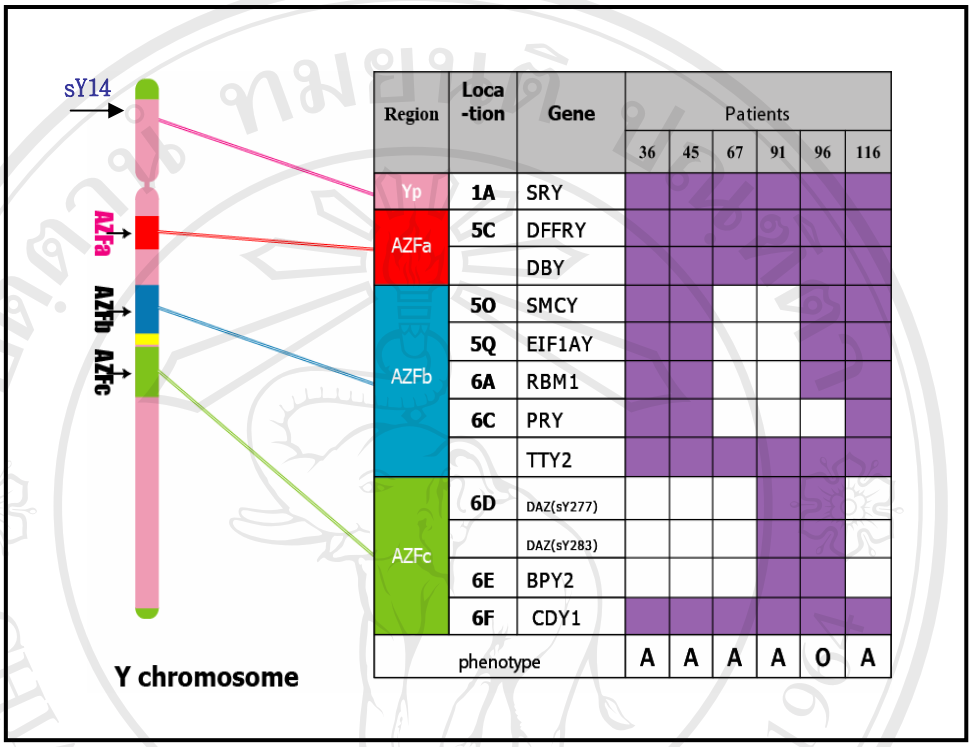

Figure 4.25 Schematic diagram of Y chromosome microdeletions in the six infertile males in this study. The positions of gene-based markers in the 4 multiplex PCRs were illustrated. Patient identifications were shown at the top of the panel. Solid boxes indicated the presences of genes and open boxes indicated gene deletion. Phenotypes were indicated as A for azoospermia and O for oligospermia.

Table 4.6 Summary of patients with regions of Y chromosome microdeletions

Patient number	Deletion regions
36	AZfc
45	AZFc
67	AZFb, AZFc
91	AZFb
96	AZFb
116	AZFc

Table 4.7 Comparison of costs between the in-house multiplex PCR assay and the Promega® kit**.**

Cost details	**In-house multiplex PCR (Baht)**	**Promega® kit (Baht)**
Cost per test		
Kit price/ test	-	3,043
PCR components (primers, dNTPs)	150	-
HotStarTaq	100	-
AmpliTaq Gold	-	190
DNA extraction	150	150
PCR consumable and electrophoresis (PCR tube, NuSieve gel, TBE buffer, ethidium bromide, DNA marker, tip)	-	397
PCR consumable and electrophoresis (PCR tube, agarose gel, TBE buffer, ethidium bromide, DNA marker, tip)	350	-
Total cost	**750**	**3,780**