VALIDATION STUDIES ON THE Y- PLEX™ 6 KIT

Joseph E. Warren, Ph.D.
Assistant Laboratory Director, ReliaGene Technologies Inc.

Introduction

The ability to analyze genetic variation found in the Y chromosome has several useful applications in the fields of Forensic Biology, Genealogical / Paternity analysis and Evolutionary Biology/ Anthropological studies. This usefulness is based on the biology of the Y chromosome itself. Genetic markers found on the Y chromosome are male specific, paternally inherited and, like mitochondrial DNA, are inherited as a haplotype. Thus, for example, in criminal investigations evidence involving female/male mixtures, multiple male assailants or aspermic samples can be analyzed with increased precision.

Despite this, Y chromosome analysis is not a widely used technique in the United States for forensic casework. One of the principle reasons is the lack of a validated kit capable of performing multiplex PCR amplifications along with an allelic ladder to insure accurate and reproducible haplotype results. ReliaGene Technologies Inc. has developed a Y STR based kit, along with an allelic ladder, capable of amplifying six loci. The development of this kit has been described previously(1). This paper will give a brief description of some of the validation studies performed by several independent laboratories on this kit.

Kit Description and Amplification Conditions

The Y-PLEX™ 6 Kit is made up of 6 loci that are labeled with two fluorescent dyes. The loci labeled with the blue dye, FAM, are DYS393 (116-132bp), DYS19 (174-210bp),and DYS389 (288-320bp). The loci labeled with the yellow dye, TAMARA, are DYS390 (174-210bp), DYS391 (245-265bp), and DYS385 (345-385). It should be noted that the loci DYS385 are two tandem duplicated loci, thus each individual will have two peaks (alleles). The kit includes the allelic ladder, the reaction mix that includes the primers, the known male positive control and a female negative control. The PCR reaction set-up is as follows:

5X Reaction Mix – 5.0 µL
AmpliTaq Gold™ (5u/µL) – 0.50µL
Template DNA (0.5- 2.0ng) – 19.50µL
Total Reaction Volume – 25.0µL

Amplification Conditions:

95oC – 10 minutes

link to-

28 cycles of-

94oC – 30 seconds
59oC – 1 minute
70oC – 1 minute
link to-

60oC – 40 minutes

link to-

4 oC – soak

note: These conditions are optimized for the ABI 9600 and/or ABI 9700 thermal cyclers.

Validation Studies were Conducted at ReliaGene Technologies Inc. by Steven Richey, BS, Patrick Cooke, Ph.D., David Bulot, BS, and Joseph E. Warren, Ph.D. All studies performed at ReliaGene used the ABI 310 Genetic Analyzer

Sensitivity Study

A sensitivity study was performed in order to assess the optimal amount of template DNA that can be reliably analyzed. This particular study utilized 0.25µL of AmpliTaq Gold™ DNA Polymerase. Analysis was carried out with the following concentrations of template DNA:

10.0ng, 7.5ng, 2.5ng, 1.25ng and 0.625ng

The results are as follows:

DYS389,391,19,390 and 393 all revealed interpretable results at all of the concentrations between 10ng- 0.625ng. DYS385 was less sensitive providing results between 10ng and 2.50ng.

Serial Dilution Study

As a result of the sensitivity study it was decided that the amount of AmpliTaq Gold™ be increased from 0.25 to 0.50µL in order to assess if an increase in sensitivity could be achieved. A serial dilution study was carried out in conjunction with the added volume of the AmpliTaq ™ Gold. The results are as follows:

3.0- 4.0ng of Template DNA

The results revealed off-scale data, many stutter peaks were called as to being alleles and off-ladder alleles were called. All of this is consistent with an overloaded sample due to too high a level of template DNA.

1.5-2.0ng of Template DNA

Overall, the results were much improved with the lower amount of DNA. There were still some off- ladder alleles and stutter peaks called.

0.75ng-1.0ng of Template DNA

Clear cut, interpretable results, were obtained from this concentration of template DNA.

0.375- 0.500ng of Template DNA

The results were highly interpretable. All stutter disappeared at this concentration and no loci dropout was observed.

0.187- 0.250ng of Template DNA

Except for a drop in RFU, the results are similar to those reported above.

0.093ng- 0.125ng of Template DNA

Level of RFU dropped, but results were still interpretable and no loci dropout was observed.

As a result of this study, the recommended concentration of template DNA was determined to be between 0.500ng- 1.50ng and the amount of AmpliTaq Gold ™ was changed from 0.25µL to 0.50µL.

Female/Male Mixture Study

Female to male extracted DNA was mixed together in the ratios of 10:1, 5:1, 2:1, 1:1, 1:2, 1:5 and 1:10. The purpose of this study was to determine first if any female DNA would amplify in a mixed sample and secondly at what level would the female DNA inhibit results from the male fraction. 1.0ng of template DNA was amplified. The results are as follows:

DYS389- 10:1-2:1- no result
1:1-1:10- male only allele detected
DYS391- 10:1-2:1- no result
1:1-1:10- male only allele detected
DSY19-10:1- 2:1- no result
1:1- 1:10- male only allele detected
DYS390- 10:1-2:1- no result
1:1-1:10- male only DNA detected
DYS393- 10:1-2:1- no result
1:1-1:10- male only DNA detected
DYS385- 10:1-1:1- no result
1:2-1:10- male only DNA detected

According to these results, DYS385 seems to be the loci most affected by the presence of large amounts of female DNA.

Forensic Mixture Study

This particular study involved mixing whole blood from two unrelated males onto sterile, cotton tipped applicator sticks in varying ratios and letting them dry for a 24-hour period. The stains were extracted organically with the resulting DNA subjected to Y STR analysis. The ratios were 50:1, 25:1, 10:1, 5:1, 2:1, 1:1, 1:2, 1:5, 1:10, 1:25 and 1:50. Approximately 1.0 ng of template DNA was amplified. The following observations were noted:

Mixtures can be detected at various loci starting at 50:1 going all the way to 1:50.
The loci labeled with FAM (DYS393, DYS19 and DYS389) were more sensitive at detecting mixtures then were the loci labeled with TAMARA (DYS390, DYS391 and DYS385).

Mixture Study with Known Amounts of DNA

A second mixture study was performed this one involved analyzing mixed samples of extracted DNA from two unrelated males. This time though, the ratios remained constant while the template DNA concentration varied. The experiment was designed as follows:

	Ratios	
	5:1	2:1
DNA Concentration	2.0ng	2.0ng
	1.0ng	1.0ng
	0.60ng	0.60ng
	0.30ng	0.30ng
	0.15ng	0.15ng

The two samples analyzed had the same haplotype at all loci except for DYS390, so that was the allele used to monitor the mixture. The results are as follows:

The minor contributor was detected at all DNA concentrations except at 0.150ng. The loci DYS390, DYS391 and DYS385 dropped out at 0.300 and 0.150ng with the loci DYS385 dropping off first. The stochastic level appears to be at a level of 200 RFU and less.

Precision Study on the ABI 310 Genetic Analyzer

A precision study was performed on the ABI 310 Genetic Analyzer by running the same samples numerous times and then calculating the mean base pair size and standard deviation for the resulting alleles from each loci. The results are as follows:

DYS389
Allele 29

N= 13
Mean=302.44bp
SD= 0.31bp

Allele 30
N=13
Mean = 306.31bp
SD = 0.77bp

DYS391
Allele 10
N= 13
Mean = 249.49
SD= 0.11

Allele 11
N= 14
Mean = 253.60
SD= 0.11

DYS19
Allele 14
N= 16
Mean = 189.66
SD= 0.17

Allele 15
N= 8
Mean = 193.39
SD= 0.20

Allele 16
N=8
Mean = 197.31
SD= 0.17

DYS390
Allele 20
N= 8
Mean = 183.39
SD = 0.13

Allele 22
N= 16
Mean = 191.60
SD= 0.18

DYS393
Allele 12
N= 34
Mean = 120.58
SD= 0.16

DYS385
Allele 9
N= 10
Mean = 356.11bp
SD= 0.11bp

Allele 13
N= 14
Mean = 370.65bp
SD= 0.25bp

Allele 14
N= 7
Mean = 374.58bp
SD= 0.16bp

Allele 15
N=11
Mean = 378.32bp
SD= 0.14bp

Precision Study on the ABI Prism® 377 DNA Sequencer

A precision study was performed on the ABI Prism® 377 DNA Sequencer by running the ladder seven times in a single gel and calculating the mean and standard deviation for each allele. The results are as follows:

DYS393
Allele 11
Mean = 119.19
SD= 0.07

Allele 12
Mean = 123.22
SD= 0.08

Allele 13
Mean = 127.28
SD= 0.06

DYS19

Allele 13
Mean = 187.66
SD= 0.03

Allele 14
Mean = 191.68
SD= 0.04

Allele 15
Mean = 195.69
SD = 0.04

Allele 16
Mean = 199.75
SD = 0.03

DYS389

Allele 28
Mean = 298.09
SD= 0.04

Allele 29
Mean = 301.99
SD = 0.04

Allele 30
Mean = 305.97
SD= 0.04

Allele 31
Mean = 309.93
SD= 0.05

Allele 32
Mean = 313.95
SD= 0.04

Allele 33
Mean = 317.94
SD = 0.05

DYS390

Allele 22
Mean = 185.88
SD= 0.04

Allele 23
Mean = 189.90
SD= 0.03

Allele 24
Mean = 193.95
SD= 0.03

Allele 25
Mean = 197.99
SD= 0.05

DYS391

Allele 9
Mean = 246.96
SD= 0.04

Allele 10
Mean = 250.93
SD= 0.05

Allele 11
Mean = 254.81
SD= 0.03

Allele 12
Mean = 258.72
SD= 0.05

DYS385

Allele 8
Mean = 344.38
SD= 0.03

Allele 10
Mean = 352.30
SD= 0.03

Allele 11
Mean = 356.09
SD= 0.04

Allele 12
Mean = 359.89
SD= 0.05

Allele 13
Mean = 363.68
SD= 0.07

Allele 14
Mean = 367.45
SD= 0.06

Allele 15
Mean = 371.23
SD= 0.07

Allele 16
Mean = 375.00
SD= 0.10

Allele 17
Mean = 378.74
SD = 0.09

Allele 18
Mean = 382.49
SD = 0.08

Allele 19
Mean = 386.19
SD = 0.05

Validation Studies Performed by Leanne Giusti, BS, Sharon Convery, and Joannie Sguelia, BS of the Massachusetts State Police Crime Laboratory

The Massachusetts State Police Crime Laboratory performed a series of four studies on the Y-PLEX™ 6 Kit. These studies were all performed on the ABI® 310 Genetic Analyzer using a rfu of 75 as the threshold for making allele calls. These studies were a sensitivity study comparing the results from DNA extracted organically versus the results from the same sample extracted with Chelex™, a tissue reproducibility study and a male/female mixture study. The tissue reproducibility study looked at blood, saliva, semen, and hair from various male donors. In all

cases, the different tissues produced the same type. The male/female mixture study failed to reveal any interfering DNA typing results from the female donor. The sensitivity study revealed an effective range of template DNA to be between 0.500 and 1.50ng. The organic extraction and Chelex™ extracted samples produced comparable results for the blood and saliva stains. The organic method produced better results then the Chelex™ method on semen stains.

Study by Pat Wojitkiewicz, Ph.D. and Mary Jones, MS of the North Louisiana Criminalistics Laboratory.

The North Louisiana Criminalistics Laboratory performed their analysis on an ABI Prism 377 DNA Sequencer with a rfu of 150 as the threshold for calling alleles. This laboratory ran several samples of unrelated males in order to optimize the Y-PLEX™6 Kit for use on the ABI Prism 377 DNA Sequencer. This laboratory found that they obtained the best results while using 2.0-5.0ng of template DNA with a reaction mixture that includes 1µL of BSA (25X). Several Off-ladder variant alleles from various loci were confirmed.

Study by Dolores Schoenbauer, MS and Ann Gross, MS of the Minnesota Bureau of Criminal Apprehension.

The Minnesota Bureau of Criminal Apprehension performed three excellent studies on the Y-PLEX™ Kit. The first study was a non-human study to determine if any of the primers would produce results on non-human DNA. The second was a male/female mixture study involving stains at various ratios analyzed at 150 and 75 rfus in order to compare sensitivity at the two rfu levels, as well as loci dropout. The final study was a population study with American Indians.

Results

Non-human DNA

DNA was extracted and amplified from the following non-human animals:

Mule Deer, Rat, Rabbit, Musk, Ox, Dog, Lamb, Chevrotain, Monkey, Horse, Pig, Cow, Elephant, Greater Kudo, Tapir, Mountain Goat, Xavier Monkey, Moose, Wolf, Llama, Tiger, Mallard, Zebra, Deer and Sheep

No amplification was observed.

Male/ Female Mixture Study

Sample	Ng DNA male	Ng DNA female	150rfu	75rfu
Female	0	5.0	No result	No result
Male	5.0	0	Full profile	Full profile
1:1	2.5	2.5	Full profile	Full profile
1:2	1.6	3.3	Full profile	Full profile

1:3	1.5	3.5	DYS389,385-nr	Full profile
1:4	1.0	4.0	DYS389,385 -nr	Full profile
1:5	0.8	4.2	DYS385- nr	Full profile
1:10	0.5	4.5	DYS389,385-nr	DYS385-nr
1:20	0.2	4.8	No result	19,389,390,385 -nr
1:50	0.1	4.9	No result	No result
1:100	0.05	4.95	No result	No result

Nr = no result

There are several observations that can be observed in the above matrix. First, no results were obtained from the female only DNA. Second, a detection level of 75rfu is more sensitive then 150 rfu. Third, DYS385 and DYS389 are the two loci that are most prone to loci dropout.

Native- American Population Study

Fifty(50) male American Indian samples that had been previously profiled by RFLP, DQA1/PM and STR analysis were analyzed for the Y STRs. The full population data is not in, but some interesting observations were noted. An individual had a tri- allelic pattern for DYS385. Two off-ladder variants were confirmed. One occurred at DYS390, allele 20 and the second one at DYS393, allele 14.

Study by John Planz, Ph.D. and Arthur Eisenberg, Ph.D. of the University of North Texas Health Science Center at Fort Worth

The University of North Texas Health Science Center at Fort Worth is currently conducting two very important studies. The first one is to develop several population databases. These databases would be from Caucasians, African-Americans, Hispanics, and Asian. The second study is to determine a mutation rate by analyzing several hundred known father-son pairs.
This work is being performed using the Hitachi FMBIO® Gel Reader with the samples being stored on and extracted from FTA™ Cards. These are two methodologies that have not been evaluated for use with the Y-PLEX™6 kit. Two important observations have already been made. One is that this kit is very sensitive on the amount of template DNA amplified. Because DNA quantitation on FTA extracted samples is not practical it was found that the primer concentration had to be cut in half in order to obtain results that were not over blown on the gel. The second observation was that in order to reach a detectable level, the amount of ladder added to the gel had to be increased four times that what was used on the ABI 310 and 377. When these changes were used, reliable results were obtained.

Overall Observations

In general, the kit has worked well so far for all of the users. The two main suggestions were that the primer concentrations should be slightly readjusted to obtain an overall better balance. The second suggestion was that the allelic ladder needs to be better balanced to cut down on the stutter peaks and split peaks that have sometimes been observed.

Future Studies

Several future studies are planned in order to fully assess the reliability of this kit. They are:

- Analysis of aspermic samples
- Analysis of non-probative casework samples
- Dnase degradation study
- Effects on varying the amplification conditions
- Identification of off-ladder variants
- Population studies and databases
- The addition of more loci in order to increase the power of discrimination of the kit

References

1. Sinha, SK, Santosh, A. Development of Multiplexed Y-Chromosome STR Genotyping System for Use with the ABI 310 Genetic Analyzer. Proceedings of the American Academy of Forensic Sciences Feb. 2000