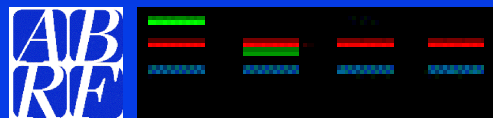


Fragment Analysis Research Group 2002 Study

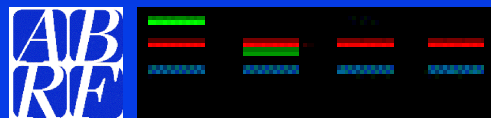
Comparing Laboratory
Protocols for
Multiplexing Markers in
a DNA Fragment
Analysis Application.



Fragment Analysis Research Group

2002 Fragment Analysis Research Group

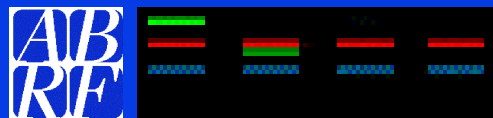
- **Doug Bintzler, Chair** University of Cincinnati
- **Duane Bartley, Past Chair** Johns Hopkins University
- **Pamela Scott Adams** Trudeau Institute
- **Yongde Bao** University of Virginia School of Medicine
- **Laura Kasch** Johns Hopkins University
- **Lynn Petukhova** The Rockefeller University
- **Caprice Rosato** Oregon State University
- **Bob Keefe** Wadsworth Center



Fragment Analysis Research Group

Presentation

1. FARG Studies
2. Study Format
3. Study Results
4. Tutorial - Optimizing PCR
5. Applying Optimization Procedures



Fragment Analysis Research Group

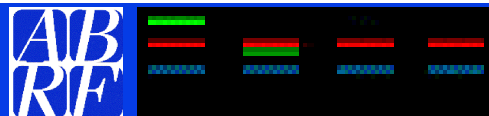
FARG - Historical

1999 General Survey

FRAGMENT ANALYSIS; A CHANGING FIELD AND A NEW COMMITTEE

Duane A. Bartley⁽¹⁾, Linda Wood Ballard⁽²⁾,
Yongde Bao⁽³⁾, Doug Bintzler⁽⁴⁾, George Grills⁽⁵⁾,
Laura Kasch⁽¹⁾.

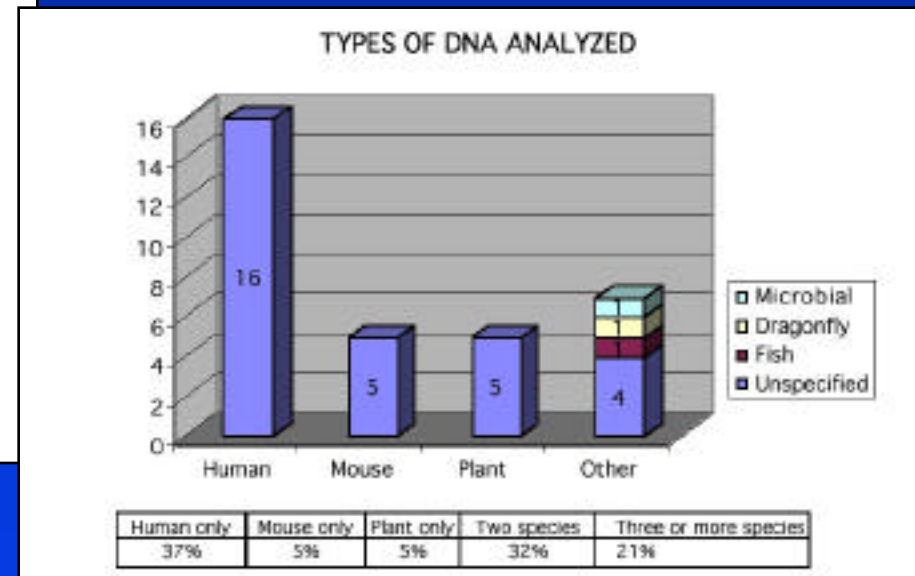
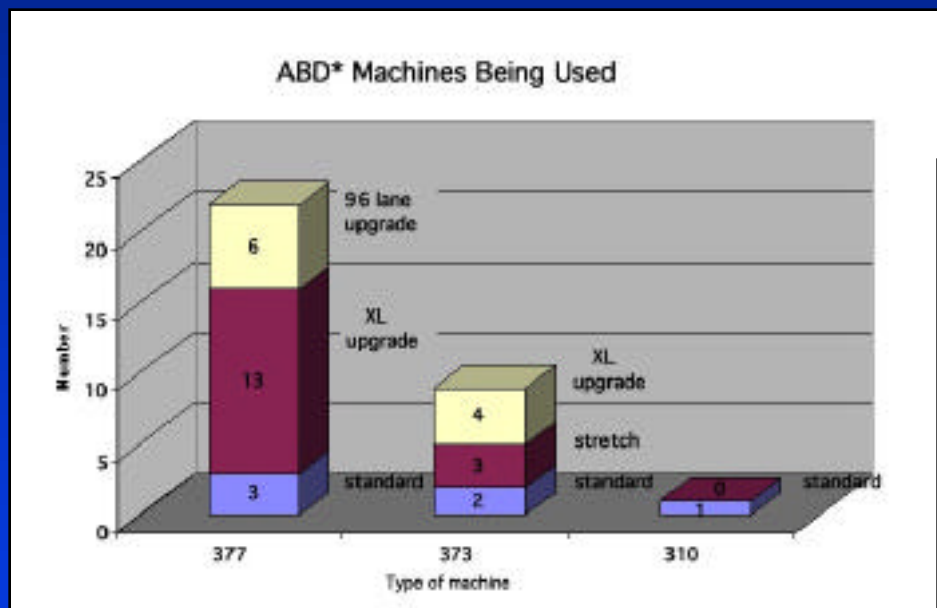
⁽¹⁾Genetic Resources Core Facility, Johns Hopkins University, Baltimore, MD, 21287; ⁽²⁾Genomics Core Facility, University of Utah, Salt Lake City, UT, 84112; ⁽³⁾Department of Microbiology, University of Virginia School of Medicine, Charlottesville, VA, 22908; ⁽⁴⁾University of Cincinnati, Cincinnati, OH, 45267; ⁽⁵⁾Albert Einstein College of Medicine, Bronx, NY, 10461.



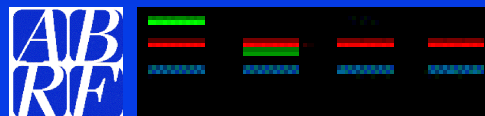
Fragment Analysis Research Group

Previous Studies

1999 General Survey



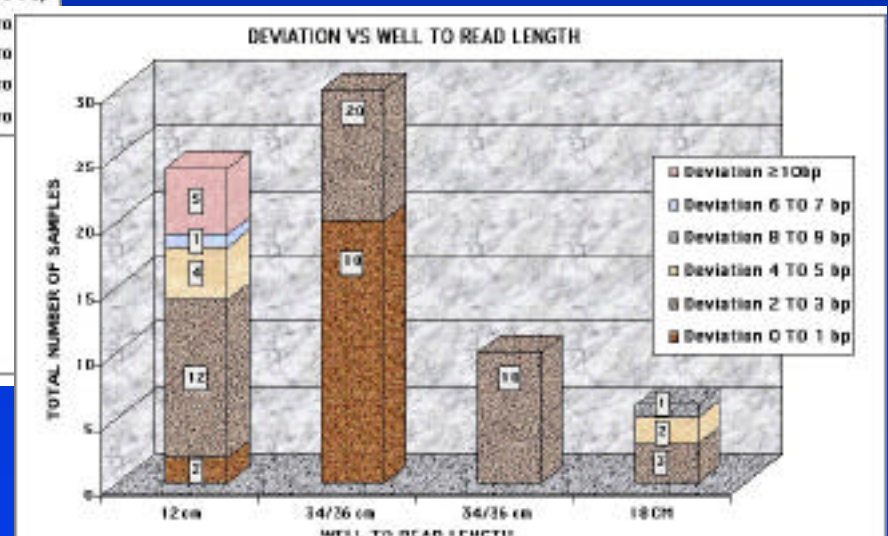
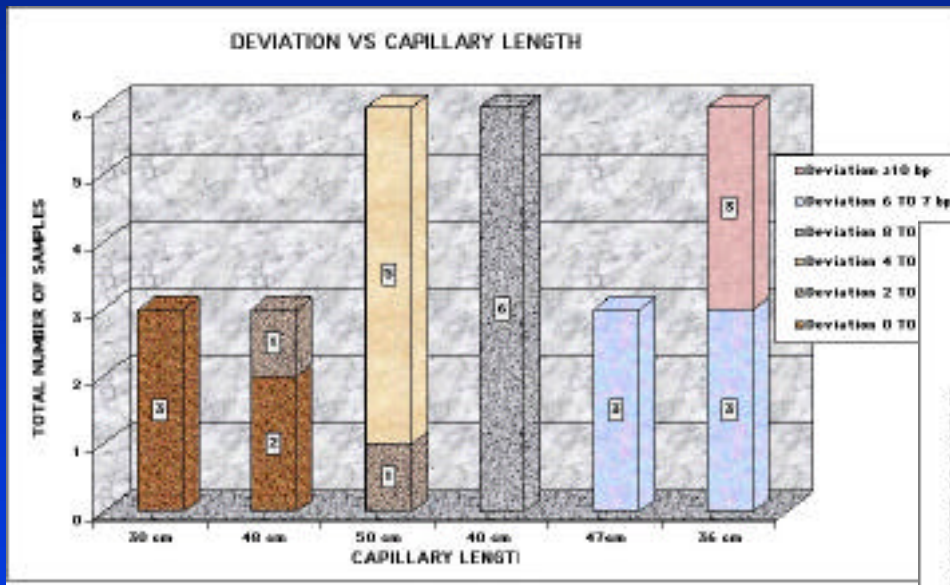
The first study conducted by FARG was an on line survey to determine the types of fragment analysis services performed by members of the ABRF. This slide shows 2 results for the study.



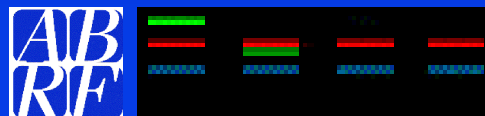
Fragment Analysis Research Group

Previous Studies

2000 Test Samples



The study for ABRF 2000 included the results from 2 samples that were sent to participants. These were load only samples. One comparison that was made from the results was the read length and accuracy.

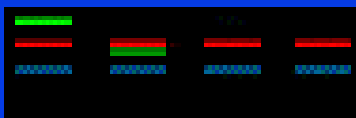
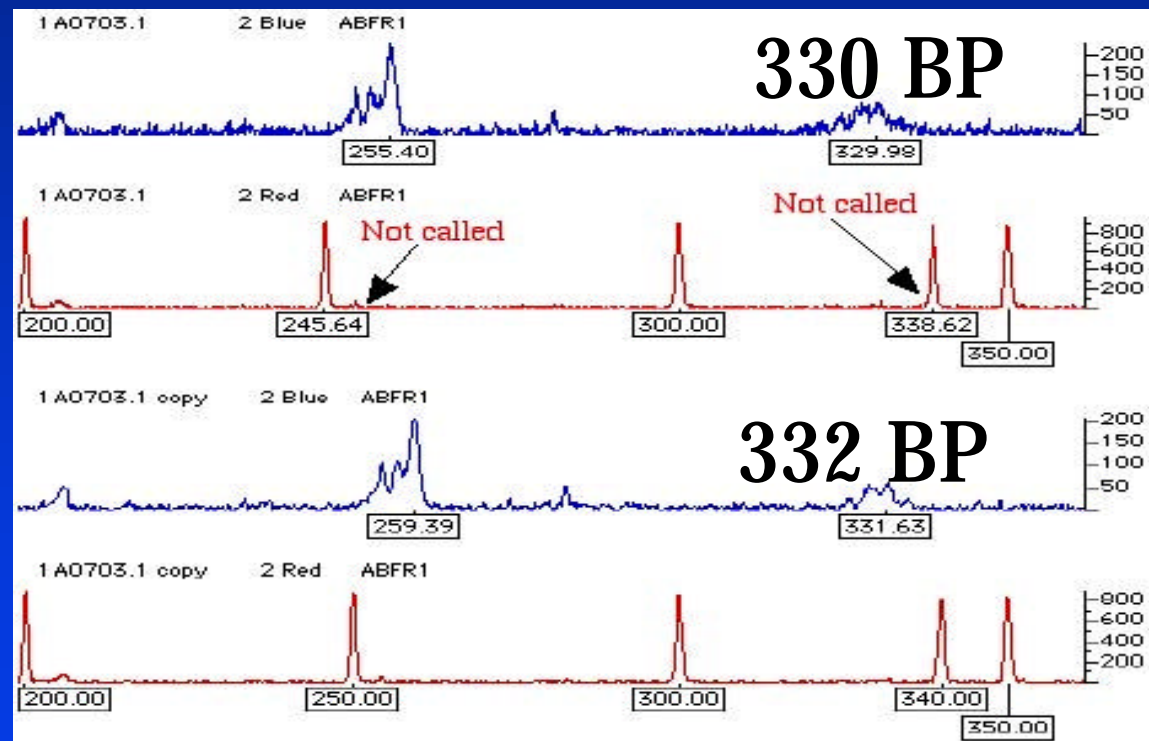


Fragment Analysis Research Group

Previous Studies

2000 Test Samples

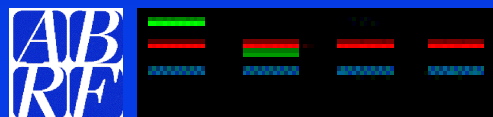
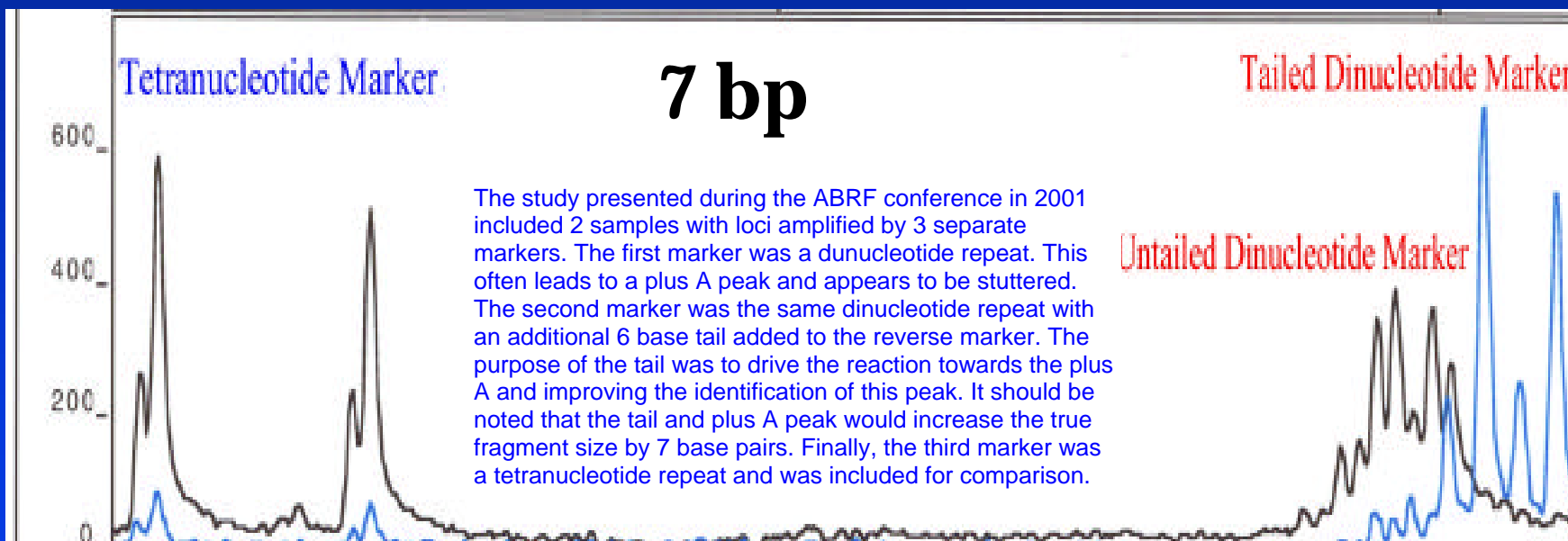
It was also observed from this study that some of the results returned to FARG were analyzed using standard values that were incorrectly called. By applying the correct base pair values to the standard, a more accurate value for the samples can be determined.



Fragment Analysis Research Group

Previous Studies

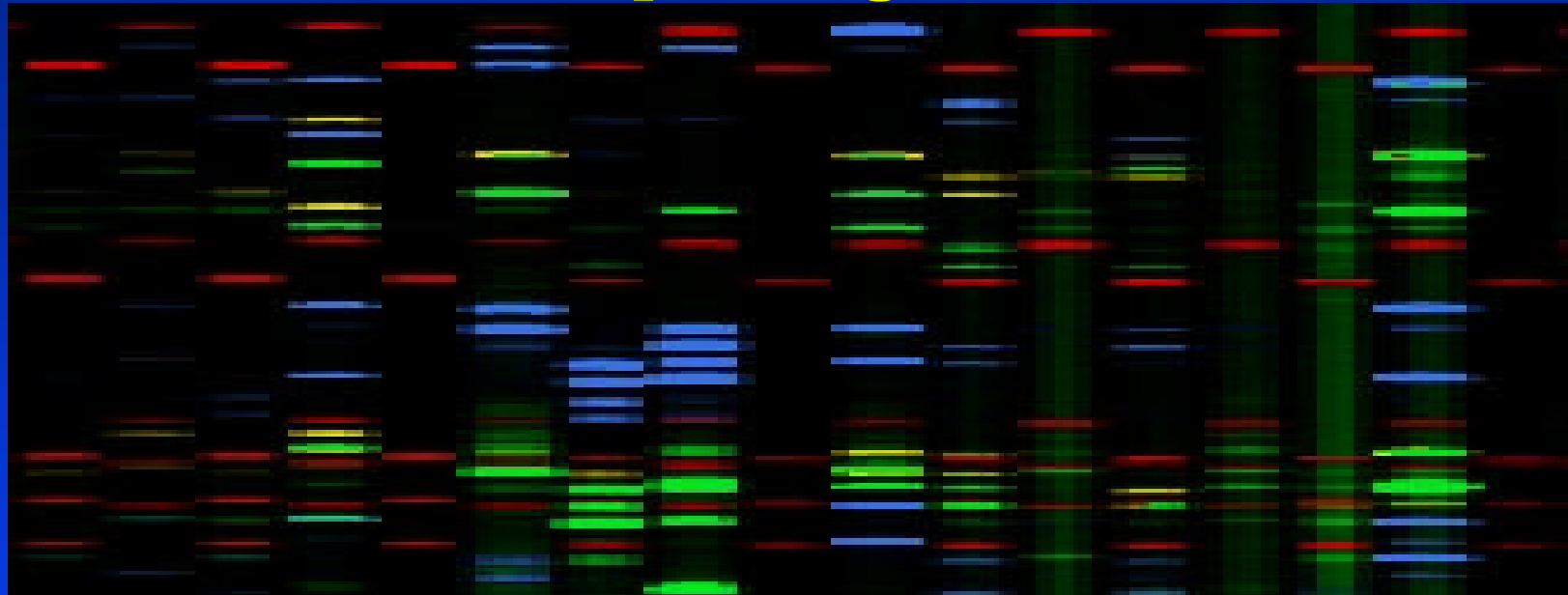
2001 Problems Associated with Dinucleotide Markers and the Plus A Peak



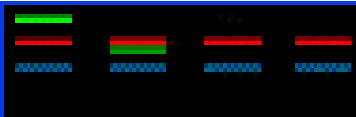
Fragment Analysis Research Group

Current Study

Multiplexing Markers



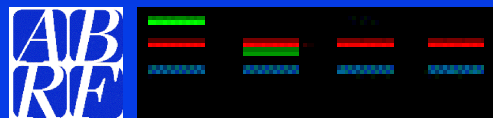
The study this year dealt with the question of multiplexing several markers into a single PCR. Study participants were sent 2 templates with 5 labeled markers. Participants could choose to amplify the templates with all five markers in a single reaction (PCR multiplexing) or amplify the templates with each marker separately and then combine the products together (post-PCR multiplexing). Results and methods used by study participants were compared.



Fragment Analysis Research Group

Goals of the Study

1. Self Evaluation and Education
2. Look at Alternative Methods of optimizing
3. To present methods that may reduce cost and time



Fragment Analysis Research Group

Study Process

1. Participants of the study requested samples on-line

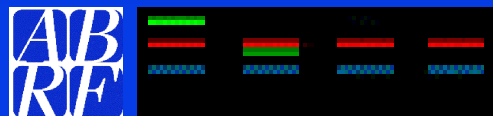
DNA Fragment Analysis Research Group Year 2002 Sample Request Form

Please complete the form below to request samples:

Mailing Address:

E-mail Address:

[Return to the FARG 2002 Study Cover Letter](#)



Fragment Analysis Research Group

Study Process

Samples and instructions sent to Requesting Facilities

WELCOME TO THE ABRF FRAGMENT ANALYSIS STUDY 2002:

Dear Colleague:

Since fragment analysis is a growing field, cost and efficiency combined with accuracy will become increasingly important. Multiplexing of markers is one way to increase efficiency. Performing separate PCR reactions for each marker and then pooling the resulting labeled products before electrophoresis is one strategy for increasing efficiency. However, it is more cost effective to pool the markers prior to PCR and perform a multiplex PCR reaction, since the cost of the Taq DNA polymerase is a major component of the expense involved. The purpose of the 2002 FARG study will be to investigate strategies for multiplexing, both pre and post PCR. A group of 5 markers labeled with FAM and HEX has been selected that will generate DNA products that differ in size and dye color. Each participant will receive 2 different genomic DNA samples and previously characterized primer sets, which have forward primers that are fluorescently labeled. The participants of the study will be requested to (1) to amplify all markers in a single reaction (pre PCR multiplex) and, if they so desire (2) amplify DNA using individual reactions for each marker, then pooling (post-PCR multiplex). The resulting marker pools are to be electrophoresed and sizes reported. A printout of a genotype file of pre PCR multiplexed material will be included as a positive control to demonstrate the results that can be achieved. PLEASE send all results, even if they are negative. *What doesn't work is as important as what does work.* An internal study within the FARG has indicated that the type of Taq and/or buffer used, may be important? The study will be used to provide researchers with possible methodologies that will assist in multiplexing which will save DNA, cost in reagents and time and also an opportunity for self-evaluation. The results from the study and method parameters used by the participants will be reviewed by FARG members and presented as a poster and a Research Group presentation during the ABRF conference in 2002.

Please go to the ABRF Fragment Analysis Research Group 2002 [Study Sample Request Form](#), to request samples.

If you have already requested samples please go to the [survey instructions](#).

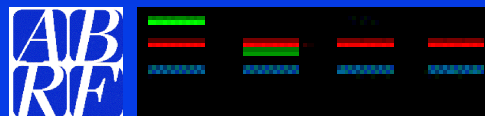
Please keep in mind that this is a survey, not a contest. It is important to receive information from as large a cross section as possible - from the oldest machines to the newest, from beginners to experts. All data is interesting and valuable. This is an opportunity for self-evaluation, to perhaps learn something new or share your successful techniques with others.

All data received by January 20, 2002 will be included in the preliminary report of the data presented at the ABRF 2002 meeting *Biomolecular Technologies: Tools for Discovery in Proteomics and Genomics*, March 9-12, 2002 in Austin, Texas in the Fragment Analysis Research Group presentation and as a poster at the meeting. The results will also be available shortly thereafter on the ABRF homepage under Research Committees/Fragment Analysis.

We hope that you have fun with these samples and find participation in this study to be a worthwhile and educational experience.

Thank you

The ABRF Fragment Analysis Research Group



Fragment Analysis Research Group

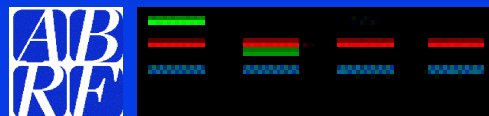
Study Process

3. Samples Tested as Multiplexed PCR

DNA Template	<input type="text"/> μl @	<input type="text"/> $\text{ng}/\mu\text{l}$
Primer Pair#DS8556	<input type="text"/> μl @	<input type="text"/> μM
Primer Pair#D8S504	<input type="text"/> μl @	<input type="text"/> μM
Primer Pair#D8S260	<input type="text"/> μl @	<input type="text"/> μM
Primer Pair#D7S517	<input type="text"/> μl @	<input type="text"/> μM
Primer Pair#D7S550	<input type="text"/> μl @	<input type="text"/> μM
Polymerase	<input type="text"/> μl	<input type="text"/> Uni
dNTP's	<input type="text"/> μl total	<input type="text"/> mM
MgCl2	<input type="text"/> μl	<input type="text"/> mM
Buffer (brand name): <input type="text"/>	<input type="text"/> μl	
Other (specify): <input type="text"/>	<input type="text"/> μl	
Total Volume	<input type="text"/> μl	

Participants could choose to amplify the products with the 5 markers multiplexed into a single reaction. The on line survey included questions based on PCR multiplexing.

<input type="radio"/> "Hot Start" <input type="radio"/> No "Hot Start"	Temp ($^{\circ}\text{C}$): <input type="text"/>	Time (s): <input type="text"/>
Denaturation	Temp ($^{\circ}\text{C}$): <input type="text"/>	Time (s): <input type="text"/>
<input type="radio"/> Step <input type="radio"/> Ramp		Ramp Time (s): <input type="text"/>
Annealing	Temp ($^{\circ}\text{C}$): <input type="text"/>	Time (s): <input type="text"/>
<input type="radio"/> Step <input type="radio"/> Ramp		Ramp Time (s): <input type="text"/>
Extension	Temp ($^{\circ}\text{C}$): <input type="text"/>	Time (s): <input type="text"/>
Number of Cycles	<input type="text"/>	
Hold	Temp ($^{\circ}\text{C}$): <input type="text"/>	



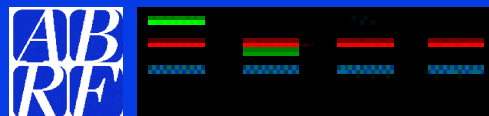
Study Process

3. Or Multiplexed after PCR

	Volume	Stock Conc.
DNA Template	<input type="text"/> μl @	<input type="text"/> $\text{ng}/\mu\text{l}$
Primer Pair	<input type="text"/> μl @	<input type="text"/> μM
Polymerase	<input type="text"/> μl	<input type="text"/> Uni
dNTP's	<input type="text"/> μl total	<input type="text"/> mM
MgCl ₂	<input type="text"/> μl	<input type="text"/> mM
Buffer (brand name): <input type="text"/>	<input type="text"/> μl	
Other (specify): <input type="text"/>	<input type="text"/> μl	
Total Volume	<input type="text"/> μl	

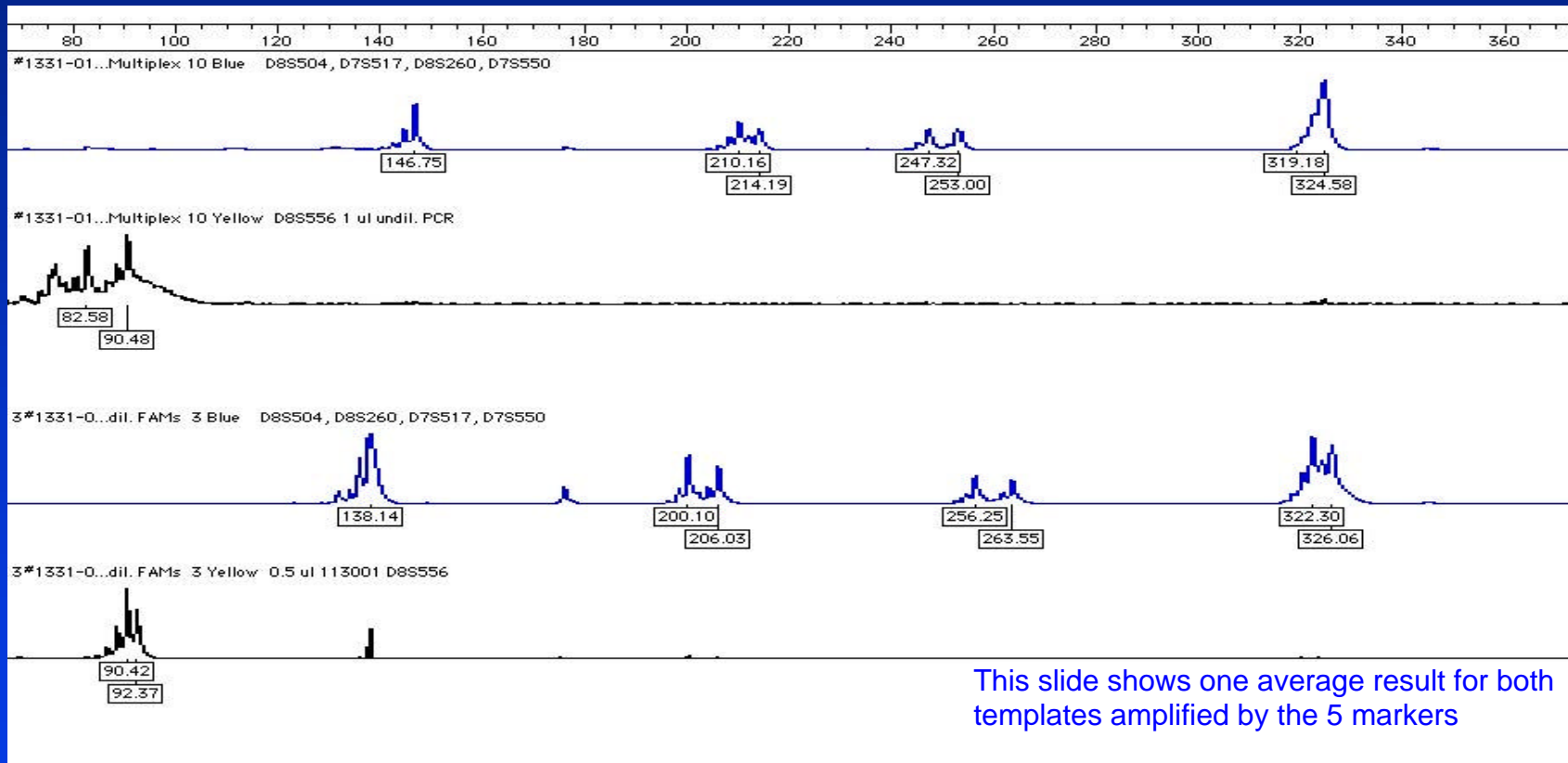
Participants could also choose to amplify the templates with each marker in separate reactions. The on line survey had a separate section for post PCR multiplexing

<input type="radio"/> "Hot Start" <input type="radio"/> No "Hot Start"	Temp ($^{\circ}\text{C}$): <input type="text"/>	Time (s): <input type="text"/>
Denaturation	Temp ($^{\circ}\text{C}$): <input type="text"/>	Time (s): <input type="text"/>
<input type="radio"/> Step <input type="radio"/> Ramp		Ramp Time (s): <input type="text"/>
Annealing	Temp ($^{\circ}\text{C}$): <input type="text"/>	Time (s): <input type="text"/>
<input type="radio"/> Step <input type="radio"/> Ramp		Ramp Time (s): <input type="text"/>
Extension	Temp ($^{\circ}\text{C}$): <input type="text"/>	Time (s): <input type="text"/>
Number of Cycles	<input type="text"/>	
Hold	Temp ($^{\circ}\text{C}$): <input type="text"/>	

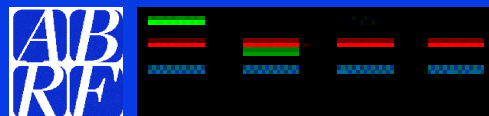


Fragment Analysis Research Group

Sample Selection



This slide shows one average result for both templates amplified by the 5 markers



Fragment Analysis Research Group

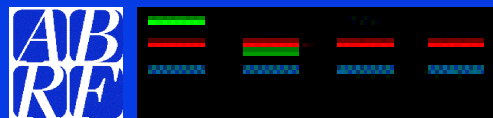
Sample Selection - Markers

FAM Labeled Markers

- D8S504 (129-141 bp)
- D8S260 (187-213 BP)
- D7S517 (239-257 BP)
- D7S550 (306-330 BP)

HEX Labeled Marker

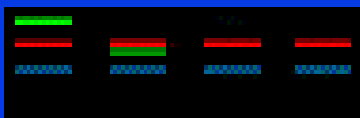
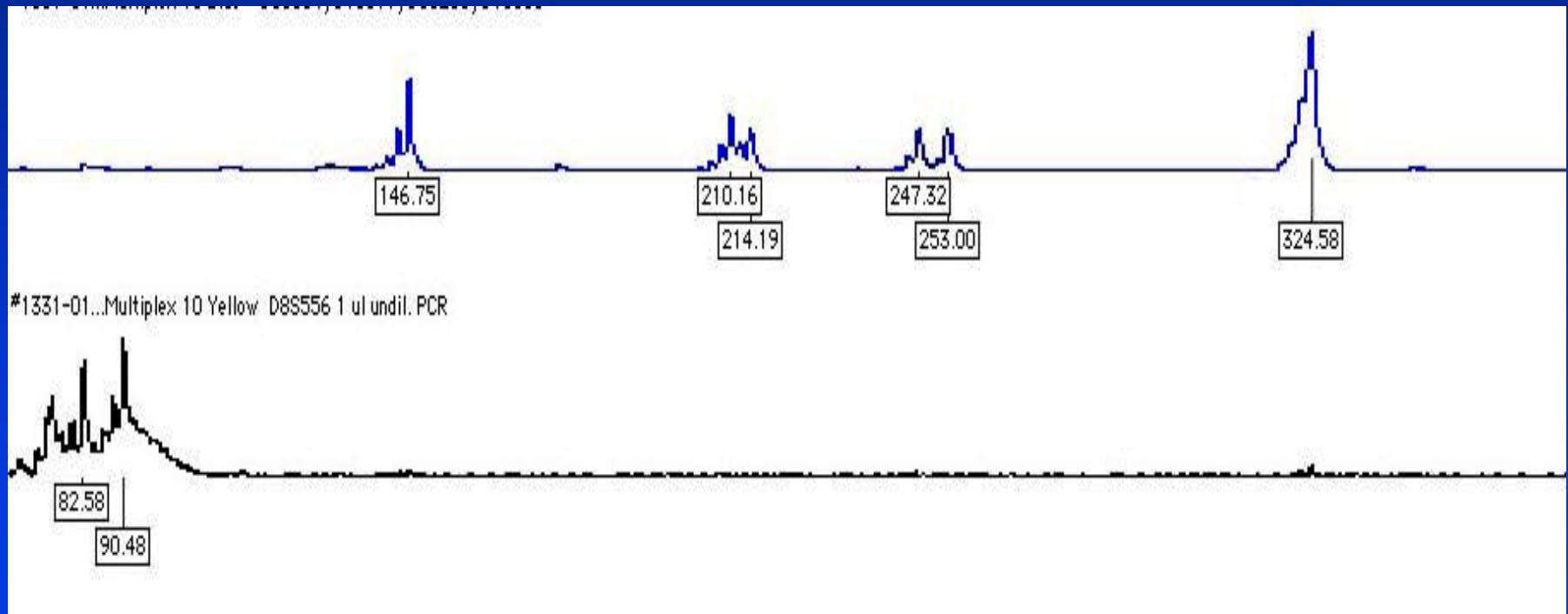
- D8S556 (71-89 BP)



Fragment Analysis Research Group

Sample Selection - Template

1331-01

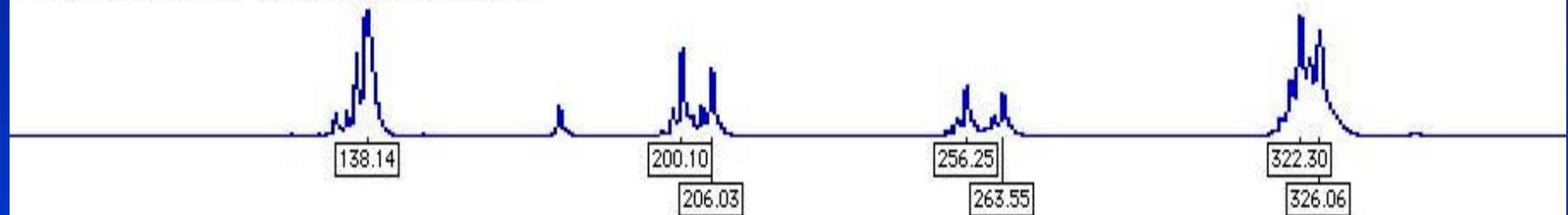


Fragment Analysis Research Group

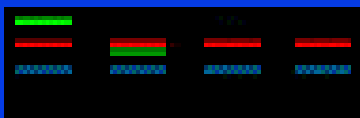
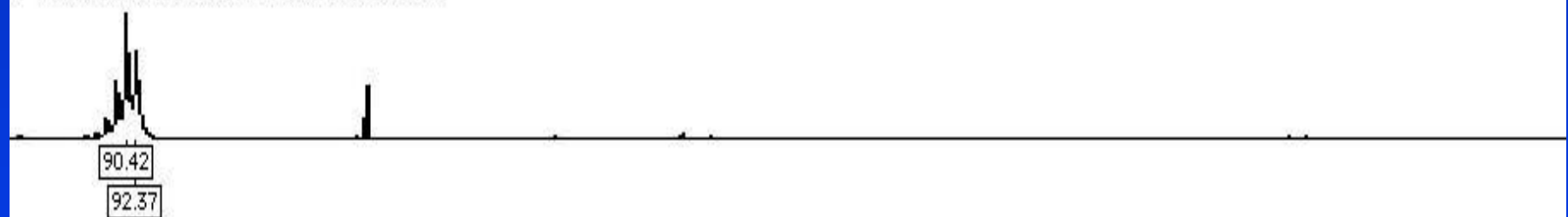
Sample Selection - Template

1331-02

3#1331-0...dil. FAMs 3 Blue D8S504, D8S260, D7S517, D7S550



3#1331-0...dil. FAMs 3 Yellow 0.5 ul 113001 D8S556

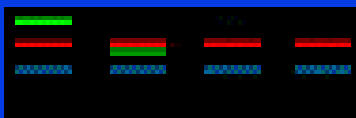


Fragment Analysis Research Group

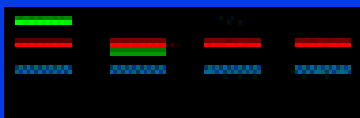
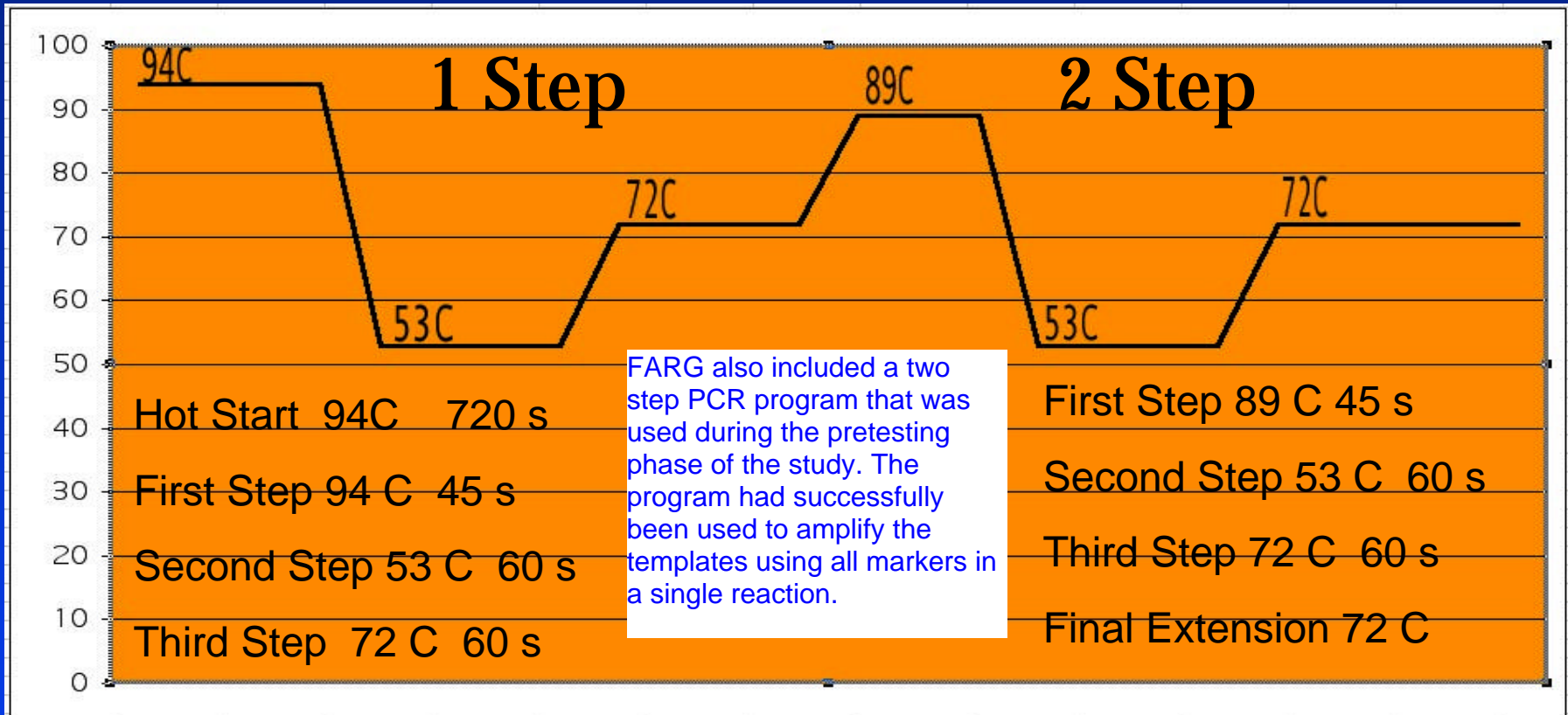
Multiplex Set-Up

Reagent	Amount	Final Concentration	
D8S504-F	0.15µl	0.15µM	
D8S504-R	0.15µl	0.15µM	
D8S260-F	0.125µl	0.125µM	
D8S260-R	0.125µl	0.125µM	
D7S517-F	0.25µl	0.25µM	
D7S517-R	0.25µl	0.25µM	
D7S550-F	0.125µl	0.125µM	
D7S550-R	0.125µl	0.125µM	
D8S556-F	0.15µl	0.15µM	
D8S556-R	0.15µl	0.15µM	
10X Buffer	1.0µl	1X	
MgCl ₂	1.0µl	2.5mM	
dNTP mix	1.0µl	0.25mM	Mix contains 2.5mM of each dNTP
Taq Gold	0.1µl	0.5U	
dH ₂ O	2.3µl		
DNA	3.0µl	60ng	1µl stock=20ng/µl
Total Volume	10µl		

FARG included a set of instructions with a complete protocol for PCR multiplexing. This slide shows the reaction mix for the given protocol.



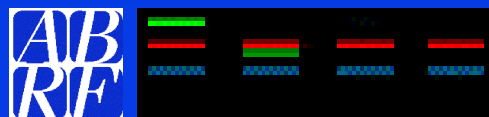
Multiplex PCR Program



Results - Different PCR Cycles

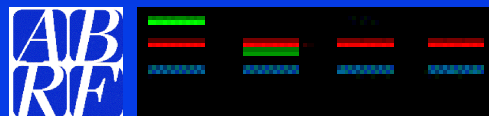
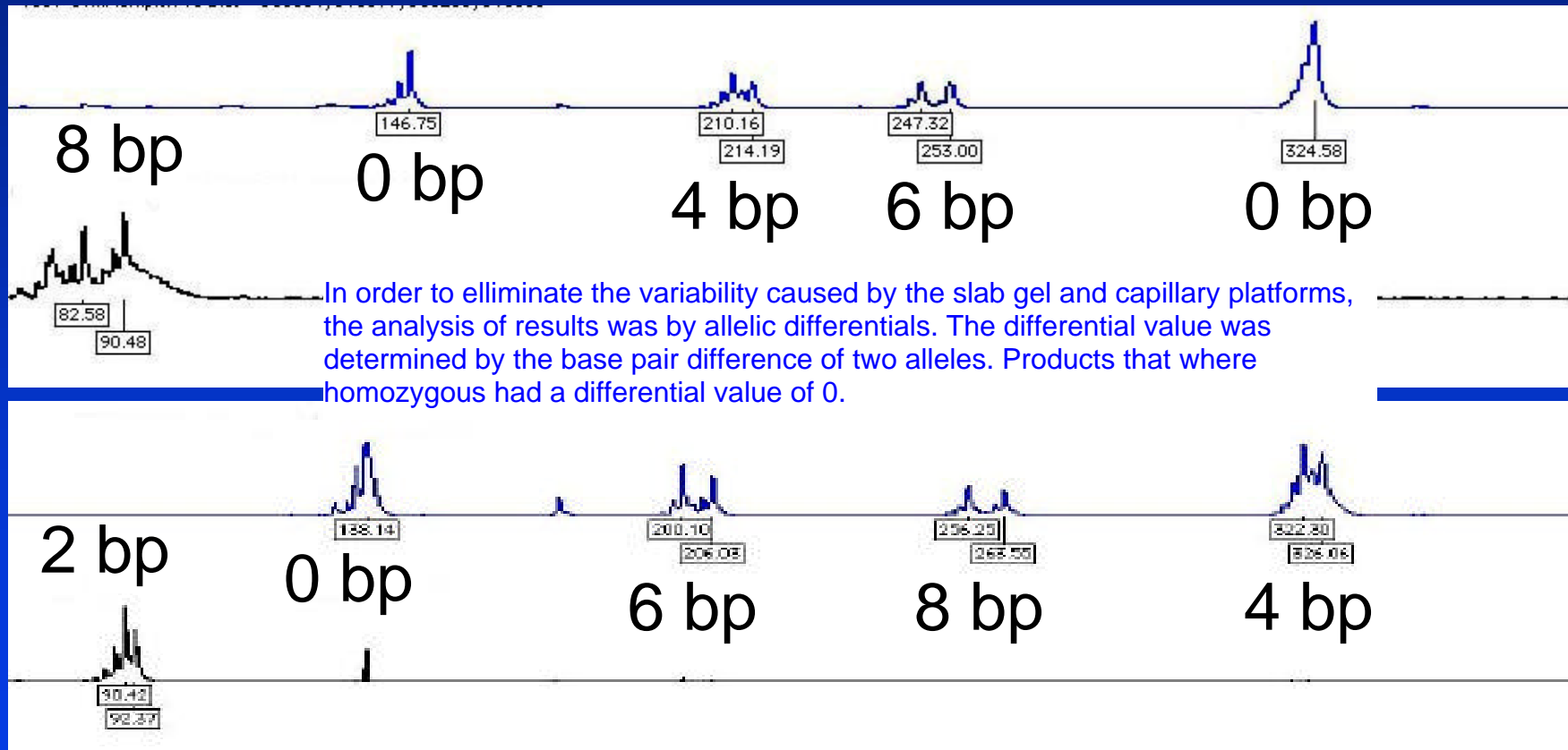
	Hot Start	1st Denature	1st Anneal	1st Extension	Cycles	2nd Denature	2nd Anneal	2nd Extension	Cycles
Program 1	94 (720 sec)	94 (45 sec)	53 (60 sec)	72(60 sec)	10	89 (45sec)	53 (60 sec)	72(60 sec)	20
Program 2	95 (720sec)	94 (45 sec)	53 (60 sec)	72(60 sec)	10	89 (45sec)	53 (60 sec)	72(60 sec)	20
Program 3	94 (120 sec)	94 (45 sec)	53 (60 sec)	72(60 sec)	10	89 (45sec)	53 (60 sec)	72(60 sec)	20
Program 4	94 (120 sec)	94 (45 sec)	53 (60 sec)	72(60 sec)	15	89 (45sec)	53 (60 sec)	72(60 sec)	20
Program 5	94 (60 sec)	94 (30 sec)	50 (30 sec)	72 (60 sec)	35				
Program 6	94 (120 sec)	94 (60 sec)	53 (60 sec)	72 (60 sec)	30				
Program 7	94 (60 sec)	94 (45 sec)	53 (60 sec)	72 (60 sec)	10	89 (45 sec)	53 (60 sec)	72 (60 sec)	15
Program 8	94 (60 sec)	94 (45 sec)	53 (60 sec)	72 (60 sec)	15	89 (45 sec)	53 (60 sec)	72 (60 sec)	20

This slide shows all of the programs that had been used by the participants in the study. Program 1 was used by the majority of the participants and was also the program given by FARG. Program 5 was a one step PCR program that successfully amplified all the products by multiplexing the 5 markers in a single reaction.

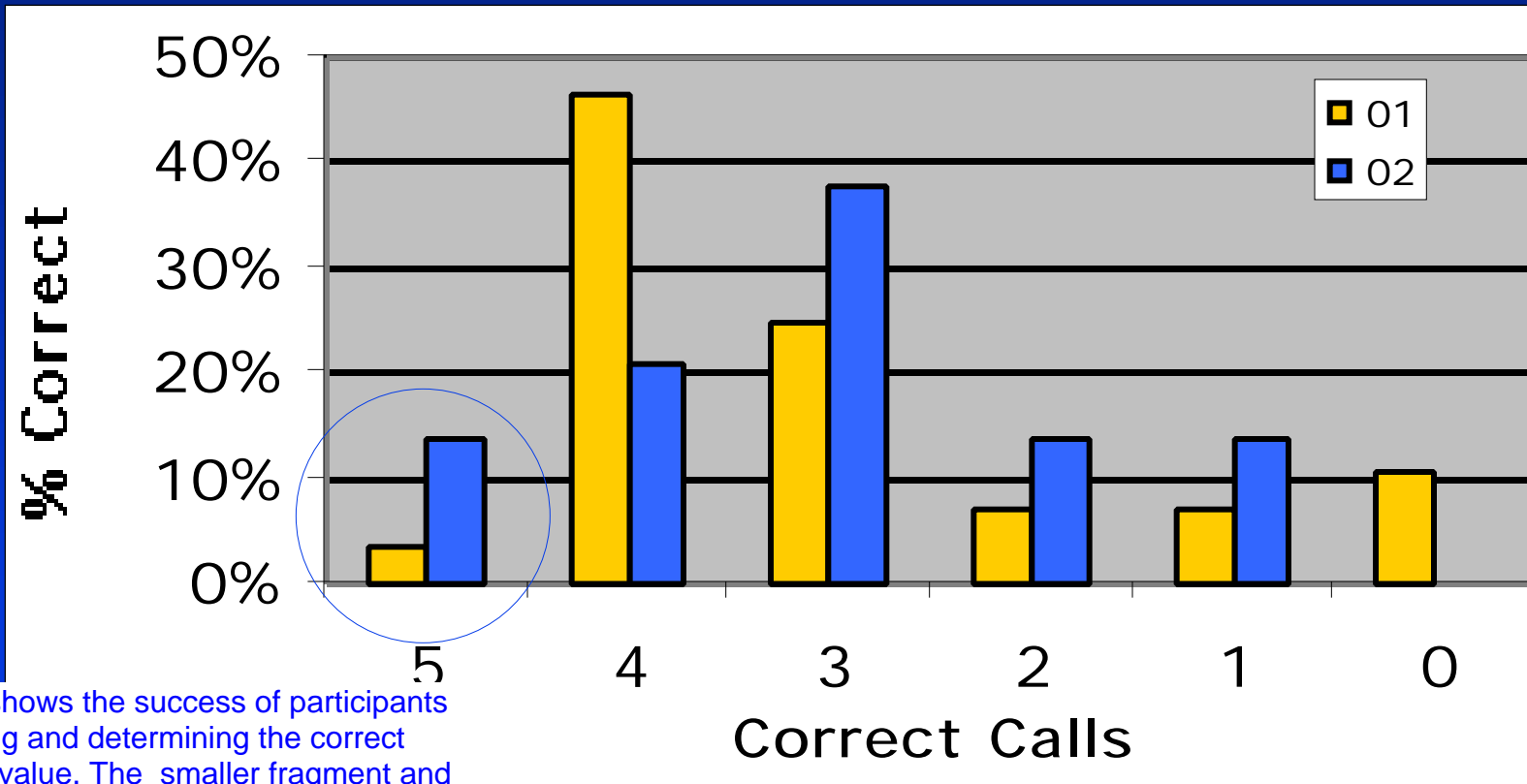


Fragment Analysis Research Group

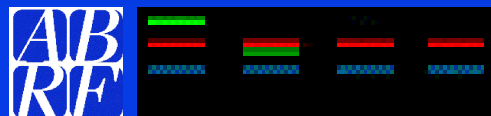
Analysis by Differentials



Results - Success by Template

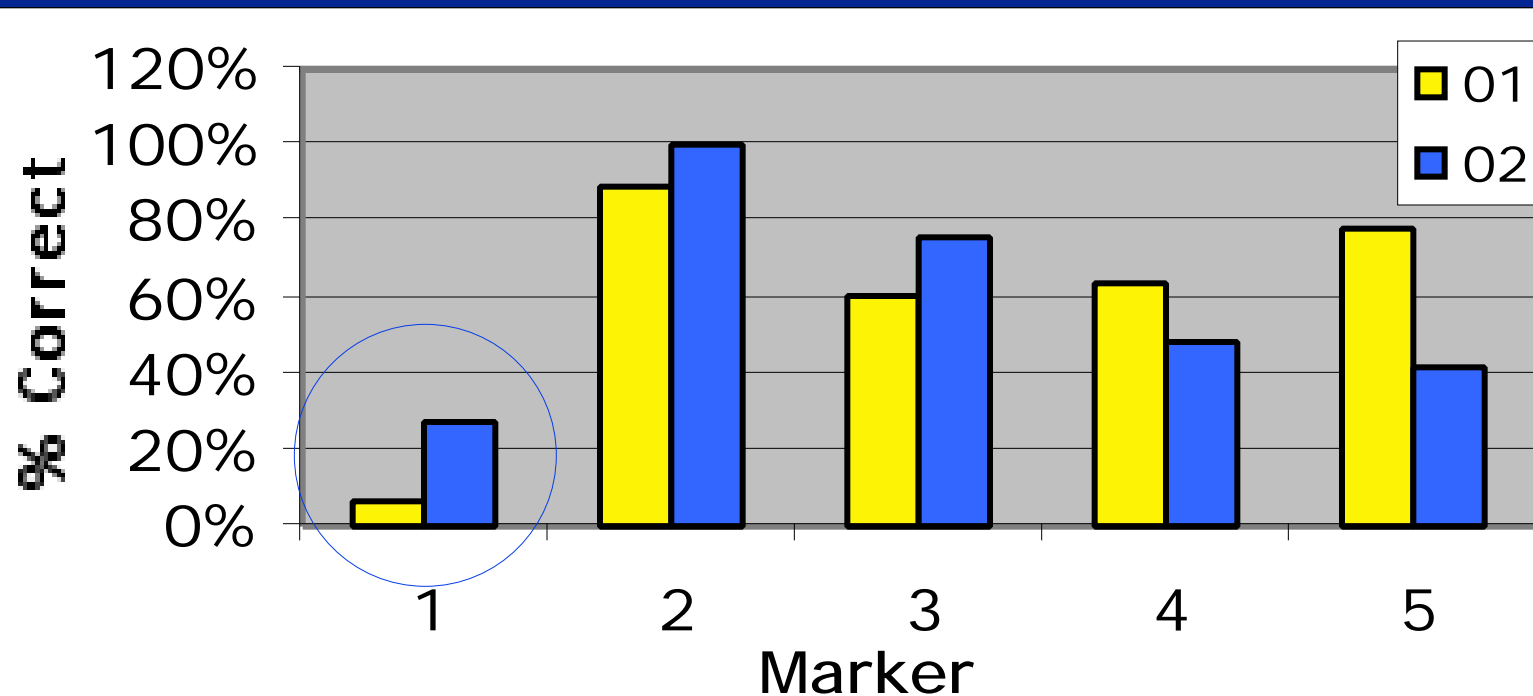


This slide shows the success of participants in amplifying and determining the correct differential value. The smaller fragment and the largest fragment appeared to be the most difficult and the least successful.

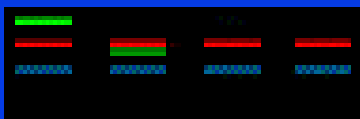


Fragment Analysis Research Group

Results - Success by Marker

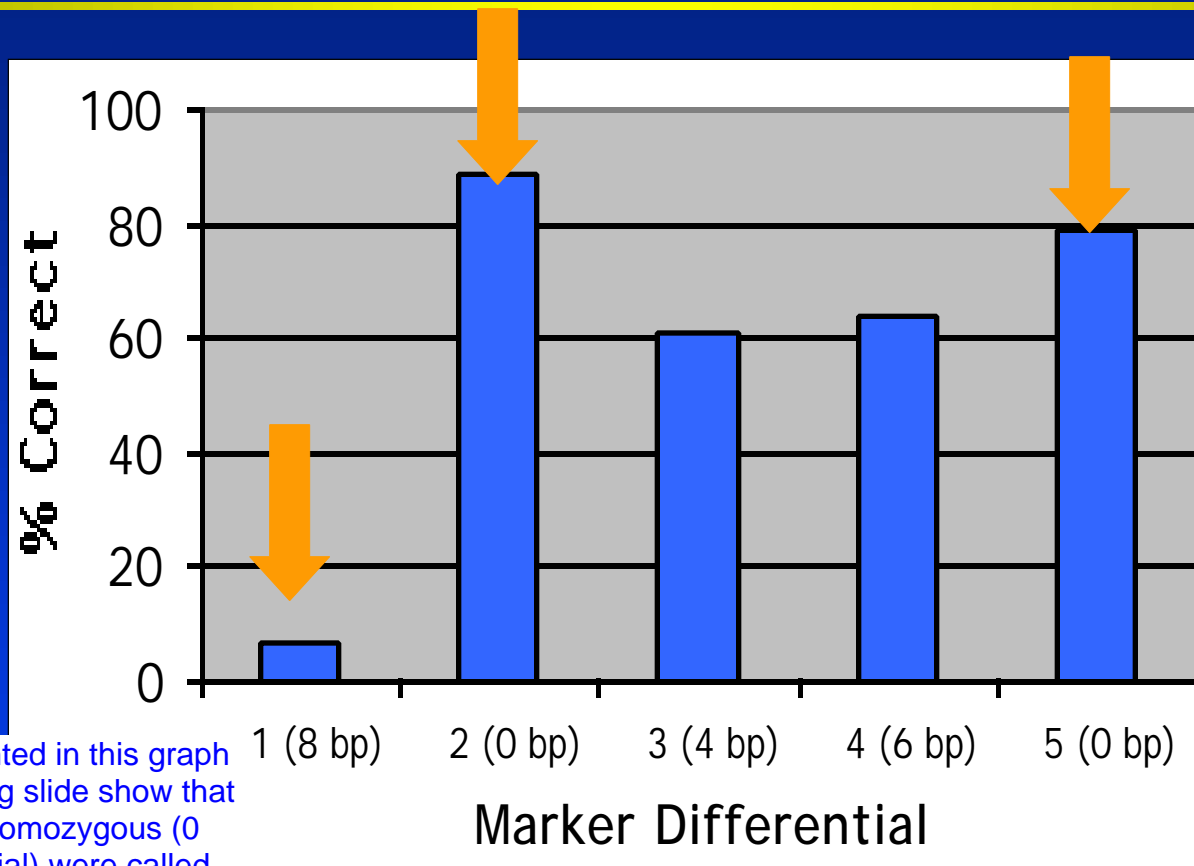


This slide shows the success of each marker based on allelic differential. The marker amplifying the smallest fragment appeared to be the least successful. This may be due to the difficulty of amplifying smaller fragments.

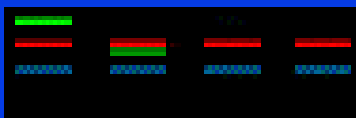


Fragment Analysis Research Group

Results - Success by Marker

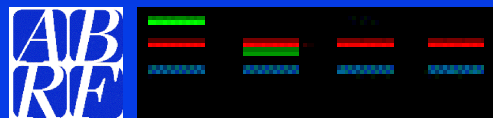
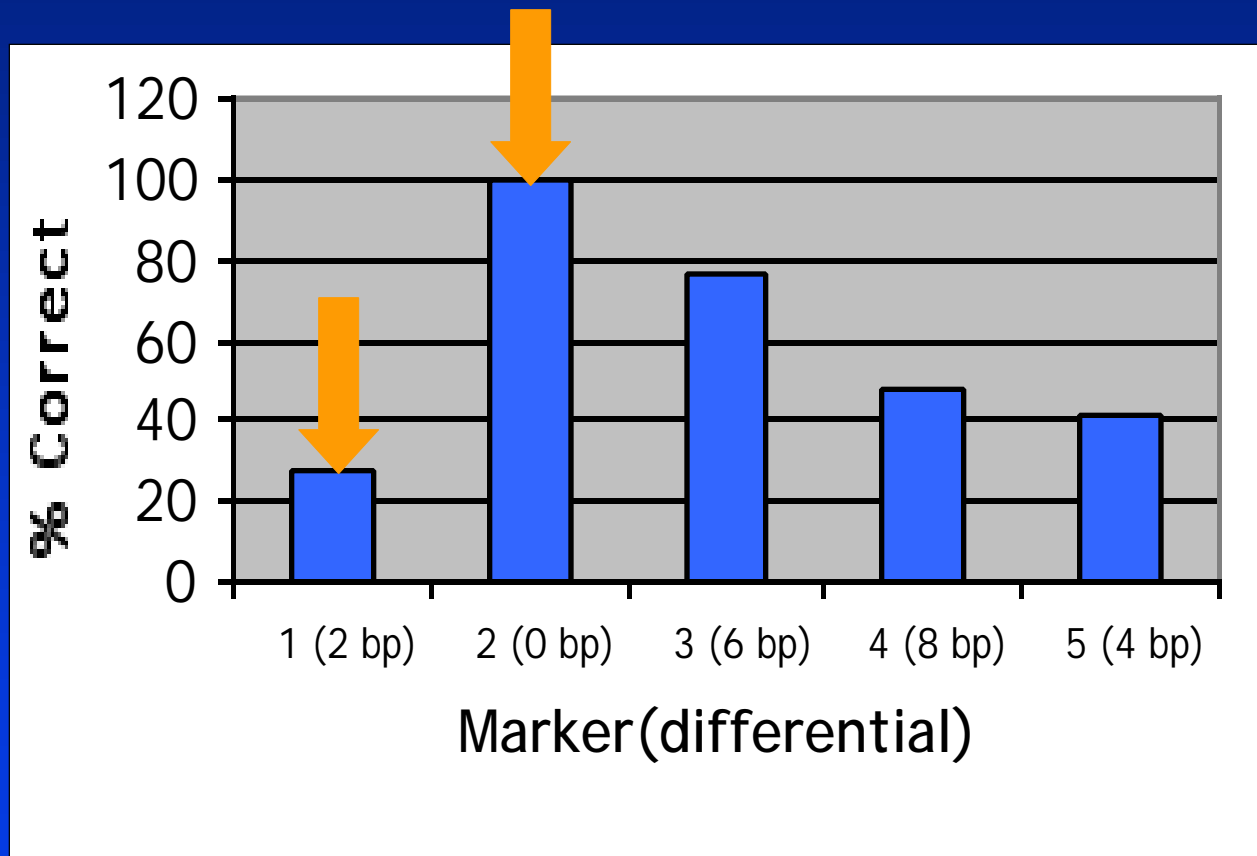


The data represented in this graph and in the following slide show that alleles that were homozygous (0 base pair differential) were called accurately most often.



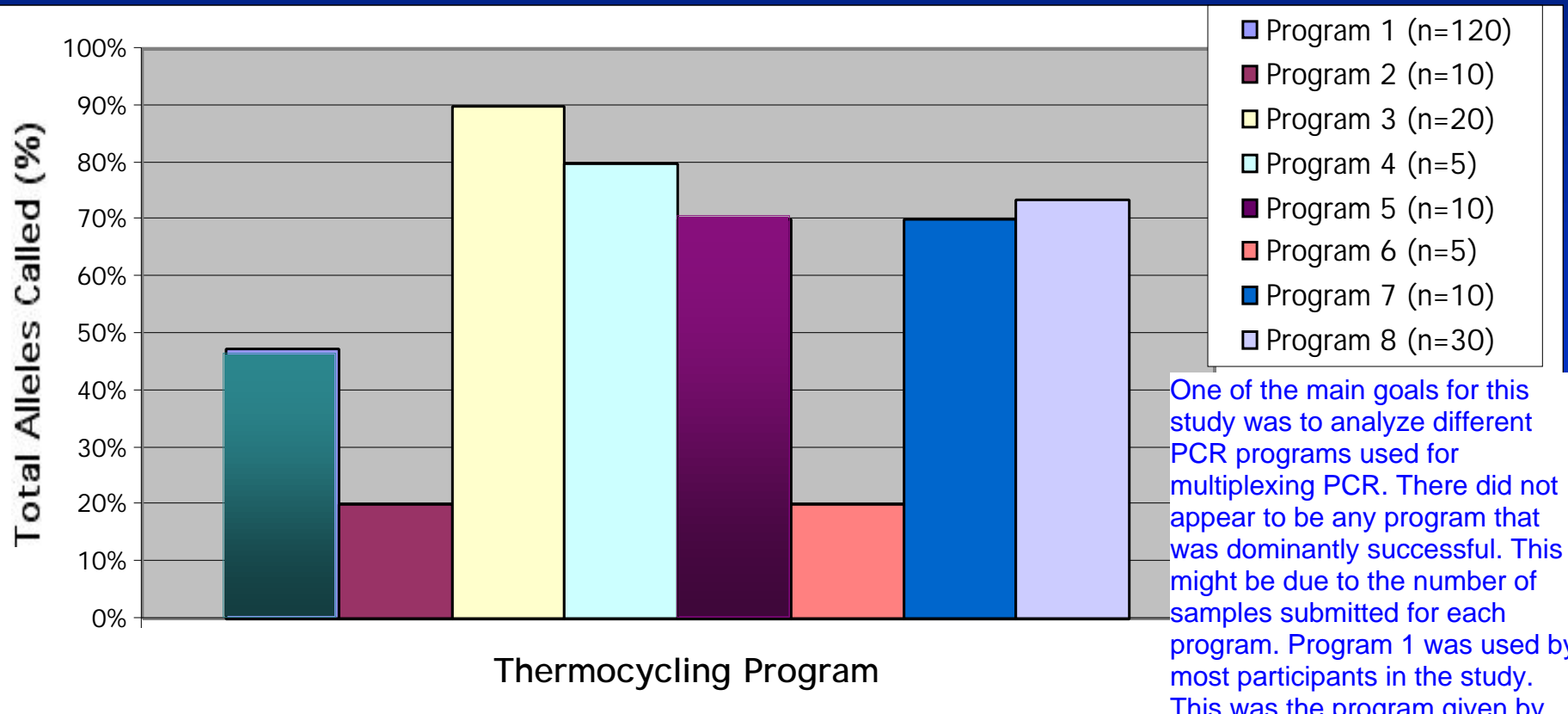
Fragment Analysis Research Group

Results - Success by Marker

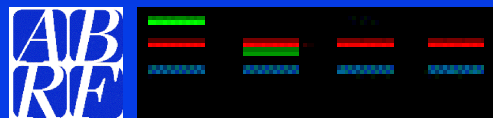


Fragment Analysis Research Group

Results - Success of PCR Program

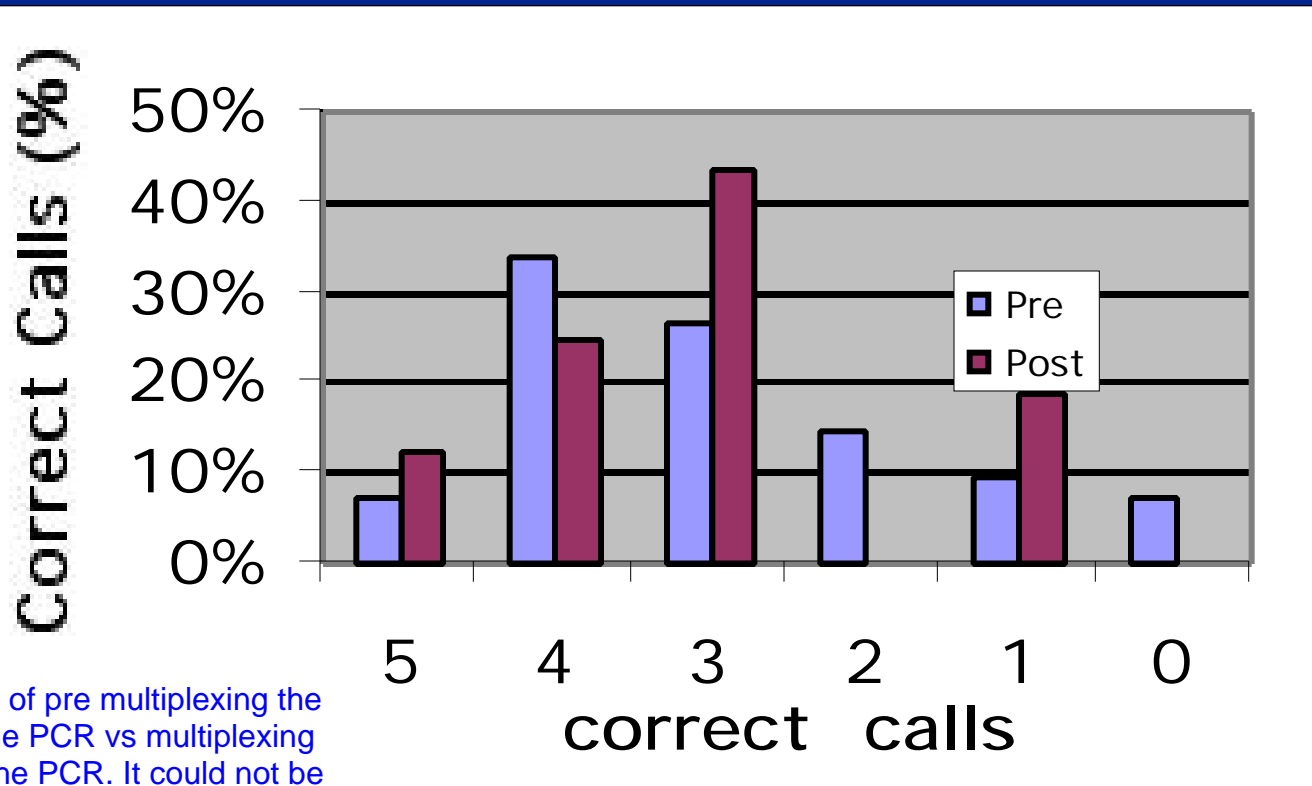


One of the main goals for this study was to analyze different PCR programs used for multiplexing PCR. There did not appear to be any program that was dominantly successful. This might be due to the number of samples submitted for each program. Program 1 was used by most participants in the study. This was the program given by FARG. Program 5 was a one step program that was also fairly successful. Program 5 and program 6 represent the 1 step programs. All other programs were 2 step programs.

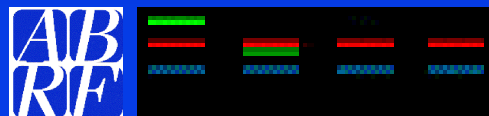


Fragment Analysis Research Group

Pre-Multiplexing vs Post Multiplexing



This is a comparison of pre multiplexing the markers directly in the PCR vs multiplexing the fragments after the PCR. It could not be determined which method produced a better result, However, multiplexing the markers together in a single PCR reaction did work for all 5 loci.

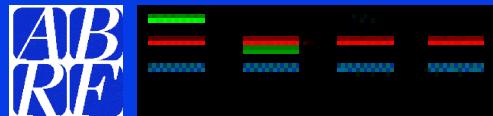


Fragment Analysis Research Group

Multiplexing

General Guidelines for Optimizing Multiplexed PCR for Fragment Analysis

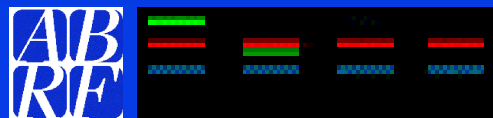
After the study data was presented, FARG gave a short tutorial on ways to improve the optimization of PCR in general as well as some helpful hints for multiplexing a group of markers directly in a PCR reaction.



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Variables for Multiplexing

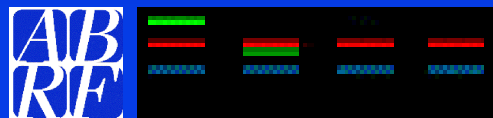
1. Amount of the Template
2. Amount of Each Marker
3. Amount of $MgCl_2$
4. The Cycle
 - Annealing Time and Temp
 - Extension Time and Temp



Multiplexing - Primers

1. Choose primer pairs that can be combined
 - T_m should be within 4 C
 - Cannot amplify the same loci
2. Mix equimolar concentrations of each primer?

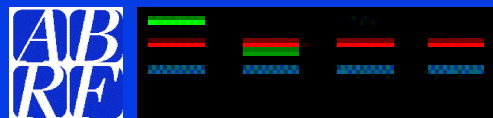
It was first recommended that the markers should be combined with equal molar concentrations in the PCR. However, it was also shown that equal amounts of primer may not, necessarily, amplify the fragments with equal energy and it might be necessary to adjust the concentrations of some of the primers.



Multiplexing - Primers

Preferential Amplification of some primers

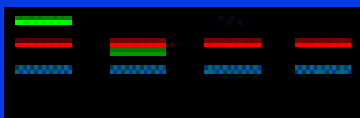
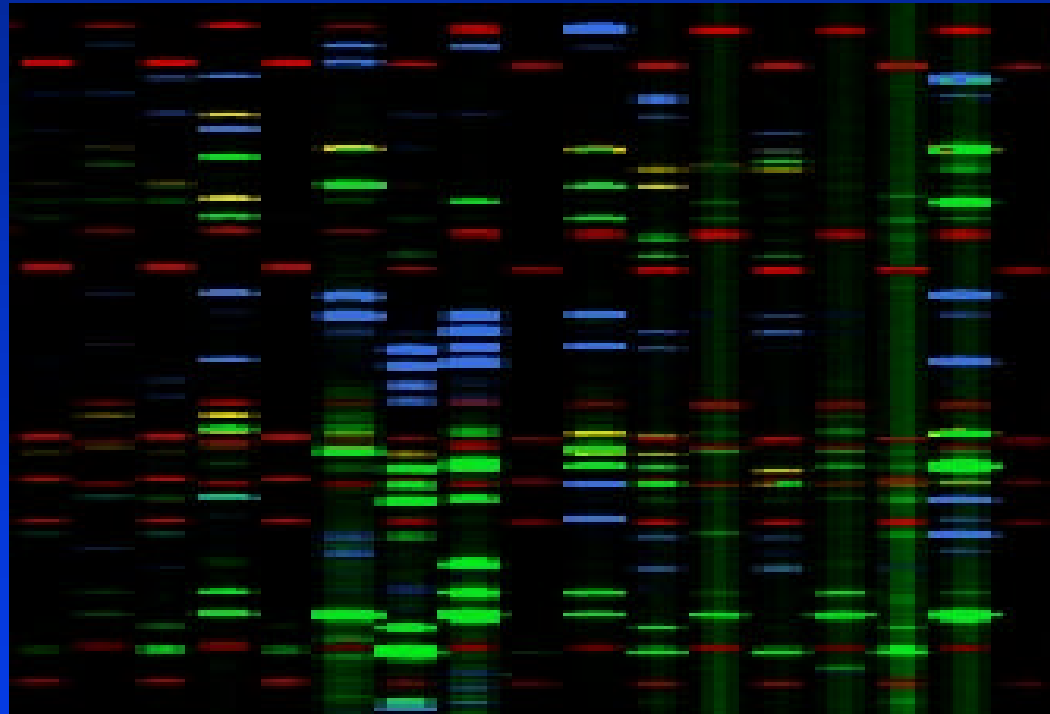
- May require adjustments to be made in concentration of some primers in the mix.
- May require adjustments in the annealing temperature and extension temperature.



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Multiplexing

Yes it Can be Done



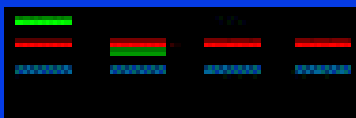
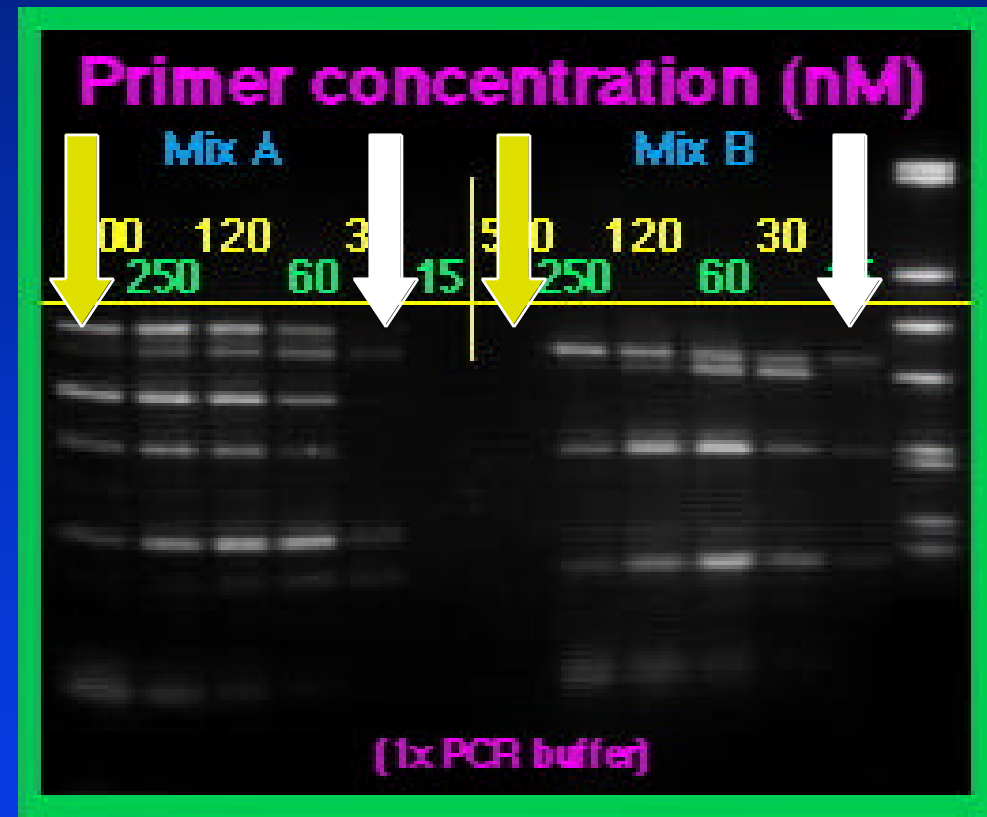
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Optimizing - Primers Concentration

Primer concentration is too high

Primer Concentration is too low

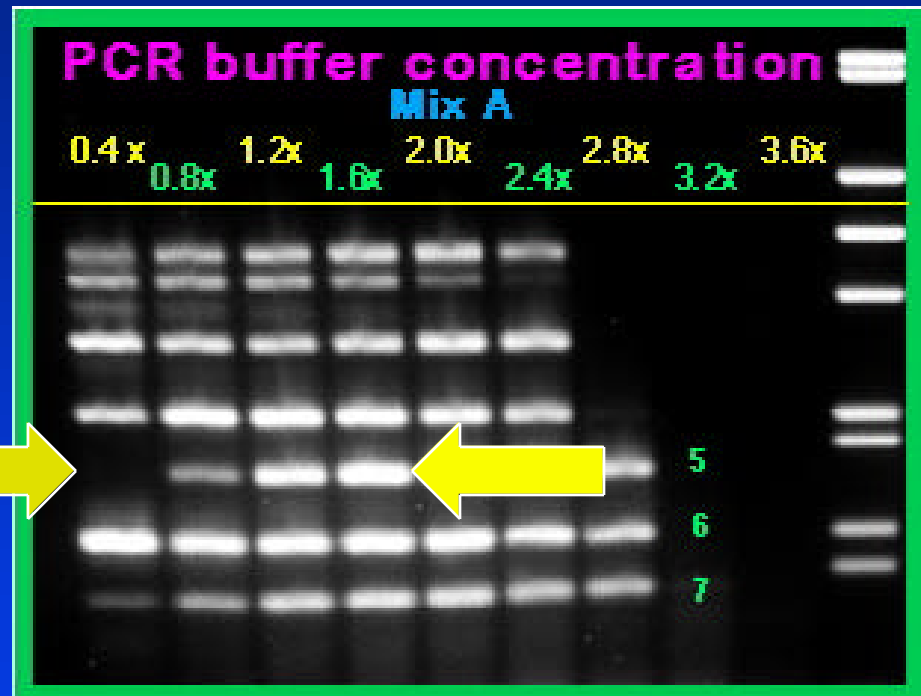
The yellow arrows mark products amplified when the concentration of primer was too high. In general this can cause preferential amplification of one allele over a second allele. The white arrows show products that were amplified when the concentration of primer was too low. This could result in poor amplification of most or all of the sample.



Optimizing - Salt Concentration

Increasing salt concentrations will slow down denaturation of larger products and favor amplification of smaller fragments (under 500 bps)

Raising KCl concentrations to 1.4 X



Increasing the buffer or salt concentration to 1.4 X for the reaction may help amplification of smaller fragments by slowing down the denaturation step. In particular, an increase of the buffer concentration increases the concentration of KCL.

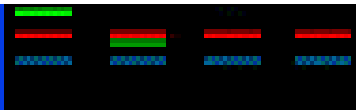


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Optimizing - Annealing Time

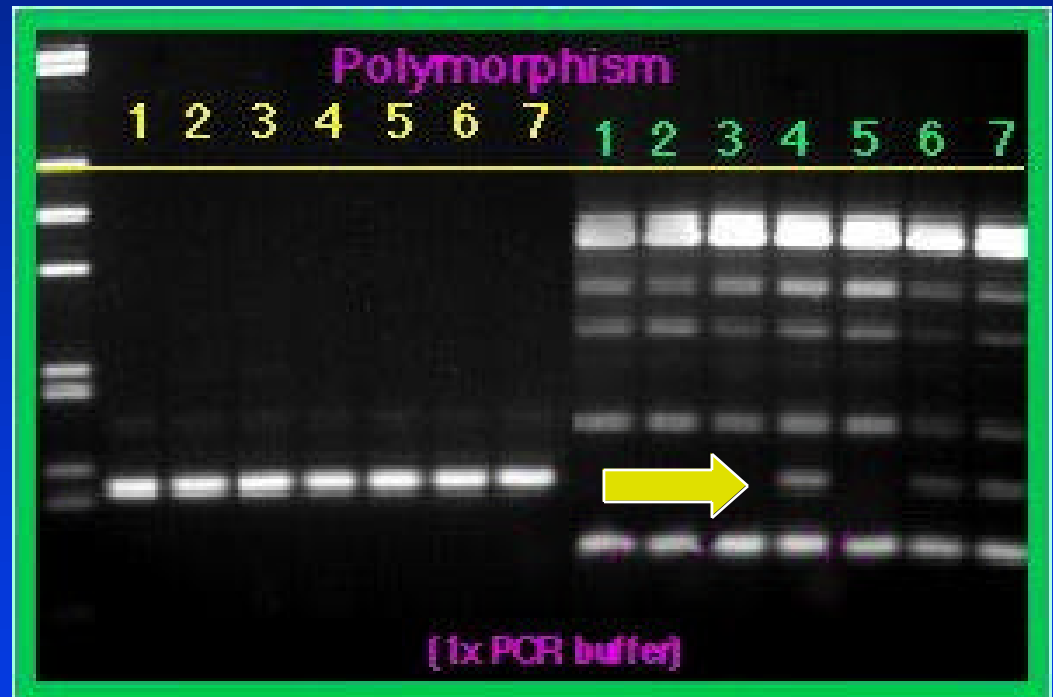
Increasing the annealing time does not influence the PCR. Longer times may increase amplification of unspecific products. Standard time is 30 to 45 seconds.

Increasing the amount of time to allow the primer to anneal to the template does not appear to improve the amplification. It was also shown that a longer annealing time can increase the incidence of non-random priming and results in unclean products.

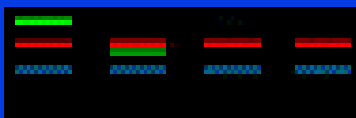


Optimizing - Annealing Temperature

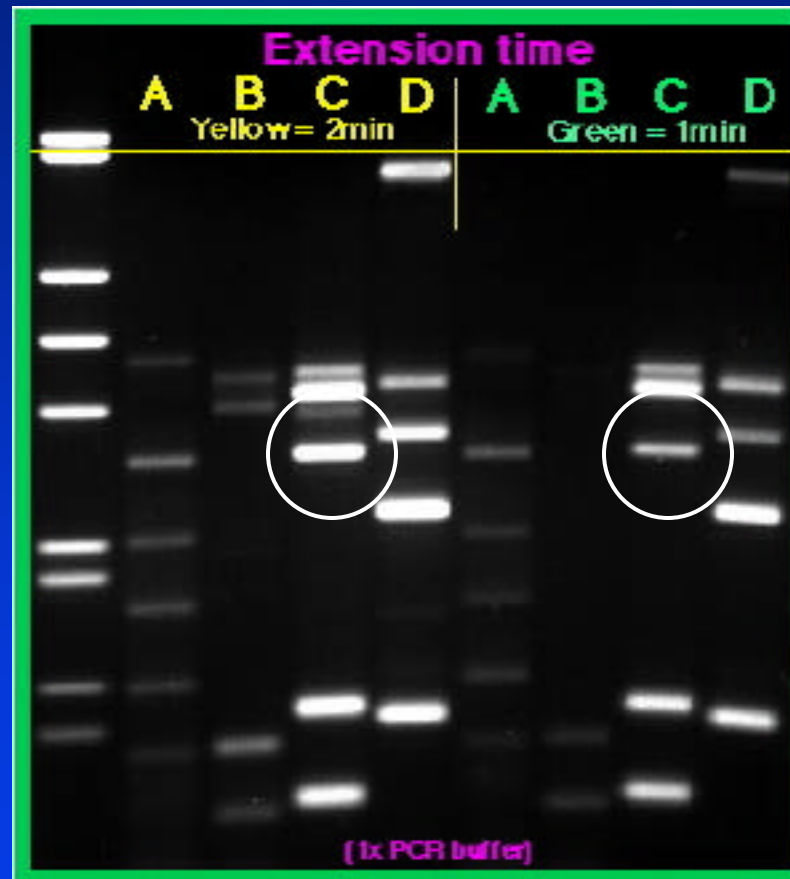
Increase in annealing temperature can prevent identification of the polymorphism



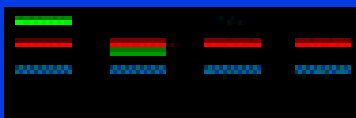
As shown by the yellow arrow, a decrease in the primer annealing temperature from 57 C to 55 C can improve the extension of the smaller sized fragment for polymorphic alleles.



Optimizing - Extension Time

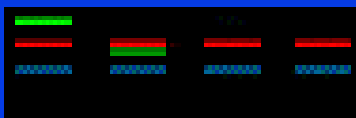
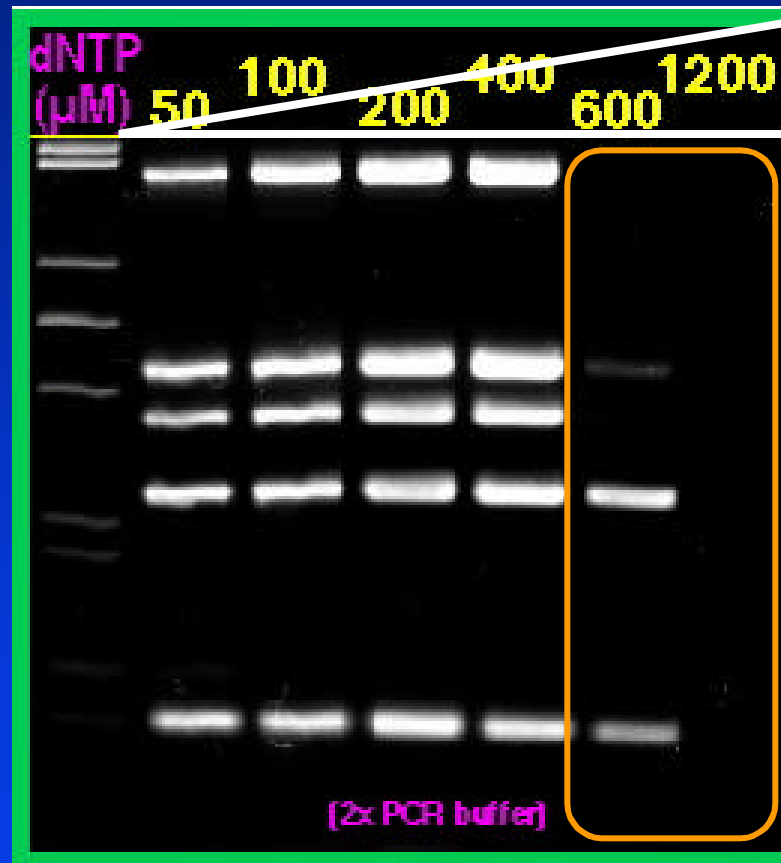


An increase in the extension time can greatly improve amplification of some products. This slide shows how an increase in extension time can improve the amplification (circled band), however, may also result in amplification of non-specific bands.



Optimizing - MgCl₂ and dNTPs

The relationship between MgCl₂ and an excess of dNTPs can result in depletion of MgCl₂ and poor amplification of certain products. This slide shows how increasing the amount of dNTPs can actually inhibit amplification.



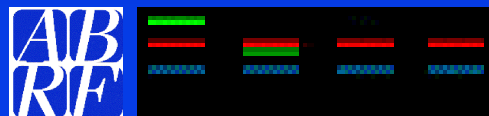
Optimizing

1. Standard Cycle

- Hot start 95 C for 9 min
- Denature 95 C for 30 sec
- Anneal 53 C for 5 sec
- Extend 72 C for 60 sec

Applying techniques for optimizing PCR to the samples used in the 2002 FARG study. The goal for setting up this set of PCR test runs was to determine if a one step PCR program could be used to successfully amplify all products.

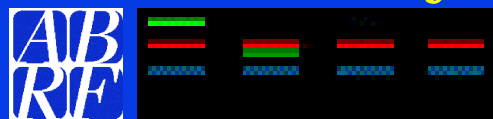
To optimize the reaction, we started with our general program shown in this slide.



Optimizing

1. Lower Annealing Temperature
2. Increase Annealing Time
3. Lower Extension Temperature
4. Increasing Amount of Template
5. Add additional $MgCl_2$
6. Add additional KCl
7. Increase Number of cycles

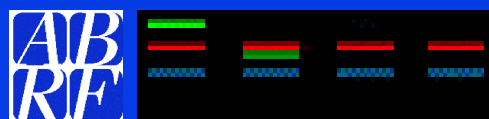
In an attempt to optimize a one step PCR and amplify the templates with all 5 markers multiplexed into a single reaction, we look at changes in the PCR program (annealing temperature and extension temperature) and in the salt concentrations



Optimizing

Cycle	Template (ul)	Primers (ul)	dNTPs (ul)	MgCl ₂ (ul) 10 mM	KCl (ul)	Buffer
Hot Start (95 - 9 min)	1.0	4.0	0.0	0.0	0.0	1X
Denature (95 - 30 sec)	2.0	4.0	0.0	0.0	0.0	1X
Anneal (53 - 5 sec)	1.0	4.0	0.0	0.5	0.0	1X
Extension (72 - 60 sec)	2.0	4.0	0.0	0.5	0.0	1X
Total - 25 cycles	1.0	4.0	0.0	0.0	0.5	1X
Final Extension (72 - 10 m in)	2.0	4.0	0.0	0.0	0.5	1X
Hot Start (95 - 9 min)	1.0	4.0	0.0	0.0	0.0	1X
Denature (95 - 30 sec)	2.0	4.0	0.0	0.0	0.0	1X
Anneal (50 - 5 sec)	1.0	4.0	0.0	0.5	0.0	1X
Extension (72 - 60 sec)	2.0	4.0	0.0	0.5	0.0	1X
Total - 25 cycles	1.0	4.0	0.0	0.0	0.5	1X
Final Extension (72 - 10 m in)	2.0	4.0	0.0	0.0	0.5	1X
Hot Start (95 - 9 min)	1.0	4.0	0.0	0.0	0.0	1X
Denature (95 - 30 sec)	2.0	4.0	0.0	0.0	0.0	1X
Anneal (50 - 5 sec)	1.0	4.0	0.0	0.5	0.0	1X
Extension (68 - 60 sec)	2.0	4.0	0.0	0.5	0.0	1X
Total - 35 cycles	1.0	4.0	0.0	0.0	0.5	1X
Final Extension (68 - 10 m in)	2.0	4.0	0.0	0.0	0.5	1X

It helped to organize the different methods in a table format



Optimizing

A:53 C E: 72 C

25 cycles

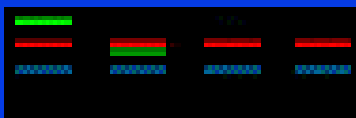
A:53 C E: 68 C

25 cycles

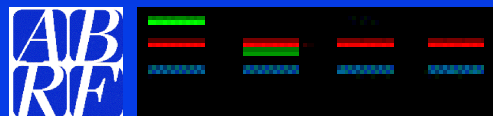
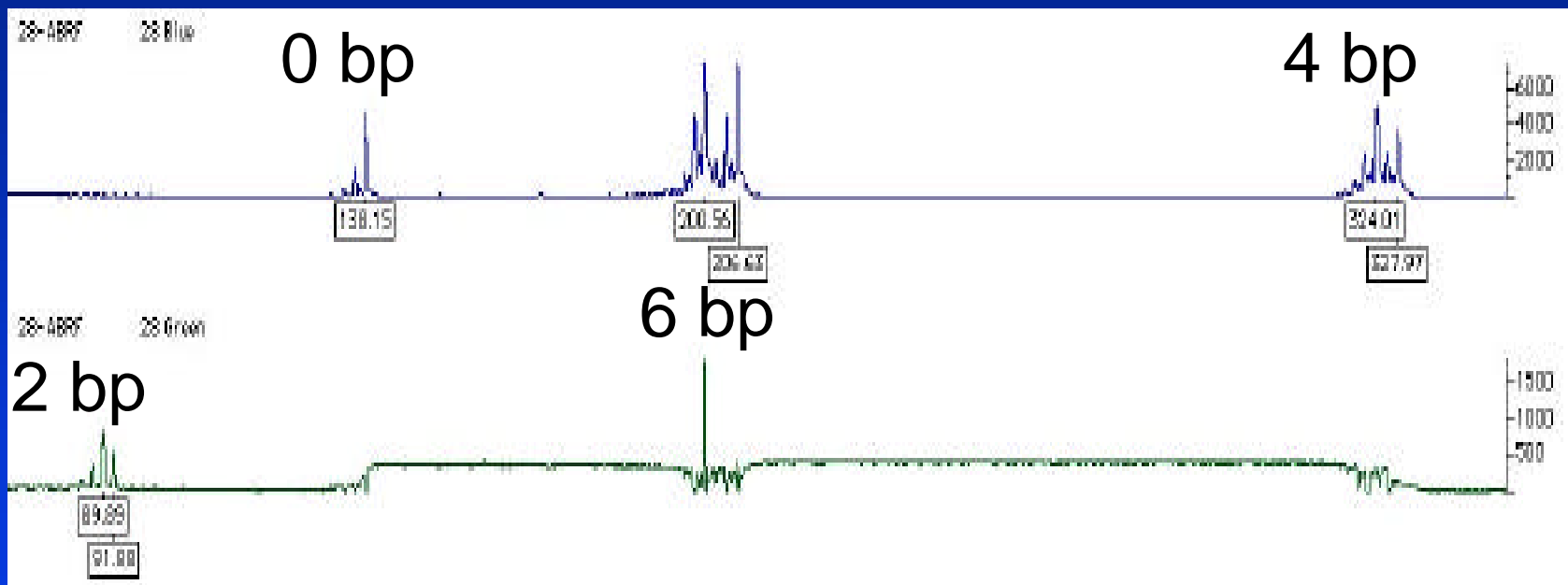
A:50 C E: 68 C

35 cycles

This slide shows some of the results of a one step program. The lower annealing temperature and lower extension temperature along with 35 cycles successfully amplified all loci in a single one step reaction.



Optimizing

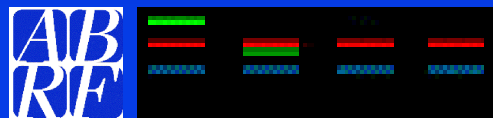


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Next Study



The FARG study for 2003 has not yet been determined. The research group has some ideas including a return to basic fragment analysis and an on line survey.



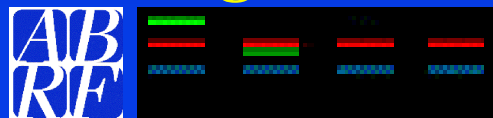
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Acknowledgements

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<http://info.med.yale.edu/genetics/ward/tavi/p04.html>



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