Final Report

A Chip-Based Genetic Detector for Rapid Identification of Individuals

National Institute of Justice

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1. Summary

Since its introduction in 1985, human DNA identification has steadily worked its way into the routine of criminal investigation. What started as a highly controversial means of fighting crime has now become an indispensable tool. Its initial value was recognized for the ability to associate crime scene evidence with known suspects. As the power of this method became evident, national databases of convicted felons were established to allow a search for suspects. These so-called cold hits, association of crime scene evidence with individuals in a database, have helped solve many crimes world-wide.

These efforts have also benefited innocent individuals who were errantly associated with crimes. Numerous convicts have been exonerated either by fortuitous review or direct solicitation of DNA evidence from a crime.

These positive outcomes have prompted a desire for expanded use of this powerful tool. One area for expansion is the broadening of legislature controlling those individuals who might be tested. Currently most states require DNA identity testing of individuals convicted of violent crimes. However many states have recently passed laws to expand this testing to all felony convictions. Recent studies have shown a strong correlation between property crimes and crimes against individuals [1]. A resource sponsored by Applied Biosciences, the DNA Legislative and Media Report, has shown a substantial increase in new legislation for expanded DNA testing passed by state legislations:

- all felon bills have gone from 5 in 2000 to more than 34 in 2005
- all 50 states require DNA testing for felons convicted of sex crimes and/or murder
- California, Louisiana, Texas and Virginia allow DNA testing upon arrest

The last development listed above has been the impetus of this project. As the use of DNA testing has increased, users have learned that expansion of its application leads to synergistic effects in fighting crime.[1] This report will address the benefits, issues and our attempt to provide technology for implementation of Record of Arrest DNA Testing (RADT).

Laws requiring DNA testing during recording of arrests face several impediments. One issue is funding: many of the laws are dependent on federal money. However, California recently passed a bill that assesses an additional fee to court costs to pay for DNA testing. Many other states are currently studying this model of payment.

While social and safety benefits achieved by preventing crime are of course paramount, it is important to track the strictly economic impact of crime-fighting methods. Beneficial use of DNA testing can most easily win public approval if an economic argument can be made that demonstrates cost savings. Studies are needed to show that record of arrest DNA testing (RADT) not only reduces crime

but also lowers overall investigational costs. The National Forensic DNA Study Report[2] recently collected data to estimate the volume of forensic DNA backlogs, but did not address the cost-benefits of DNA testing. Further studies providing econometric data would be useful tools for prioritizing DNA technology development and gaining pubic support

A further economic consideration is efficient use of lab space. The cost of increasing the size of forensic DNA testing laboratories is highly prohibitive. This means that methods requiring equipment that have a large footprint (like fluid handling robotic platforms) need to be redeveloped towards the goal of miniaturization and automation. This will permit a greater efficiency of use of current space.

Reducing labor costs can be achieved through the greater use of automation provided by integrated, miniaturized systems. Great steps towards improved efficiency are currently being made through the use of robotic systems. However these systems require high sample volume to be practical. This is due to the large capital expense development and maintenance costs associated with robotics. A better solution would be the miniaturized devices supported by this grant, which integrate automation into a disposable product.

Reduced labor costs could also be realized with interrupting the criminal patterns of serial criminals or recidivists, therefore eliminating the cost of investigating their future crimes. This is achievable through obtaining DNA profiles of suspects at the time of arrest since many minor crimes are committed by those responsible for serious offenses. Rapid RADT could further reduce costs by providing evidence to

support probable cause, so a suspect could be charged before expiration of a holding period. This would eliminate the cost of finding and re-arresting the suspect. Apprehending a charged suspect is likely one of the higher risk duties required by a law enforcement officer. Benefits will also be realized by reducing the number of times this duty is required to be performed.

In addition to funding, the lack of appropriate technology is also an impediment to practical RADT. Current means of DNA identity testing often depend on methods that require high levels of skill and experience. Compartmentalized rooms with separate tasks and highly trained individuals are standards for DNA testing today. Such facilities are highly efficient as centralized processors of biological samples, but not every police station has timely access to these. Very few police stations have the space or resources to set up such labs for their own use. To realize the potential benefits of RADT, DNA identification needs to be self-contained, have a rapid time to result and be easy to use.

While RADT is a primary target for the device being developed with the support of this grant, there are other forensic and national defense applications that will benefit from such a system. Identification of disaster victims in remote sights could be facilitated by self-contained and mobile instrumentation. These same attributes would also support crime scene investigations. Such devices could additionally provide unique advantages for tracking individuals suspected to be a threat to U.S. security. The 9/11 attack means that we have a new perspective on national security. As such, there have been proposals to implement DNA testing at ports of entry into the U.S. It

is clear that current technology would have difficulty meeting the time-to-result requirements of that goal.

In Summary, the benefits offered by point-of-use forensic DNA testing include: 1) crime prevention by taking recidivists off the streets, 2) lower investigative costs by preventing crime, 3) lower investigative costs by providing timely evidence thereby preventing premature release of arrestees, 4) lower infrastructure costs by more efficient use of currently available space, 5) lower personnel costs by reduced training requirements, 6) enable broader application of forensic DNA testing.

The purpose of this research grant has been to investigate development of a cost-effective, rapid and integrated system for human genetic identification. The proposed system has been shown to: amplify and analyze target loci directly on individual array sites, and provide easily interpretable results. This has been accomplished with both STR and SNP loci.

This project has resulted in the development of numerous assays for genetic variants relative to forensic investigations. These include STR and SNP loci. Assays have been developed for use with PCR amplification (STRs) and anchored SDA (STRs and SNPs). Ultimately, these efforts resulted in a panel of 22 SNP loci featuring automated amplification and analysis. The SNPs were selected from Y-chromosome, autosomal and mitochondrial loci.

2. Introduction

Issue Addressed

Active DNA chip technology offers easy-to-use assays for SNPs and STRs. This research project supported development of sample-to-answer devices with the goal of making DNA identification technology capable of operation by anyone who can take fingerprints. Integration and ease-of-use will permit rapid turnaround and short chain of custody for forensic samples, enabling DNA information to be applied during the critical early hours of an investigation.

The Human Identification CODIS and international databases contain allelic information of short tandem repeat genetic variants. Discovery of STRs by Alec Jeffreys in the early 1980's and refinement of Jeffreys' concept in the early 1990's represented a breakthrough in genetic analysis because the recently developed technology of DNA amplification, PCR could be applied to these variants. Previously used RFLP variants were not amenable to PCR so the combination of STRs and PCR constituted a broad based breakthrough in human identification. However, due to the molecular nature of STRs, analysis has been limited to sieving methodologies. While this method supported rapid acceptance of DNA identification as a crime-fighting tool, it is limited. Typical sieving methods include porous gels through which DNA follows a tortuous path, allowing precise size separation to occur. Size resolution of a single base difference can be routinely attained.

However sieving methods have limited applications. Protocols used when this grant was initiated included slab gels. These are cumbersome to use and must be fabricated on-site by a skilled user. Data interpretation requires experience and NIJ has sponsored efforts to automate this phase of DNA testing (Cybergenetics True Allele®). The current standard method is capillary electrophoresis. This method reduces the burden of matrix formulation and increases the ease of data interpretation. Yet these improvements still limit DNA analysis to highly trained professionals using sophiscated equipment. Also, the tests are not well integrated and require separate instruments to carry out amplification and analysis. Mobility is also limited.

The purpose of this research grant has been the development of an integrated system for human genetic identification. The overall goal is to provide a device for Record of Arrest DNA Testing (RADT) in suitable police precinct or state labs, thereby technically enabling DNA testing of arrestees in a short time period. Nanogen's development of on-chip amplification systems for STRs and SNPs (through grants provided by NIJ and others) provides a foundation for high efficiency analysis. This is accomplished by automation and miniaturization of many cumbersome techniques and through the use of highly multiplexed testing. The delivered protocol has been developed for the Nanogen's next generation 400 pad chip, which can be used on a portable or benchtop device. We report here that we have developed protocols for integrated multiplex-amplification and

analysis STR and SNP loci that exceed the capacity of any current methodologies or products.

3. Project History

This project was initiated following a presentation at the Air Force Research Laboratory in Rome, NY. Dr Michael Heller, the scientific founder of Nanogen, was discussing some recent work done by Dr. Ronald Sosnowski on electronic stringency of DNA hybrids. Dr. Sosnowski's work showed that DNA hybrids could be distinguished by the length of a single-strand overhang, even when the complementary sequences were identical. It was further discussed that this length discrimination could have applications in human DNA identification. At that time the standard genetic variant for discrimination of DNA was the Restriction Fragment Length Polymorphism (RFLP), and there was interest in whether electronic could identify these genetic variants. After some additional government contact, Drs. Heller and Sosnowski met with Tom Bode Sr and Dr. Kevin McElfresh from The Bode Technology Group (TBTG), where the feasibility of applying Nanogen technology to DNA identification was discussed.

At a subsequent meeting, Dr. McElfresh brought up the possibility of using Nanogen's technology to discriminate a different type of genetic variant, the Short Tandem Repeat (STR). It was his belief that the attributes of STRs, most notably their amenity to PCR amplification, would allow it to supplant RFLPs as the standard variant for human DNA identification.

Since the STRs being used for DNA identification had repeat units of four bases, pilot studies were performed to determine whether electronic stringency was capable of length discrimination with a precision of four nucleotides. It quickly became apparent that this specification was too challenging for the state of the technology at that time.

In completing these pilot studies, Dr. Sosnowski began contemplating other methods of achieving STR discrimination. In discussions with Gene Tu, another Nanogen scientist who along with Dr. Heller laid the groundwork for Nanogen's technology, a novel hybridization method for distinguishing STRs was invented. This invention resulted in U.S. patent 6,395,493 which served as the basis for US 6,753,148 and US patent applications 20020115098 and 20030073122.

Grant funding managed by TBTG supported development of initial assays for CSF, TPOX and TH01 (CTT). These assays used PCR product amplified from kits or primers developed at Nanogen. Assays were performed in one of two formats: amplicon down or capture down (fig 1). Analysis was performed on Nanogen's electronically active microarray. Nanogen's technology allows the localization and concentration of charged molecules to permit manipulation of molecular interaction(fig 2). It was determined at this time that electric-field driven hybridization was necessary for appropriate hybridization of the amplified STR loci (NAR paper[3]). These studies culminated with a concordancy study performed by Dr. Jim Schumm at TBTG. This study found one discordant call out of 214. Data analysis algorithms used at Nanogen would have designated

the discordant call as a no-call, however the appropriate threshold values were not incorporated into the software used by TBTG (fig 3).

In 1996, Nanogen applied to NIJ for funding of a new assay for integrating amplification and analysis. This assay was to be based on work with Strand Displacement Amplification (SDA) which was being developed at Nanogen for microbial analysis. Nanogen had modified this amplification technology to enable *in situ* or anchored amplification at an electronic test site. We were able to demonstrate that purified microbial genomic DNA could be electronically hybridized to amplification primers immobilized at a test site and amplified. The resulting attached amplicon could then be interrogated for sequence variation.[45] The NIJ research proposal envisioned this integrated amplification and analysis as the basis for a portable device capable of rapid DNA tests with little user intervention.

A research grant was awarded to Nanogen for the development of anchored SDA assays for human identification. Dr. Mike Nerenberg was the Principal Investigator of Phase I. Using on-going development of anchored SDA for bacterial identification, this grant was to support development of tests capable of discriminating genetic variants, particularly STRs, from human genomic DNA. Successful achievement of this was a significant accomplishment for two reasons: 1) the human genome is about 100 times more complex than bacterial genome, and 2) developed assays were only capable of detecting the presence or absence of an identifying locus, not a subtle genetic variant within a locus.

Complexity is a major factor in any DNA test. In more complex genomes, the probability of randomly finding sequences that are partially complementary to the probe increases. Specific probe hybridization can be attained, but it takes the probe longer to find its most stable state, since it must entertain false suitors that keep getting in the way.

Detecting the presence or absence of a specific probe is less demanding than differentiating variation within a sequence. Stringency must be applied in both cases, but precision is less of an issue for simple detection.

4. The STR Hybridization Assay

From a molecular mechanistic viewpoint, STRs are a unique genetic variant. Theories regarding the origin of these multi-allelic variants include a mechanism whereby slippage between strands within the repeat region during replication results in generation of new alleles with differing numbers of repeats.[6] [7] This intrinsic hybridization characteristic creates difficulties for amplification and analysis with complementary probes. For example, the THO1 locus is generally present in the population with alleles of 5-10 repeat units[8]. That means that analysis with a conventional complementary probe could require a repeat region of 40 bases. However, such a probe, even with unique flanking sequence is relatively stable with just 20 complementary base pairs. Therefore there are many conformations that would allow the probe to bind to a target incorrectly.

Our solution to this issue was to use the electronically active microarray to make each test site a spot for detection of an individual allele. This allowed each allele to be tested individually. In addition to the microarray allowing individual analysis, we also took advantage of the properties of electric field facilitated hybridization. Because the same field that concentrates the target DNA at a test site can simultaneously remove partially hybridized complements, a high level of specific hybridization is attainable[9]. The enabling factor of the assay was the splitting of the complementary probe into two parts: one of which is immobilized at the test site and the other labeled (fig 4). By using this configuration, we were able to identify the number of repeat units in a specific target. This format is the basis for all STR assays discussed.

5. Anchored Strand Displacement Amplification

Strand Displacement Amplification (SDA) is an exponential amplification method that uses opposable primers[10]. It differs from PCR in that replicating strands are separated by the actions of the polymerase, rather than repeated cycles of thermal denaturation. Advantages include no requirement for precise and rapid temperature changes and short amplification times. Disadvantages are additional reagents and a more complex reaction. Originally developed by Becton-Dickinson, several solution SDA products are on the market, including those utilizing real-time SDA. A market advantage for commercial use of SDA is a licensing agreement that is more favorable to developers and customers than

the Roche PCR agreement. Nanogen holds a license to develop SDA on its electronically active microarray.

While BD has investigated the use of SDA probes immobilized on microbeads, Nanogen has used probes with amplification primers immobilized on electronic test sites. This method, dubbed anchored SDA (aSDA), enables target amplification and analysis to occur at the same site. This method is ideally suited to a microarray system, since the target is immobilized at the analytical site, and buffer changes do not require re-isolation and application of the target. Once target material has been immobilized any sequence analysis using complementary probes may be performed. Nanogen has demonstrated analysis of STRs and SNPs. These developments have been disclosed in US patent 6,531,302.

While microarray test sites are capable of isolating specifically addressed molecules, all sites share the same solution. Some products of aSDA are free in solution. Therefore one concern regarding the application of aSDA on a microarray is that of carryover or cross-contamination between test sites. This is clearly an issue when the same sequence is being analyzed for multiple samples. But carryover between sites can also affect tests for different sequences if the amount and is significant or especially if there is some cross-reactivity between the sequences. Since carryover between different samples being analyzed for the same sequence is the more sensitive condition, we addressed this issue in a publication[11] attached as an exhibit. These results demonstrate that for Factor V gene, a sequence that displays robust

amplification, ten samples of all possible SNP genotypes could be reliably discriminated. These results indicate that it is highly unlikely that carryover is an issue with the genetic panels developed for human DNA identification.

One of the challenges of this project has been transitioning from methods and materials that function in the research lab, to methods and materials furnished by manufacturing. In order to meet yield and other efficiency requirements, processes must sometimes be changed in manufacturing. While these changes are validated for their effect on commercial products, it is not feasible to also check the effects on all products still in research. This has created several significant impediments to progress in this project.

The approach we have taken is to continually improve the robustness of the assay by expanding the boundary limits of performance. That is, the assay has repeatedly been improved to provide greater adaptability to varying conditions that were not under our control.

Guidelines for optimum assay performance have been developed and were continually improved as a particular parameter or experimental variable was controlled or understood better.

Apendix I shows an example of such guidelines developed throughout the project that was established and was used by all participants and researchers on the grant consistently until a new finding or implementation of changes was agreed and approved. This assured that all the results could be easily compared and a status of assay development was understood at all levels of assay management and performance. Appendix I provides generic guidelines for

designing the discriminators for the anchored SDA including the melting temperature calculations as well as recommended concentrations of reagents and salts.

Appendix II shows typical conditions used for PCR amplification of SNPs and STRs.

The development of each particular SNP followed established protocols that were optimized throughout the project, and an example of these protocols is given in Appendix III. These protocols comprised a choice and preparation of oligonucleotides including on-chip and nested primers, bumper primers (biotinylated and non-biotinylated), discriminators and ratio of references. The first optimization steps included testing of discriminators at varying concentrations and temperature. The following development step involved testing of series of designed discriminators using PCR amplicons prepared from genomic DNA samples (NIJ# samples). Ampllicons were denatured and their concentration determined on Agilent Bioanalyzer 2100. They were prepared as dilutions in a series of concentrations and addressed electronically on our cartridges. The genotypes were recorded using green and red fluorescence and calls were compared to known genotype results for each sample. Once the best discriminators were determined using this approach testing of on-chip amplification was pursued. Combinations of primers and/or nested primers were addressed at varying concentrations on the cartridges. The best amplification primers are chosen based on criteria that included primers that yielded correct genotypes as the PCR amplicons, produced highest signals and showed no

signals on primer-only pads (no target addressed). The procedure was repeated until one combination of primers satisfied those criteria.

Once the discriminators and primers were determined for a particular SNP, validation of best primer combinations was performed using a series of genomic DNA samples (NIJ# samples) prepared at concentrations that commonly ranged from 0.5 ng/µl to 20 ng/µl. The sensitivity of the assay was thus achieved and correctness of genotyping confirmed by comparison with the PCR amplicons generated from same samples. If needed, some samples were sent for DNA sequencing that provided further confirmation of results.

Each individual SNP assay development followed those procedures, and once accurate results were confirmed, the newly developed SNP assay was added to the multiplexed SNP panel. This commonly required further optimization, often further varying of primers and discriminators design to prevent overlapping with existing and established multiplexed panel assay.

Appendix IV shows typical experimental conditions of the SDA workflow on the Nanogen instrument. Typical electronic addressing conditions are summarized as well as typical concentrations of primers, targets and ratio of references used in the anchored or on-chip SDA amplification assays. The procedure also emphasizes enzyme and other reagent concentrations for the on-chip amplification and amplification conditions such as temperature and time. A separate set of optimizations is normally performed in the detection step involving denaturation, reporter mix addition and imaging at varying optical conditions.

As the individual SNP assays were developed, and capability to increase the number of multiplexed SNPs we improved our understanding of mechanisms of the anchored SDA processes occurring on the chip. A number of approaches based on understanding of anchored SDA mechanism were tested and used to optimize the assays. These included using different nicking enzymes, number of bumper primers and/or nesting primers, excluding bumper primers, or for instance repeating the amplification process on the same chip.

One mechanism in the assay that might affect performance is nicking of the restriction site. Nicking provides an extendable site for the polymerase. Allowing the restriction enzyme to cut both strands of the hybrid would result in a loss of the template strand. The original assay uses α-thiol dNTP to limit cutting by the restriction enzyme. The thiolated nucleotide is selected so that the SDA restriction site is modified on only one strand. That way a restriction enzyme can be manipulated to nick only one strand of the hybrid. However, the restriction enzyme is capable of cutting through the thiolated site. And the α-thiol dNTP is not the natural reagent of the polymerase, so it likely produces some decay in the processivity of the polymerase. α-thiol dNTP also adds to the complexity and cost of the reagent mix.

New England Biolabs (NEB) has engineered enzymes that have one of their cutting domains inactivated. This means that α-thiol dNTP is not needed in the mix. After trying several NEB nicking enzymes, we settled on BbvC 1B. It has a 7 base recognition site which means that the recognition is not frequently found in the genome, which is an important factor.

Some recent advancements in assay performance have also resulted in a more general improvement to assay expandability. The issue of complexity was discussed earlier in a comparison between bacterial and human genomes. Complexity can also be an issue when working with large numbers of amplified loci. That problem is addressed in this system by sequestering amplification at individual test sites. However, standard SDA protocols require bumper primers in solution (fig 5). As the number of loci being amplified increases, so does the number of bumper primers and complexity becomes an issue.

Therefore we asked whether it would be possible to eliminate bumper primers. It is theoretically possible, if bridging between double-stranded amplification primers occurs (fig 6). The function of the bumper primers is to displace the genomic strand of DNA. This exposes the extended single-strand and allows complementary polymerization, including the nicking site. This process activates the nicking site and permits exponential amplification to occur.

The same result can be attained if an unextended amplification primer can invade the end of the extended hybrid. In order to get amplification with anchored amplification, bridging between extended single-stranded primers and unextended primers must occur. Since DNA hybrids open up most often at their termini[12], it is possible that primer bridging by invasion can occur (fig 6).

Using some of our more robust SNP loci, we attempted to do aSDA without any bumpers. An example of those results is shown in Figure 7. We then began adding more loci from the panel developed. We found that several weaker loci yielded either no signal or not enough signal to allow for genotyping.

Since elimination of bumper primers is highly desirable, we tested other methods of improving non-bumper aSDA. A method that was successful turned out to be relatively simple. After 30 or 45 minutes, we replaced the SDA reagent mix and allowed the reagent to proceed for another 30 or 45 minutes. This resulted in significantly more signal (fig. 8). There are likely multiple reasons for this a result. While the polymerase is thermophilic, its activity declines with time at higher temperature. This occurs less rapidly at 50° C where the assay is run, but its performance does decline. As mentioned above, there are no thermophilic nicking enzymes available that support anchored SDA. Therefore we could only use a nicking enzyme that that has an activity optimum at 37°C. So enzyme activity is replenished with replacement of the reaction soluion.

A further complication is that this assay relies on the ratio of activities of the polymerase and the nicking enzyme. Too much polymerase activity results in extension and displacement of randomly nicked sites in the genomic DNA. Too little nicking activity might result in the same outcome. Too much nicking activity can cause a double-strand cut, resulting in loss of signal. Since the activities of the polymerase and the nicking enzyme decline at different rates, the optimal activity ratio can be lost rapidly. This situation would be aided by use of a compatible thermophilic nicking enzyme, a product that NEB is developing.

Another reason for observed improvement in assay performance following replacement of the reaction solution may be the removal of unwanted side reactions that can accumulate in the solution phase. Solution SDA does not always prepare unique product. If a sample from solution SDA is run on a gel

and stained with ethidium bromide or other nucleic acid stain, many bands will be seen. In anchored SDA, solution amplification reactions can also occur. With time, they can dominate the anchored amplification reactions and rob the anchored reaction of enzymes and reactants. Replacement of the reaction solution removes the majority of the solution amplification templates and "resets the clock" in favor of anchored amplification.

This simple modification to the protocol permitted robust amplification of 22 SNP loci without the need for bumper primers. However, we had indications that a complex panel might require bumpers for selected loci.

6. STRs – On-chip SDA and analysis

The original of this project was to use Nanogen's active DNA chips to analyze length polymorphisms in loci containing short tandem repeats. To enable this, a novel method of STR analysis using hybridization probes was invented (U.S. patent 6,395,493). This invention was discussed above. Using PCR, we developed assays for 8 STR loci. Since this work was started before the announcement of loci selected for CODIS, a few of the developed loci were not in the CODIS thirteen. Also we found that development time increased significantly as we moved away from the simpler loci (CTT) towards ones with higher numbers of alleles and high frequencies of microvariants (D21S11). Through the suggestion of reviewers at NIJ, we ultimately decided that completion of all thirteen CODIS loci would consume too many resources,

especially when combined with anchored SDA. Therefore we began to consider other uses of the technology developed for DNA identification applications.

The use of SNPs for human identification was just coming under consideration at this time. It was suggested that Nanogen proceed with SNP analysis, incorporating anchored SDA into the assay. Those efforts are discussed below.

We also discussed using STRs in combination with SNPs as a means of creating a method that might yield results that could be back-compatible with STR databases (fig 9). The initial plan was to include six STR loci with a panel of SNPs, as a demonstration of the feasibility of this approach. We developed assays for three loci (fig 10) and designed reagents for another three (table). Later in the project, development of SNP assays became a priority and we focused on those.

7. SNPs – On-chip SDA and analysis

The major attraction of this system is the ability to combine amplification and analysis on the same medium without the need for movement of the analyte. Therefore it was determined to be valuable to use other genetic variants even though their applicability to human identification was not yet validated.

At the onset of the SNP phase of the project, it was not clear what a SNP panel should look like. It was determined with NIJ that a representative panel

would be developed consisting of: Y-chromosome SNPs, mitochondrial SNPs and autosomal SNPs. Y-SNPs had just been described by Mike Hammer, Peter Gill, Peter Underhill and others, but their genomic distribution was not yet fully understood. After many Y SNP assays had been developed for this platform, it was determined that their greatest value lies in identifying populations rather than individuals. They have remained on the multiplex panel. Analysis of Y markers has a technical advantage because there is only a single copy in an individual's genetic make-up. However one of the Y loci, P25, is present in multiple copies. Three copies have been documented (STRbase). Prior to this discovery we were having difficulty understanding our results with P25, which often gave sample specific ambiguities. It is now clear that the assay was reflecting what was present in the sample.

The use of autosomal SNPs for human identification was also a novel concept at the time the initial SNP was being considered. However it seems that the value of autosomal SNPs for this application has increased since the original plan. Because of the growing interest in the possibility of using autosomal SNPs for human identification, they became the priority in the later stages of the grant. The use of SNPs for human identification has been well-supported in Europe by organizations like the Forensic Science Service and the SNPforID consortium. Peter Gill at the FSS and Angel Carracedo and Peter Schneider at SNPforID have led the investigations in this area. At the time we were selecting autosomal SNPs, Peter Gill had submitted a list of suitable candidates to STRbase and

these served as the basis for our panel. Orchid has also established a 70 locus panel and has made information on those sequences available as well.

Analysis of mitochondrial SNPs is a well established tool for forensic identification of biological material. Because of the biology of mitochondria, mitochondrial genomes can be present at a thousand-fold greater excess than nuclear DNA. Therefore it is highly useful in analyzing ancient DNA or DNA subjected to extreme levels of trauma and degradation. However the mitochondrial genome is riddled with variants, likely because of the low level of selective pressure on the integrity of it's genome. Heteroplasmy , where different cells can have alternative mitochondrial genetic variants further complicates analysis.

As might be expected, amplification of mitochondrial was not an issue even with DNA isolation methods optimized for nuclear DNA. However developing probes for analysis was challenging. Only a single HVII variant is included in this SNP panel because of theses challenges. We felt that it was more important to obtain the highest level multiplicity of loci possible, and that the autosomal SNPs were a good combination of utility and practicality.

The project concluded with a panel of 22 SNPs combining mitochondrial, Y chromosome and autosomal SNPs (Table 1). To our knowledge, this is the largest multiplex that is simultaneously amplified for forensic applications (fig. 11).

8. Instruments

Three different instrument systems were used during the course of the grant: the Molecular Biology Workstation with 100 pad chip, the portable NIJ box with a 400 pad chip and the Nanochip 400 which include fluid handling robotics.

The Molecular Biology Workstation was Nanogen's first instrument product. It consists of a system of two boxes, controlled by a single Central Processing Unit (fig 12). The disposable part of the system is a 100 pad chip (fig 13). Four of these chips can be addressed on the loader box while a single chip is being analyzed on the reader box. The loader therefore is a preparative device that can create user-defined microarrays.. Microarrays can be created from multiple sources: capture oligos, amplified material or any other charged molecule. There are three major buffer reservoirs. Other reagents such as oligos, amplified DNA, enzymes and other buffers can be applied by a fluidic handling robotic system through a microtiter plate. Although there is no temperature control on the loader, stringent hybridizations can be carried out by using chemical stringency or electric field stringency. Analytical probes can also be applied using the loader with subsequent imaging and analysis carried out on the reader.

The CPU is contained in the reader and programming for both boxes can take place there. Programming is facilitated by a Graphic User Interface that displays the microtiter plate grid and the electronic microarray grid. The user can click and drop reagents contents of microtiter wells to any variation of electronic

microarray pads. The user is warned when multiple use of wells or pads is attempted. Programs can be stored as templates for re-use with different samples.

The reader has an optical imaging system that accommodates the two most common fluorescent probes: red and green. The optical system detects fluorescence and translates image data to quantitative data via a Photo Multiplier Tube. That data can then be interrogated by numerous mathematical algorithms. Internal analysis algorithms have been written for some applications, but data can also be exported to spread sheet programs like Excel. The CPU can be networked with in-house systems to permit export of data and reports.

Another individual aspect of reader function is temperature control. This feature, combined with real time imaging allows melt curves to be generated. This method can be useful when interrogating sequences that are difficult to discriminate.

The need for a chip capable of analyzing more complex panels or more samples led to the development of a new disposable and instrumentation to handle it. Market analysis determined that a 400 pad chip (fig 14) would have optimal utility. Also, in order to reduce the cost of the instrumentation, more functions were transferred from the instrument to the chip. These include current switching and temperature detection. The flow cell volume remains the same meaning the total amount of reagent used can in some circumstances be reduced 4-fold relative to the 100 pad chip. Additionally, a new feedback mechanism was incorporated that permits current measurement at each pad,

allowing for more precise control of electrical field effects on molecular reactions. Starting with a breadboard device, new instruments were developed in parallel with chip development.

Designing a new instrument for commercial sales is an expensive and time-consuming effort. This grant benefited from the leverage of this program since SNP analysis also constitutes a significant health care market. Originally called simply the Next Generation Instrument, the NanoChip 400 combines the features of the MBW reader and loader into a single box (fig 15). Footprint, cost and weight have been significantly reduced. Although the NanoChip 400 had not yet been launched at the time of this report, we did perform anchored SDA SNP analysis on this instrument (fig 16).

The major amount of the work presented in this report was performed on a smaller device designed for a Dual Use Science and Technology awarded to Nanogen. Using the basic design and expertise gained from the DUST project, we built four mobile boxes for the exclusive use by NIJ personnel. These devices offered the flexibility needed to pursue assay optimization, and the ability to have access to many instruments was an asset.

Several features of the NIJ Box have applications for forensic applications. Designed as a field instrument (fig 17), it is portable and able to withstand drop tests. Power sources can be either AC or DC so that batteries may be used. This portability means that this device could be taken to a scene of mass destruction. It is also possible that the instrument could fit into a sophisticated crime scene investigation van. The tradeoff with the NanoChip 400 is the level of

automation. The NIJ Box has no fluidic handling robot, so the user is afforded less walk-away time than when using the NanoChip 400. The automation level of the NanoChip 400 is also desirable in preventing operator errors in running the assay.

9. Permeation Layer

One of the most unique and critical aspects of Nanogen's technology is the permeation layer. It serves multiple functions to enable the use of electric fields to facilitate DNA analysis (fig 18). The permeation layer serves as a structural element in providing scaffolding for attachment chemistry. This permits the DNA to be distributed above the electrolysis occurring on the surface of the electrode. A good distribution of DNA means that sufficient numbers of molecules will be localized in regions that will have the proper biochemical conditions to promote DNA hybridization.

The initial permeation layer was made up of agar. However because of the difficulty of reproducibly manufacturing agar coatings on electronic chips, other polymers were investigated. Numerous iterations of a polyacrylamide based permeation layer have been investigated. While manufacturability has been a priority, the anchored SDA assay had made it clear that the pore size of perm layer is very important. This is due to the fact that enzymes need to access DNA within the matrix of the perm layer. So the perm layer must be dense enough to provide a sufficient number of binding sites, yet open enough to allow

access to the DNA by enzymes. Unfortunately, we did not find any methods that were able to directly measure the pore size of the perm layer. Therefore any change in the perm layer needed to be evaluated by aSDA experimentation.

10. References Cited:

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2. “National Forensic DNA Study Report”, 2004, No. 203970 N.P Lovric et al.

3. “Rapid, high fidelity analysis of simple sequence repeats on an electronically active DNA microchip”, Radtkey R, Feng L, Muralhidar M, Duhon M, Canter D, DiPierro D, Fallon S, Tu E, McElfresh K, Nerenberg M, Sosnowski R, Nucleic Acids Res. 2000 Apr 1;28(7):E17.

4. “Anchored multiplex amplification on a microelectronic chip array”, Westin L, Xu X, Miller C, Wang L, Edman CF, Nerenberg M., Nat Biotechnol. 2000 Feb;18(2):199-204.

5. “Antimicrobial resistance and bacterial identification utilizing a microelectronic chip array, Westin L, Miller C, Vollmer D, Canter D, Radtkey R, Nerenberg M, O'Connell JP., J Clin Microbiol. 2001 Mar;39(3):1097-104.

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8. STRbase - http://www.cstl.nist.gov/div831/strbase/

9. “Rapid, high fidelity analysis of simple sequence repeats on an electronically active DNA microchip”, Radtkey R, Feng L, Muralhidar M, Duhon M, Canter D, DiPierro D, Fallon S, Tu E, McElfresh K, Nerenberg M, Sosnowski R., Nucleic Acids Res. 2000 Apr 1;28(7):E17.

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12. “Kinetics for exchange of imino protons in deoxyribonucleic acid, ribonucleic acid, and hybrid oligonucleotide helices”, Pardi A, Tinoco I Jr., Biochemistry. 1982 Sep 14;21(19):4686-93.

11. List of Figures

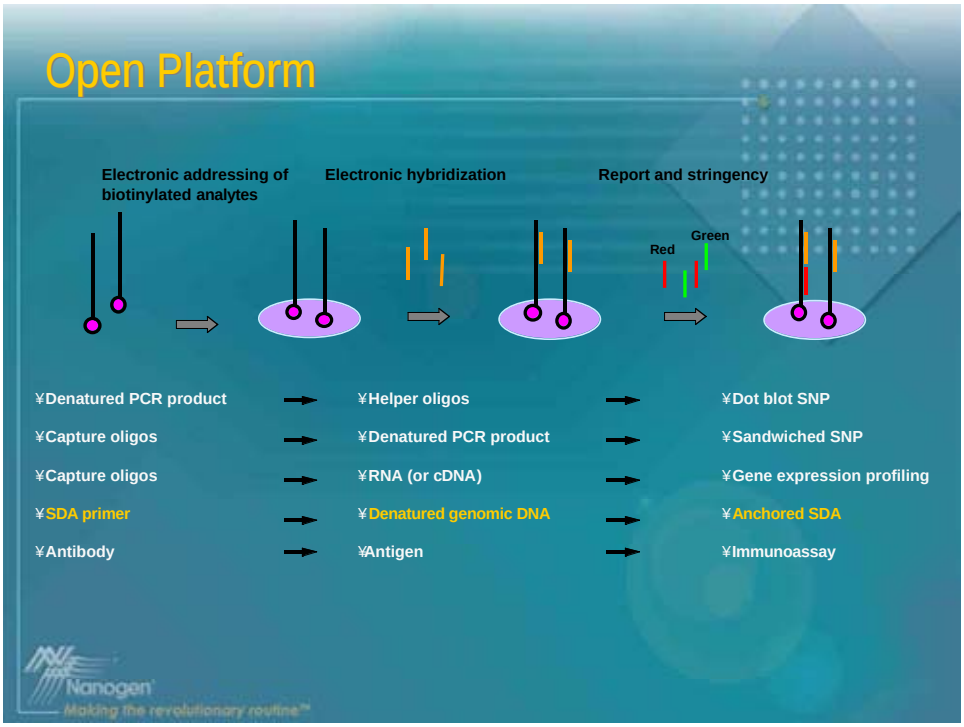


Figure 1 – Examples of different electronic Microarray assay formats.

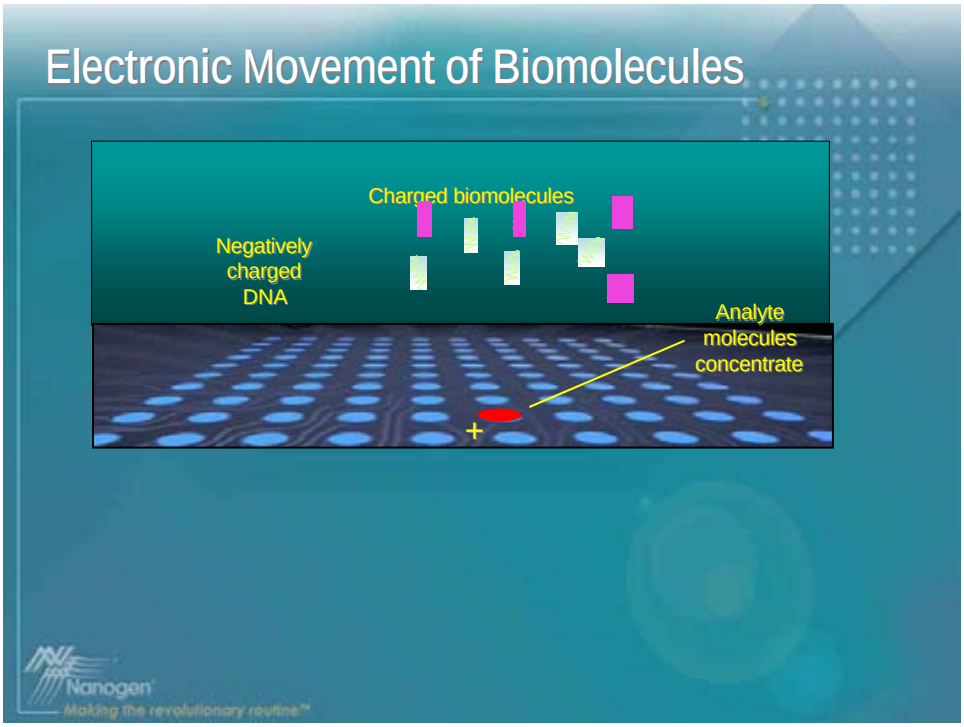


Figure 2a – Charged molecules (DNA is negative) are placed in solution over the microarray.

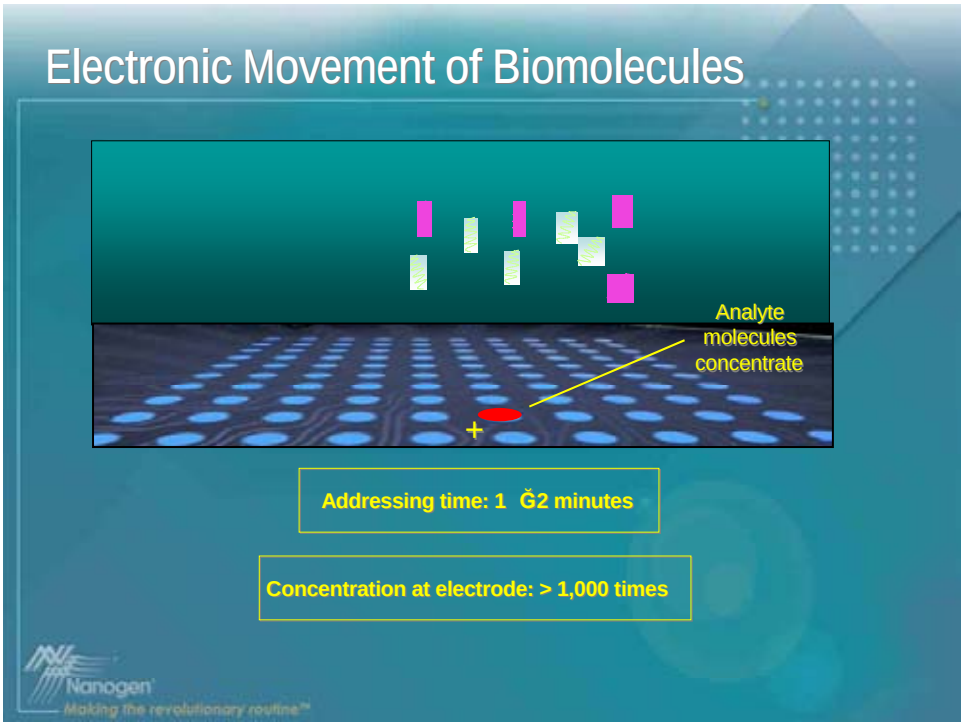


Figure 2b – An individual pad is activated to attract oppositely charged molecules to the site.

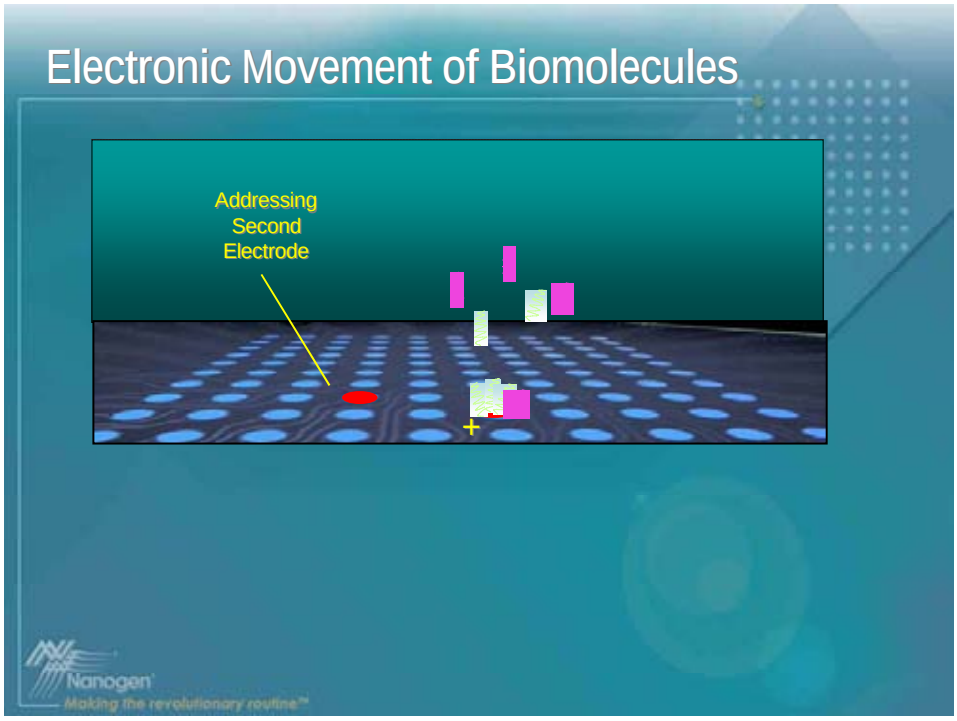


Figure 2c – Molecules concentrate to an equilibrium concentration. Additional sites may be activated

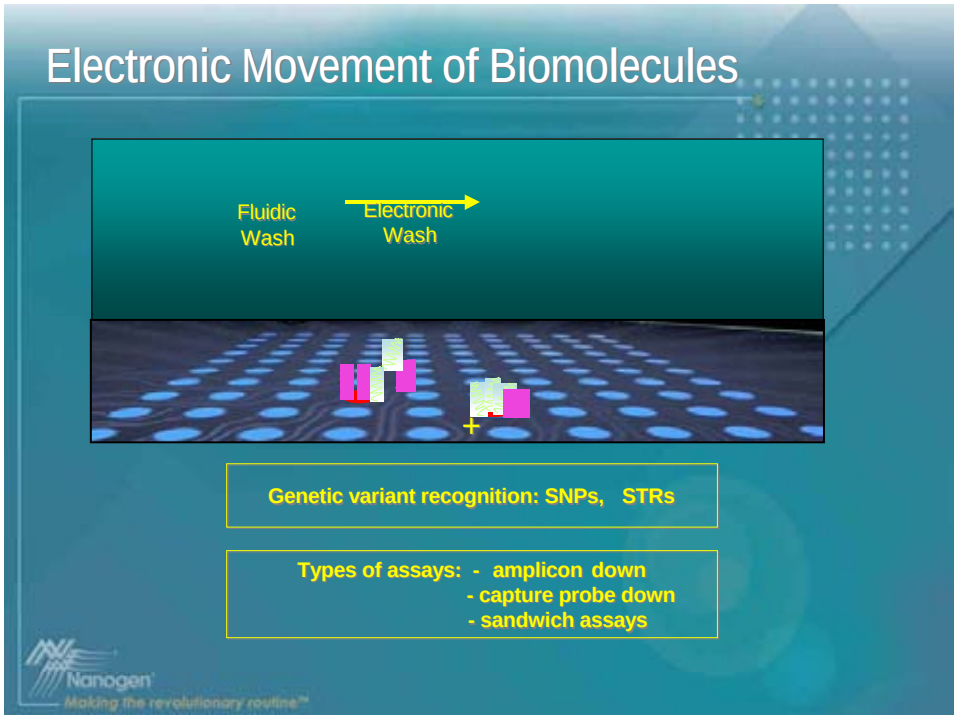


Figure 2d – Electric field concentration of charged molecules drives an attachment reaction between moieties on the mobile molecules (biotin) and complementary entities imbedded in the permeation layer (streptavidin)

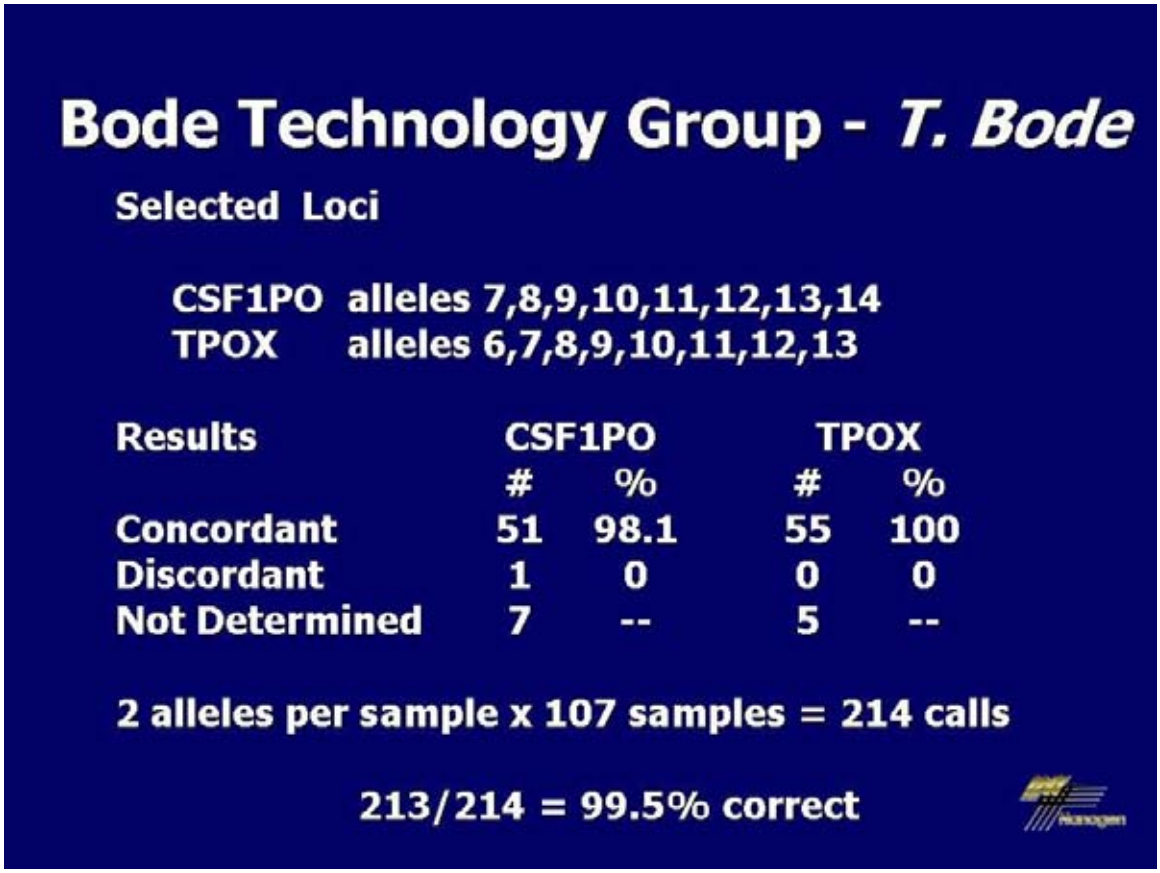


Figure 3 – Results of a concordancy study performed under the direction of Jim Schumm at The Bode Technology Group

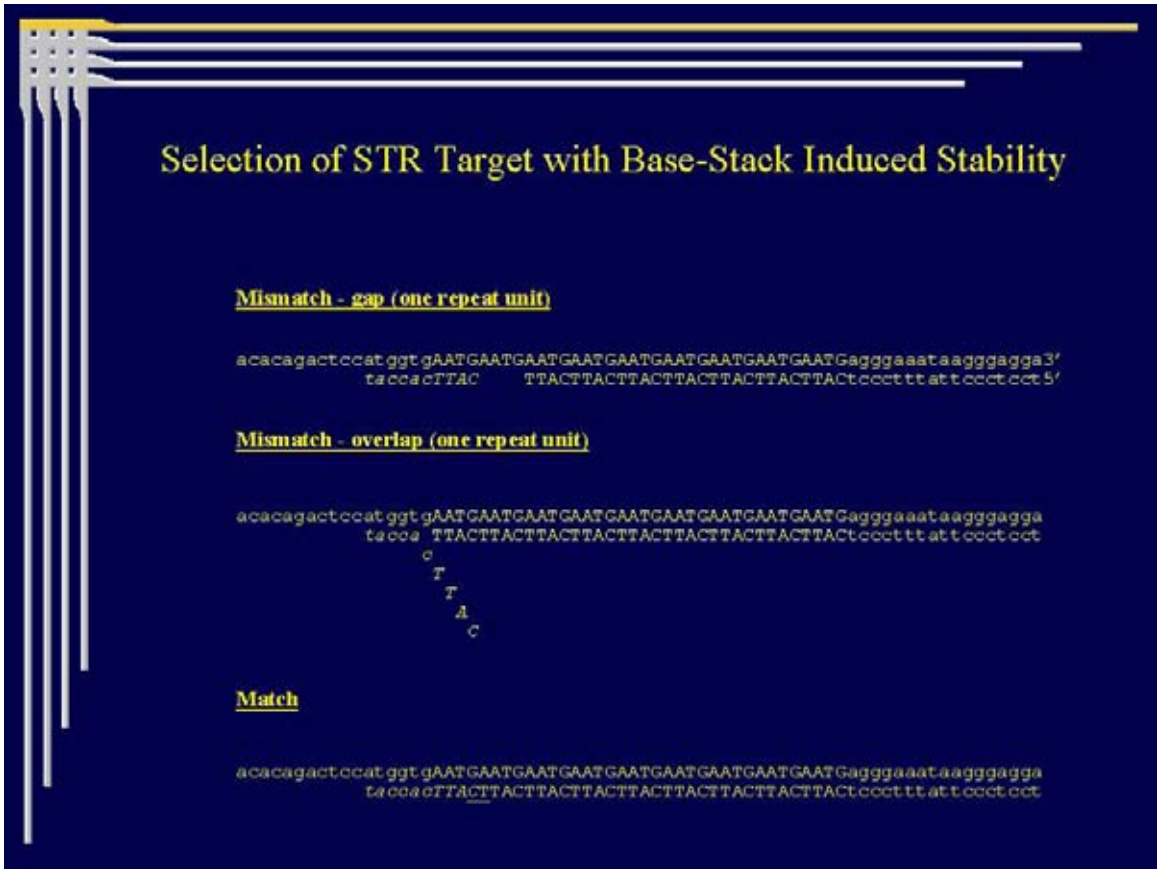


Figure 4a – Depiction of two types of mismatch and a match hybrid complex

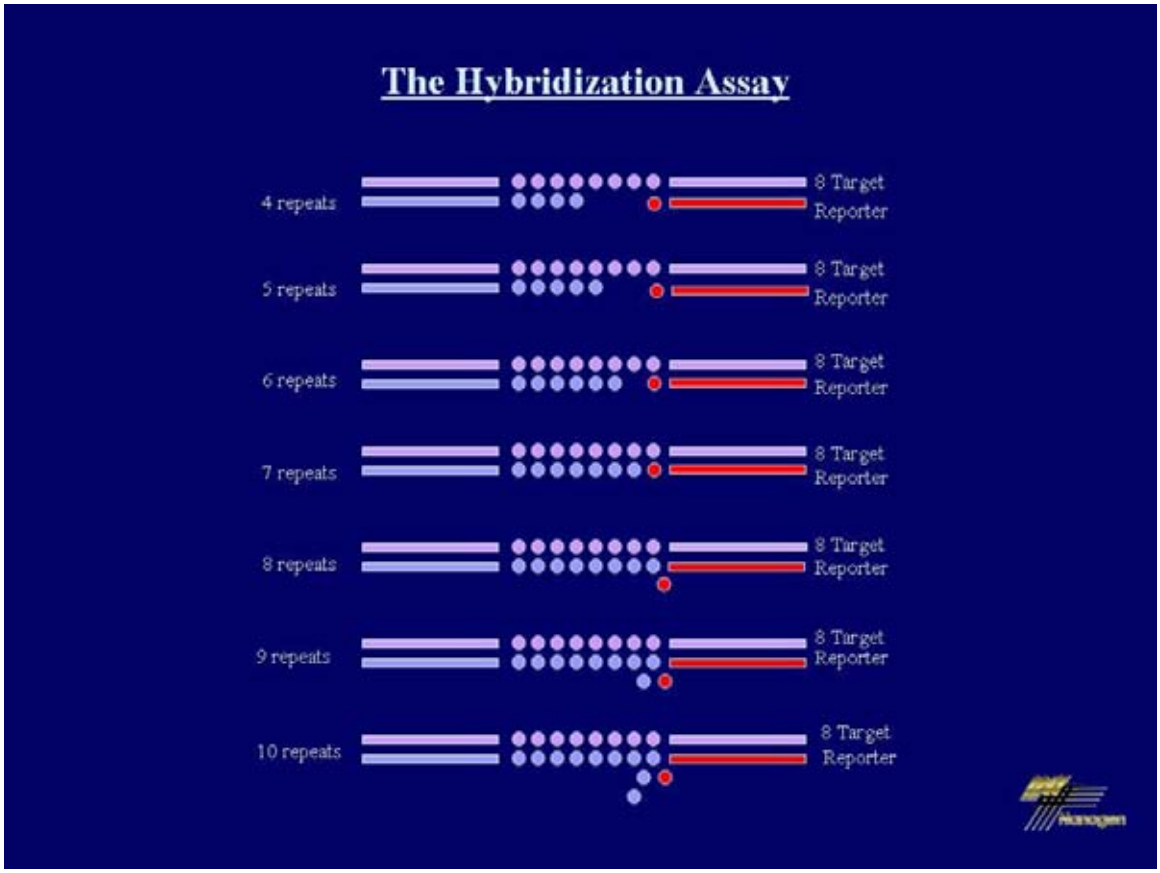


Figure 4b – Use of an array to distinguish STR alleles. Individual test sites are specific for a different allele

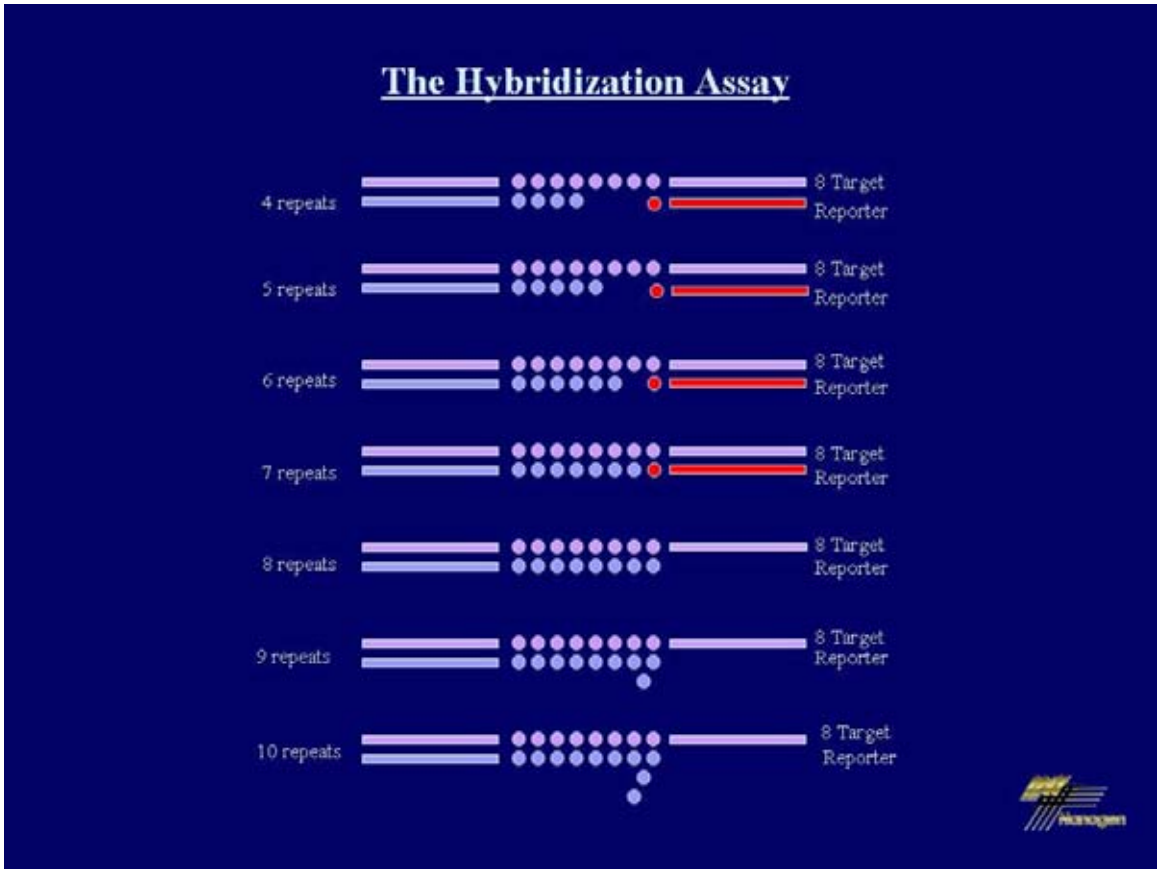


Figure 4c – Application of initial stringency conditions deselects reporter from the least stable hybridization complexes

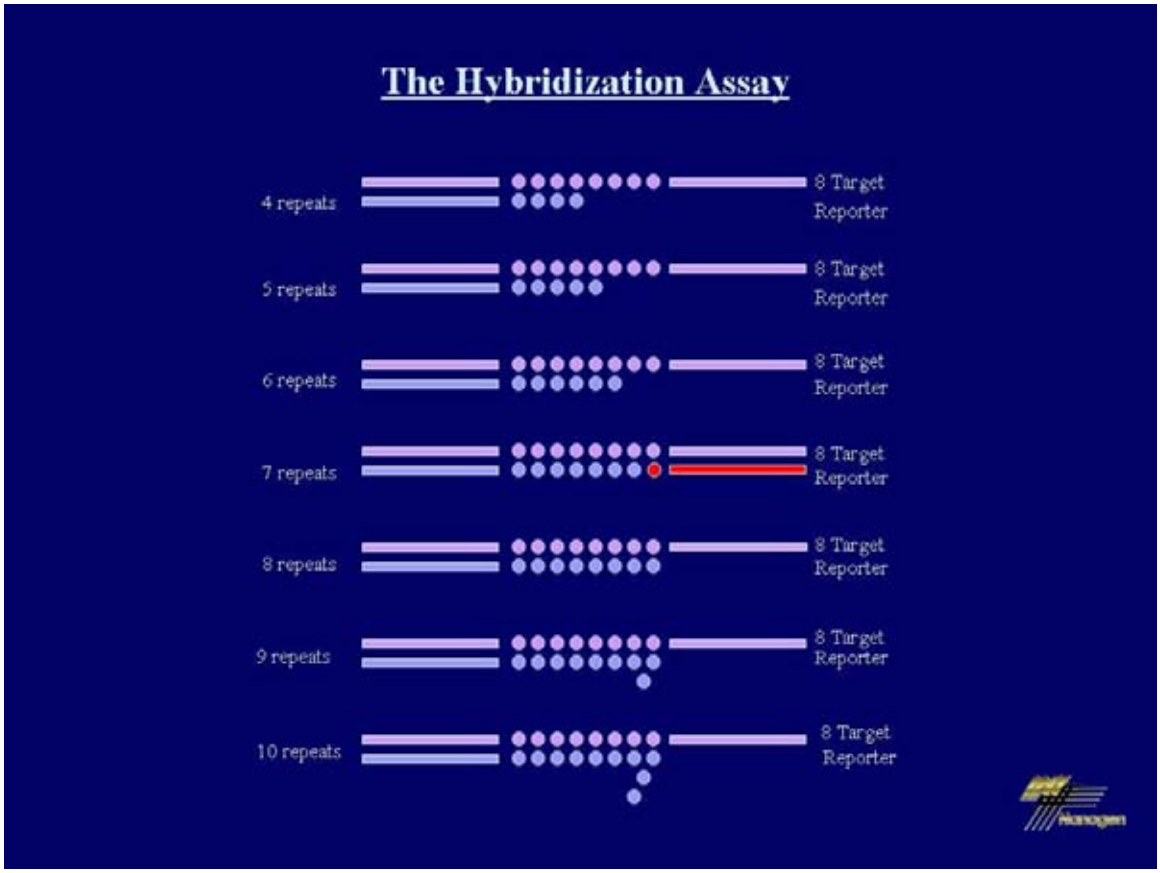


Figure 4d – Continued application of stringency removes the next stability class of reporters. Sites with reporter remaining at this point indicate a match. Knowledge of the capture oligo addressed to that site provides an algorithm with data necessary to make an allelic determination.

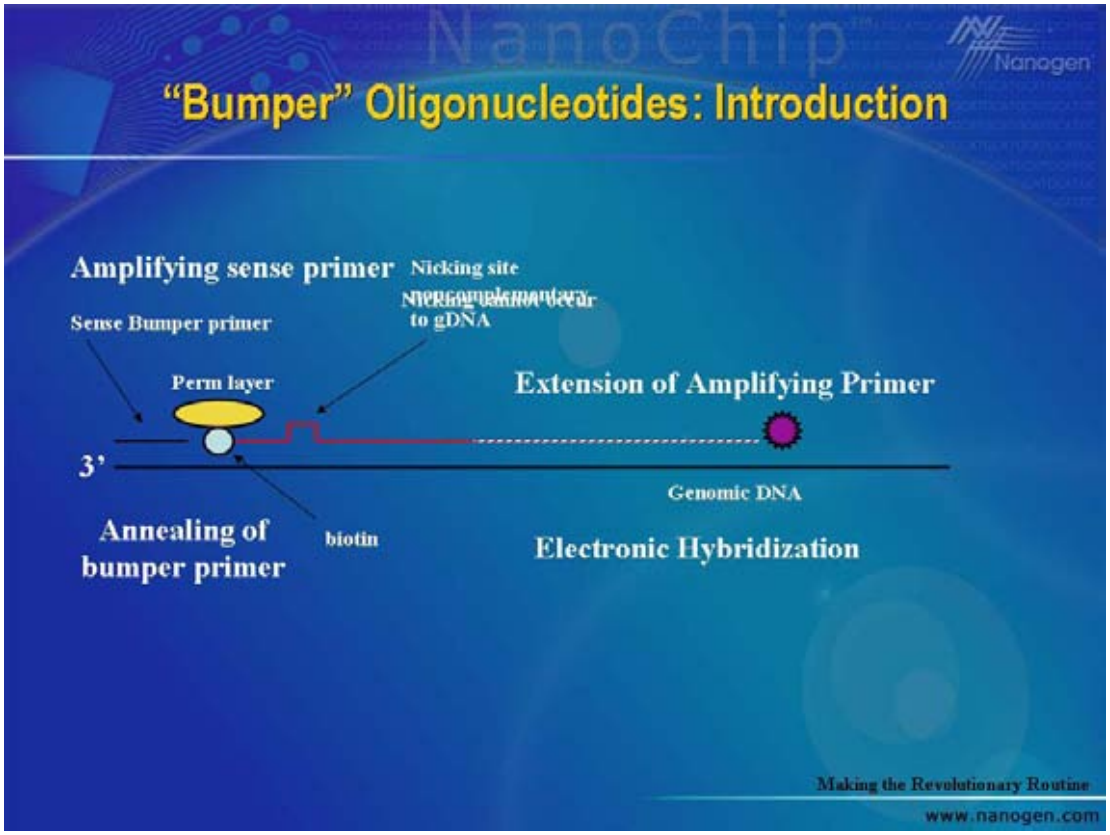


Figure 5a – A bumper primer is designed to bind downstream from the amplification primer sequence and provide a template for the polymerase.

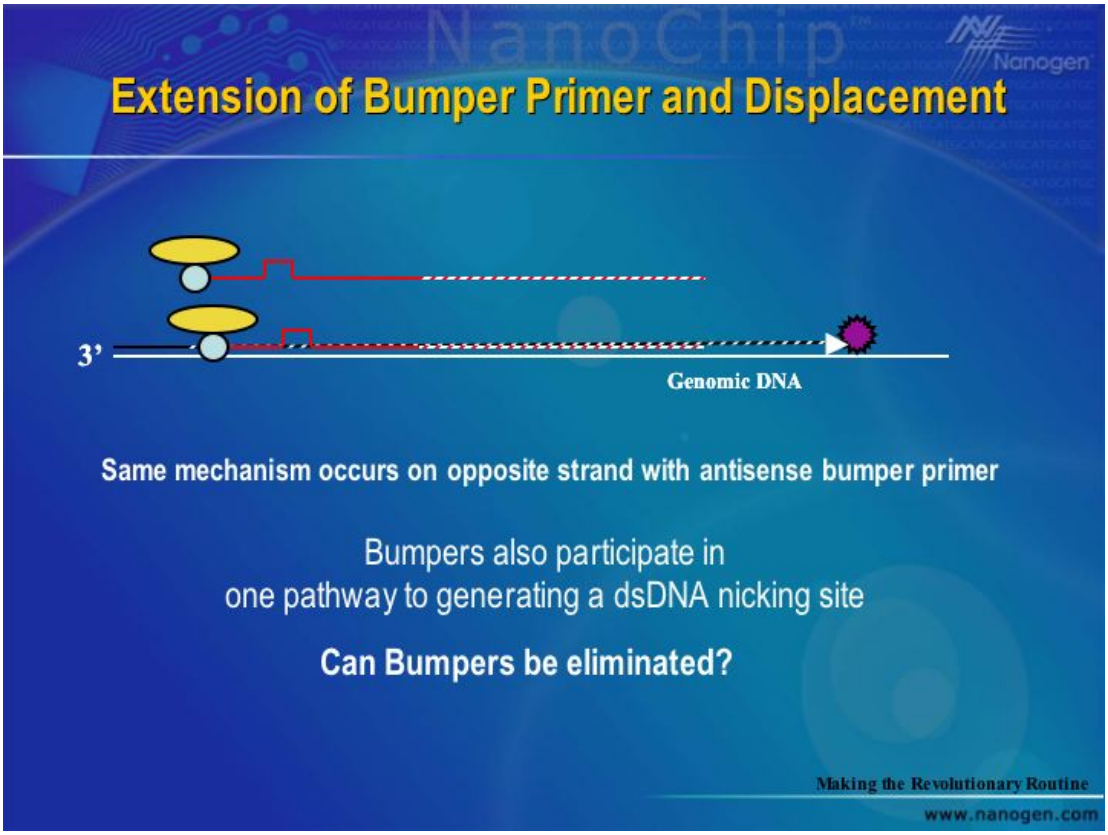


Figure 5b - This extension will displace ("bump") the gDNA strand and allow either the complementary amplification primer or bumper primer copy the extended strand and nicking site. Once a double strand copy of the nicking site has been made, the nicking enzyme will provide a nicked template and bumpers will no longer be needed.

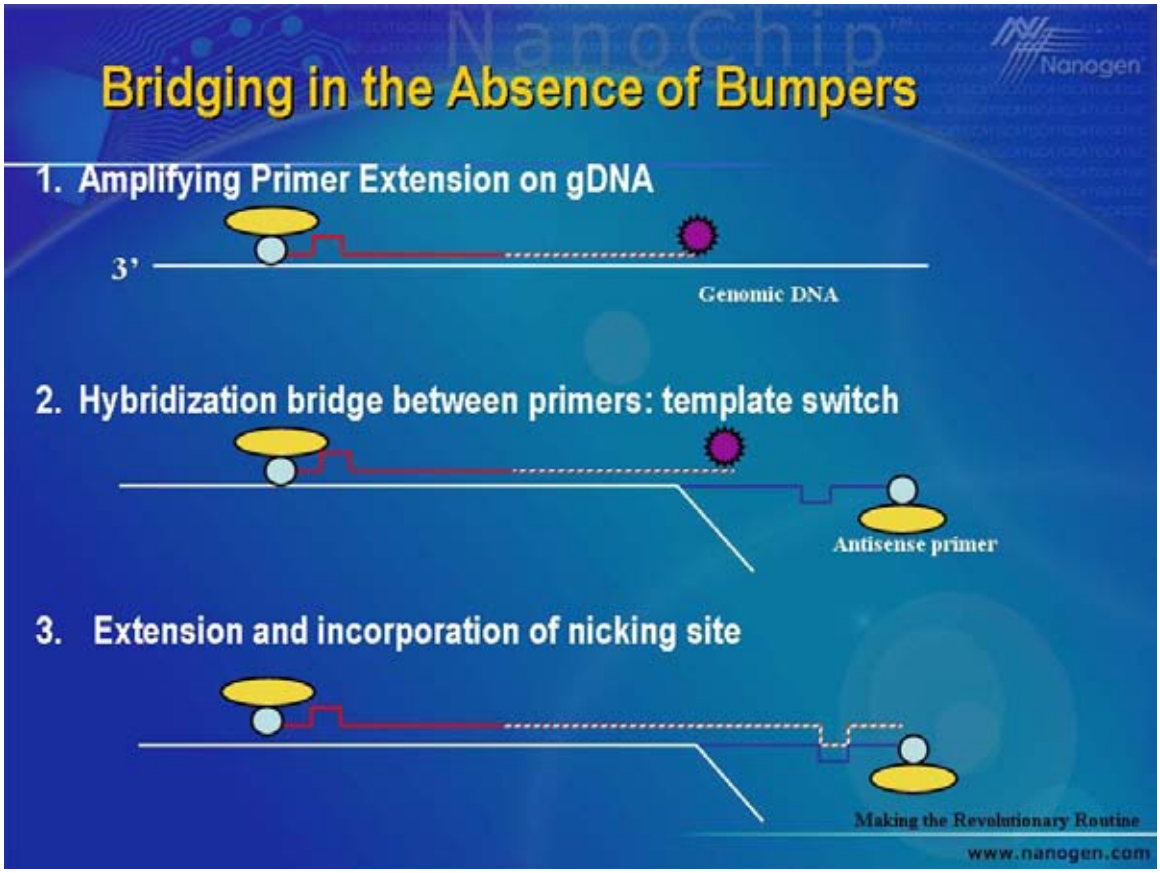


Figure 6a – 1) Genomic DNA hybridizes to a primer and is extended by the polymerase. 2) The end of the extended duplex "breathes". This partial dehybridization permits an antisense primer in close proximity to hybridize and provide a template for the polymerase. 3) The polymerase can now use the antisense primer as a template and incorporate the nicking site.

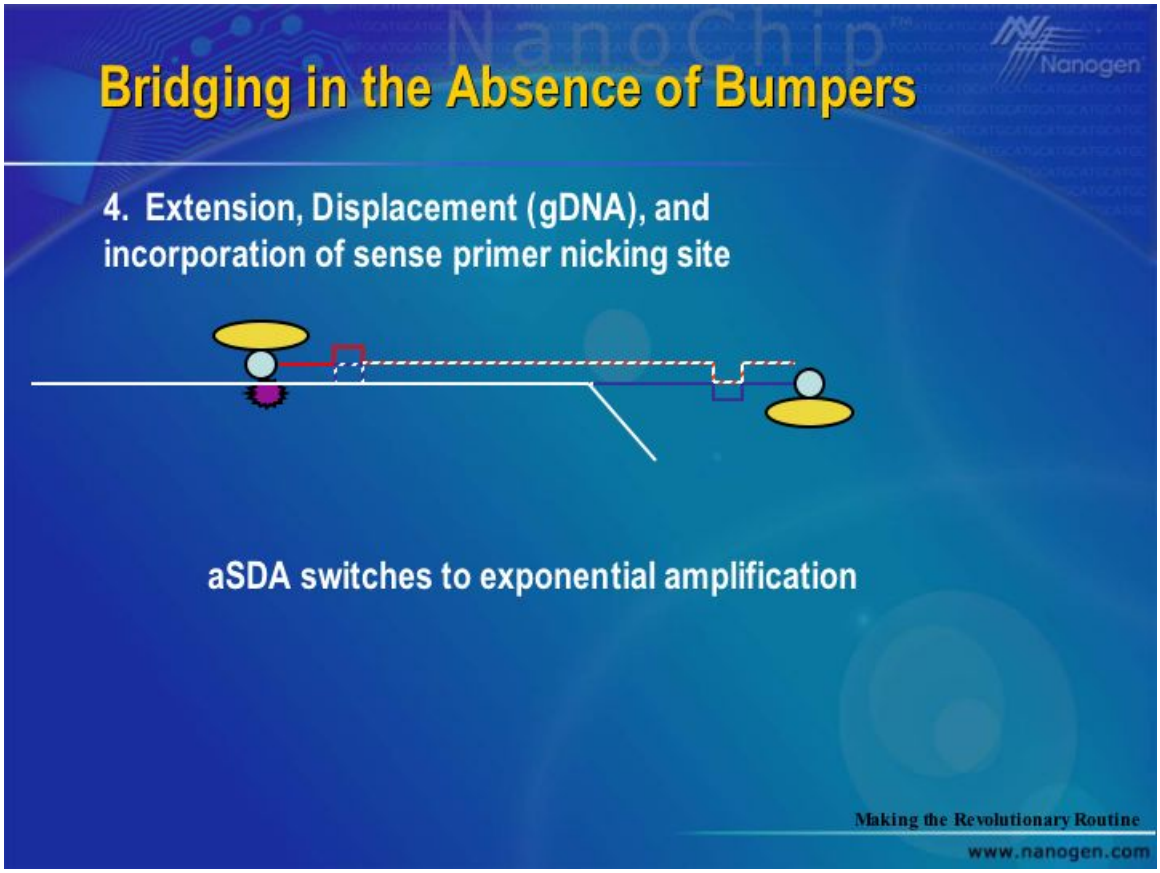


Figure 6b – 4) The polymerase may also extend from the end of the antisense primer, incorporating the complementary nicking site.

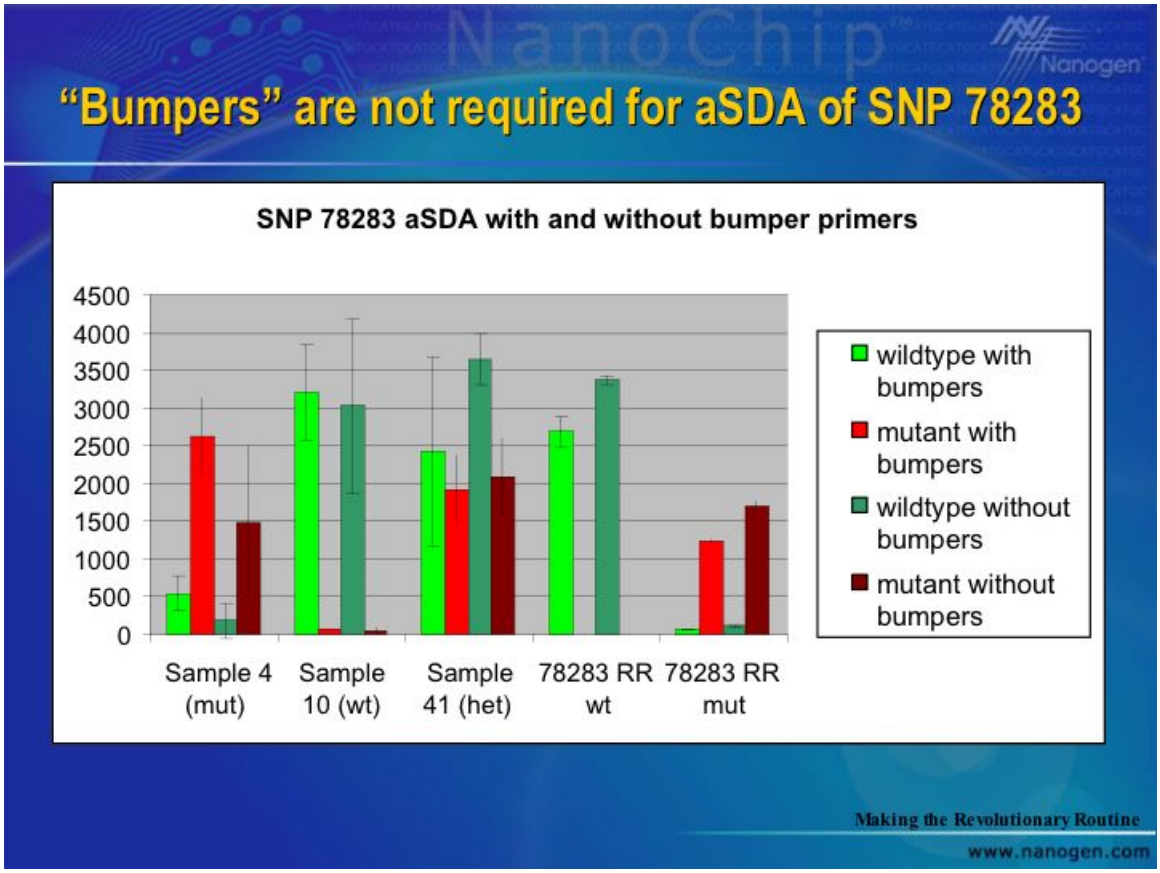


Figure 7 – Amplification of a SNP locus without the use of bumpers. Three different samples were analyzed on the same chip either with or without bumpers as indicated. RR – Ratio Reference standards.

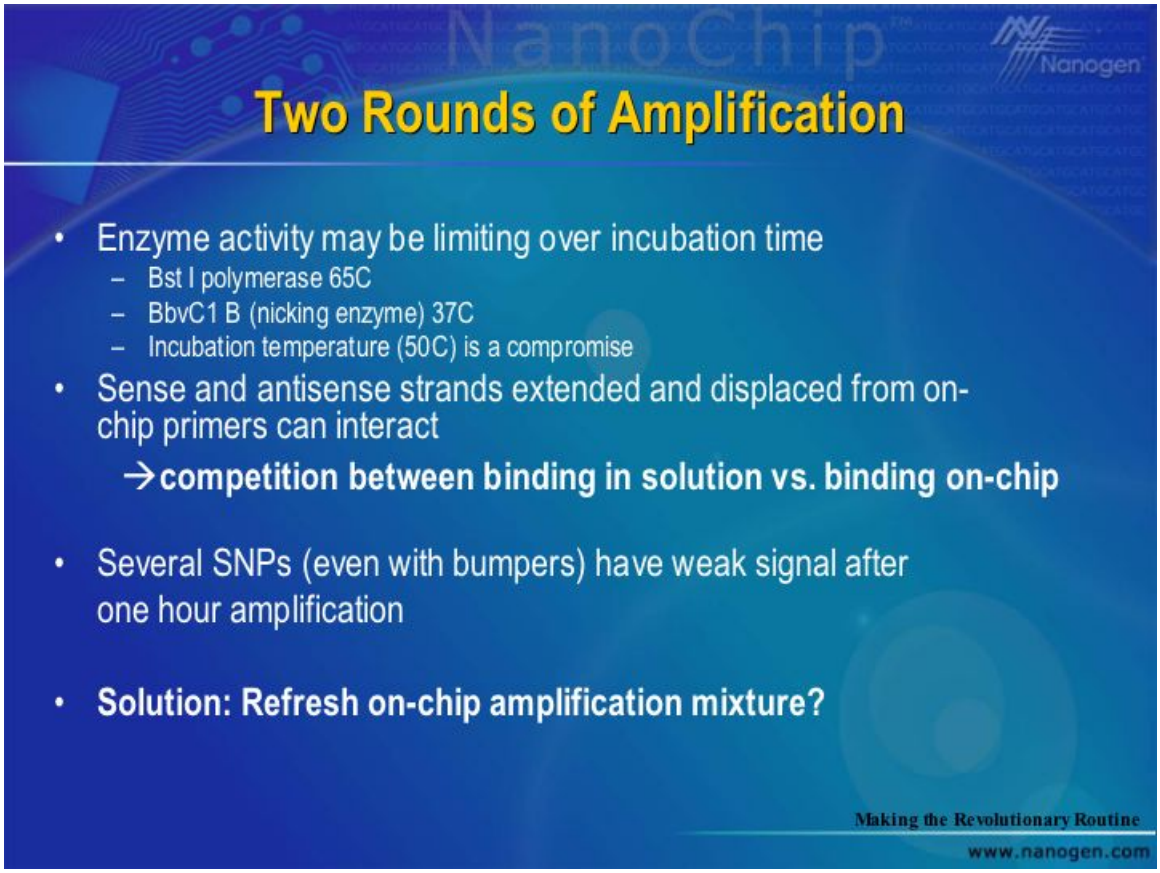


Figure 8a – Reasons for double rounds of amplification

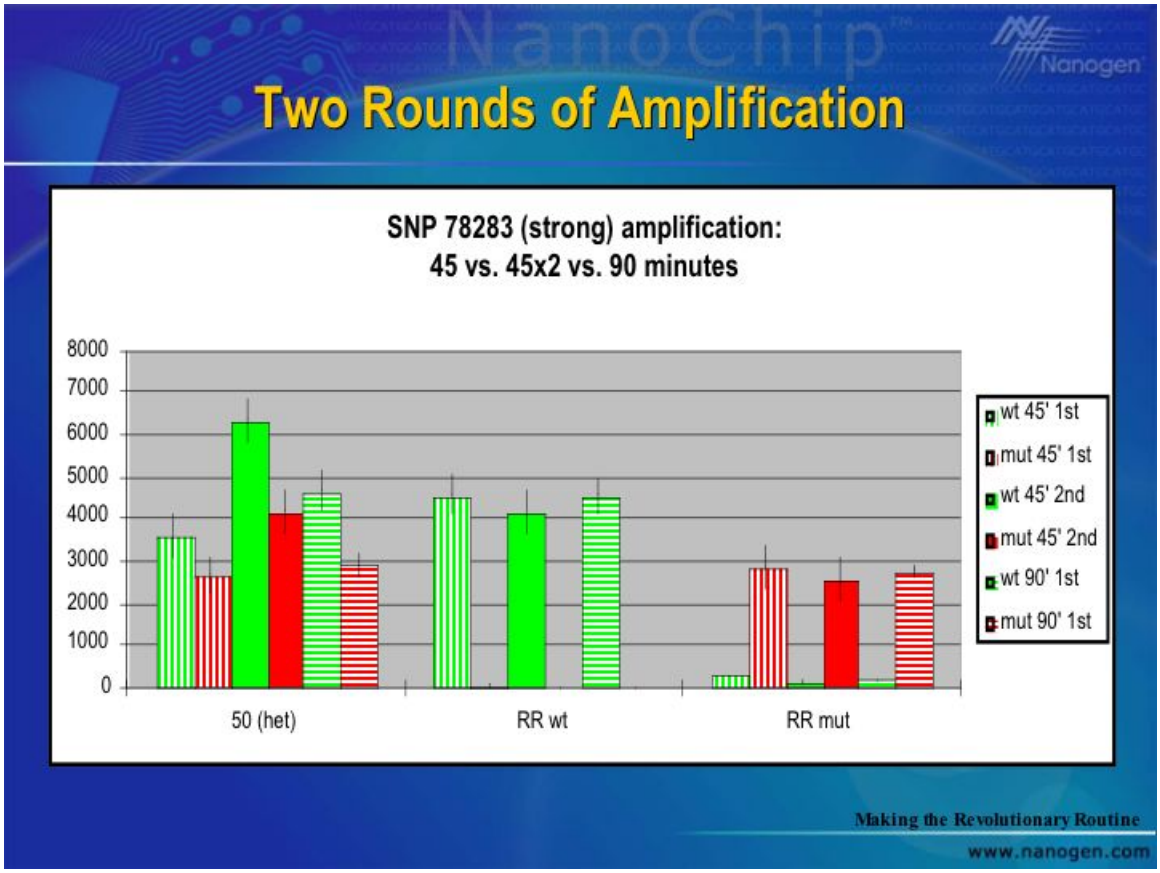


Figure 8b – Comparison of amplification time protocols (without bumpers). SNP 78283 amplifies well with all protocols.

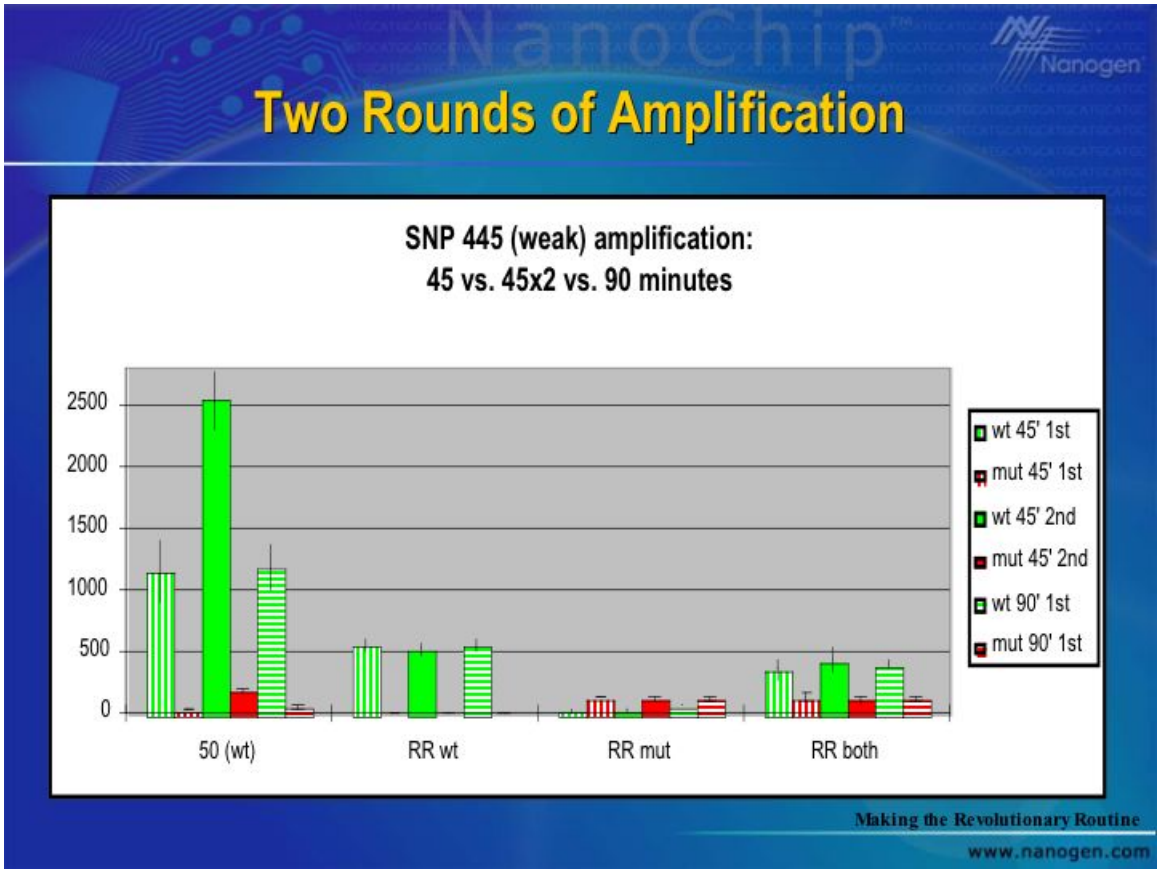


Figure 8c – Comparison of amplification time protocols (without bumpers). Locus TSC1342445 benefits significantly from two rounds of amplification.

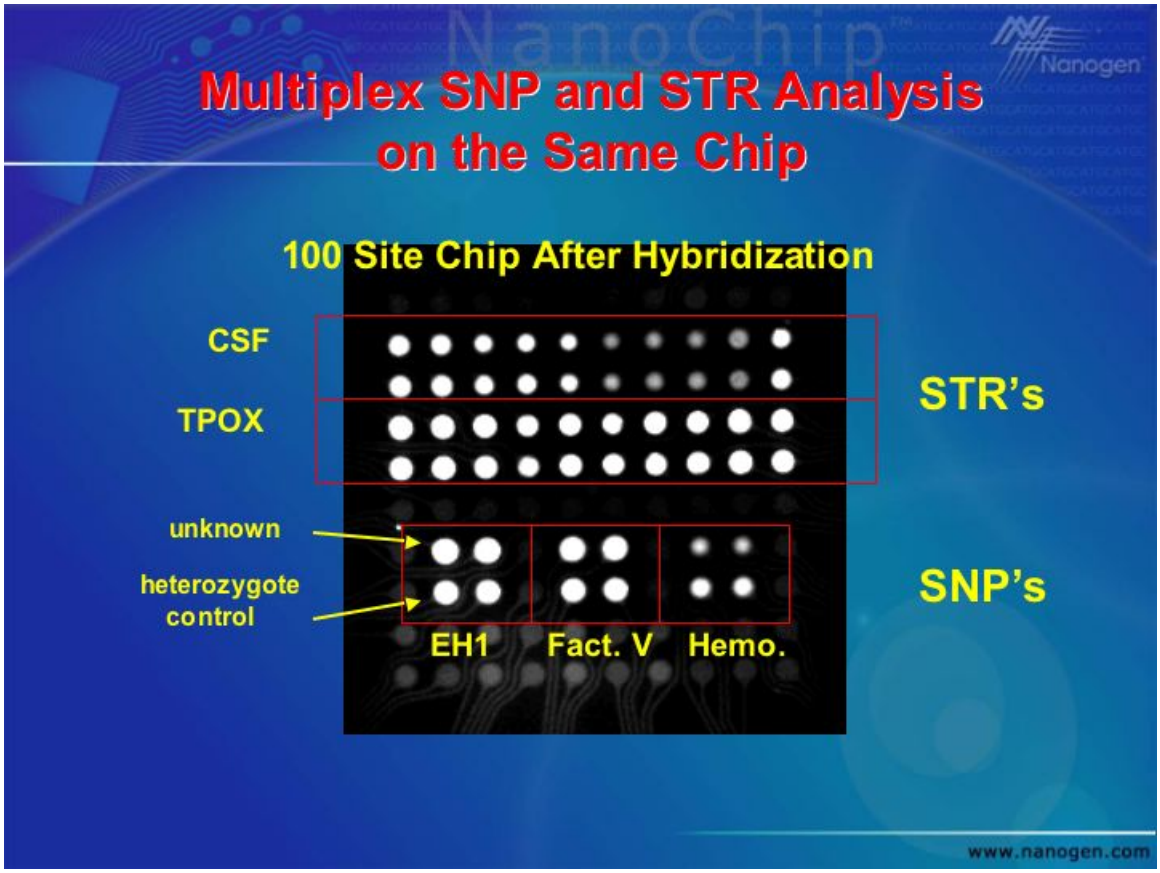


Figure 9 – Multiplex SNP and STR Analysis on the Same chip a) – Image of fluorescence detected as a result of hybridization of targets and probes.

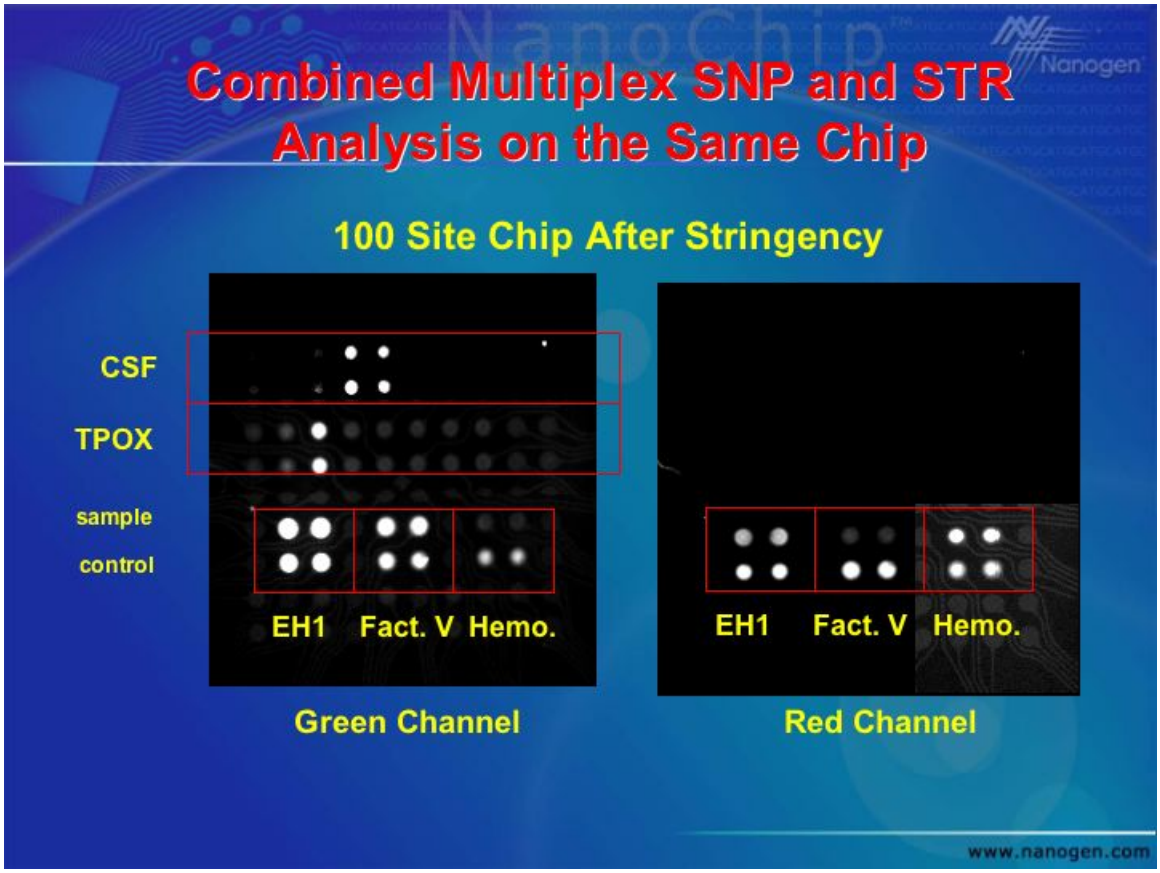


Figure 9b – Image of fluorescence after stringent conditions were applied. Fluorescence remains at pads with the greatest stability of the complex, indicating the target length (see text).

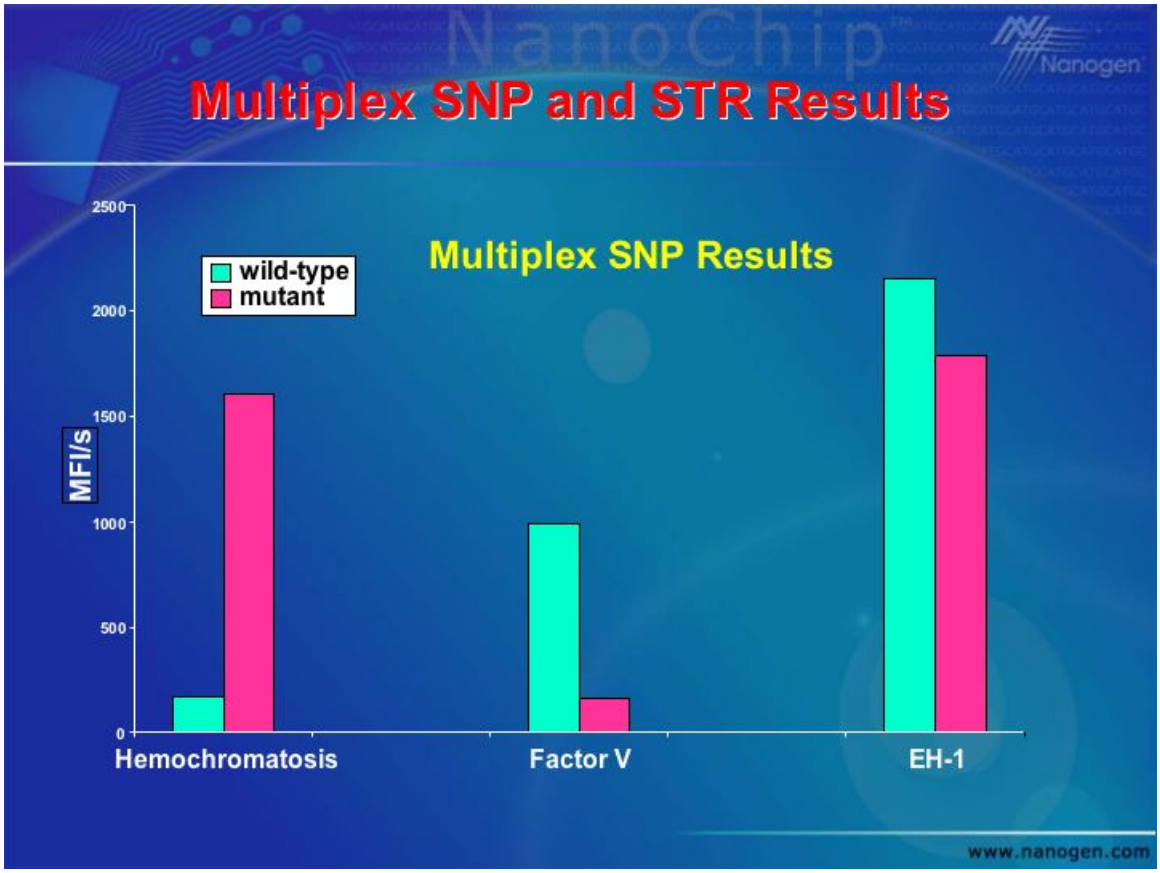


Figure 9c – Histogram representing the fluorescence remaining on SNP pads after stringency.

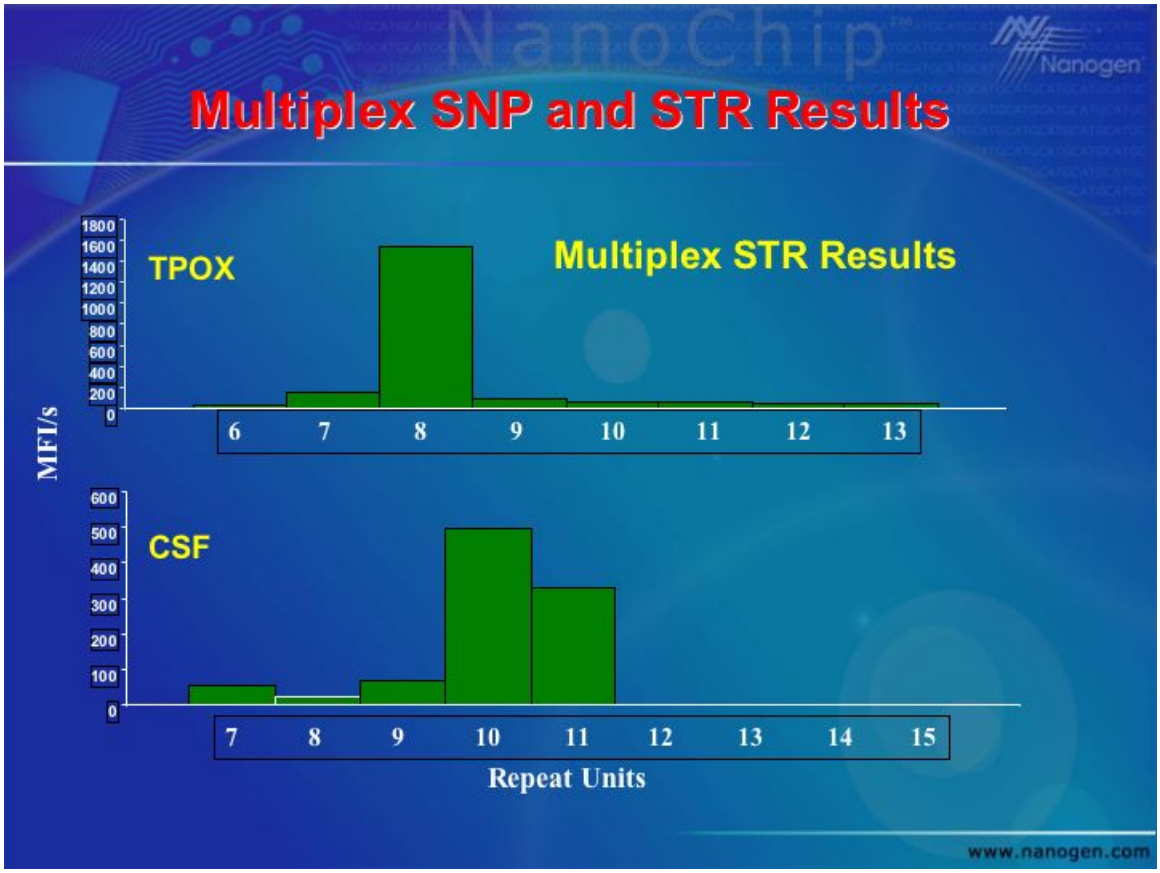


Figure 9d – Histogram representing the fluorescence remaining on STR pads after stringency.

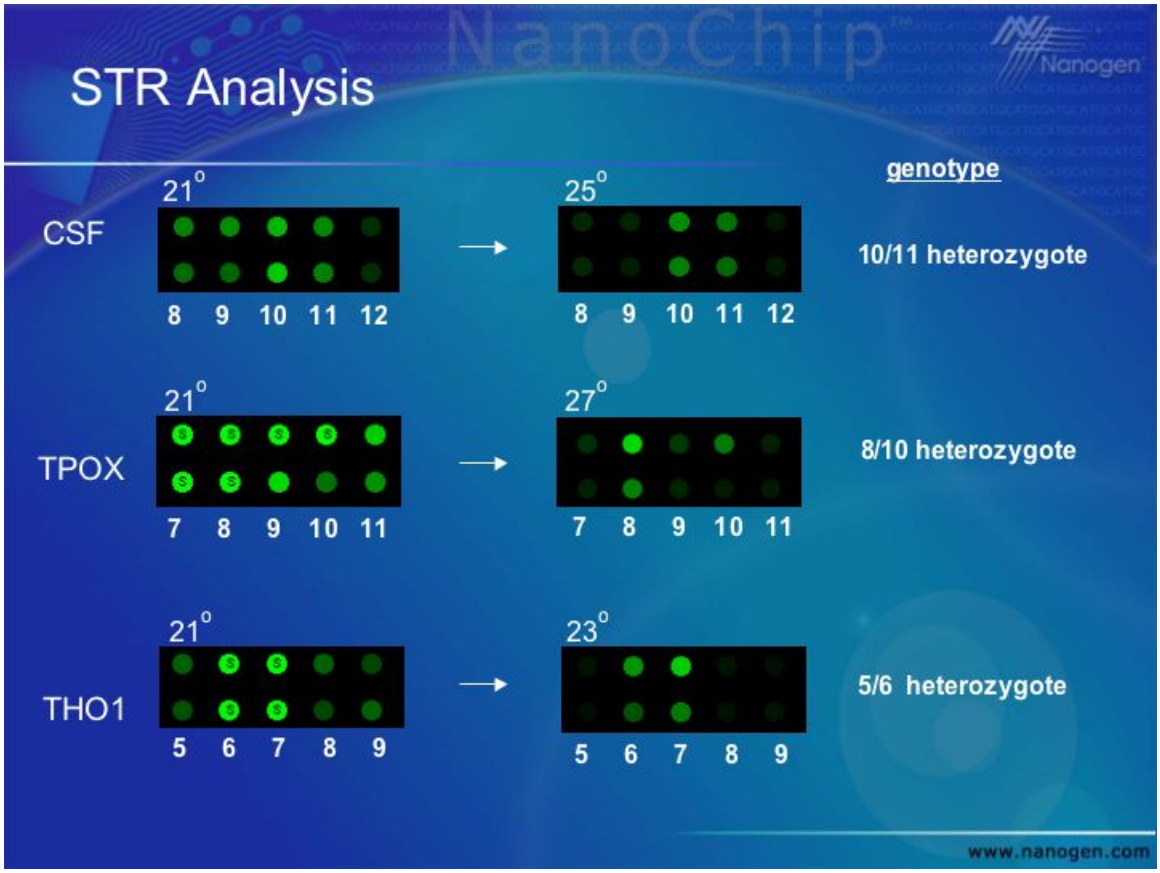


Figure 10a – Psuedo-Image of aSDA amplified STR loci on a chip. Images on the left side are post-amplification and labeling, but prior to thermal stringency. Images on the right are post-stringency. Thermal stringency was applied as a ramp-up in temperature, with continuous data acquisition.

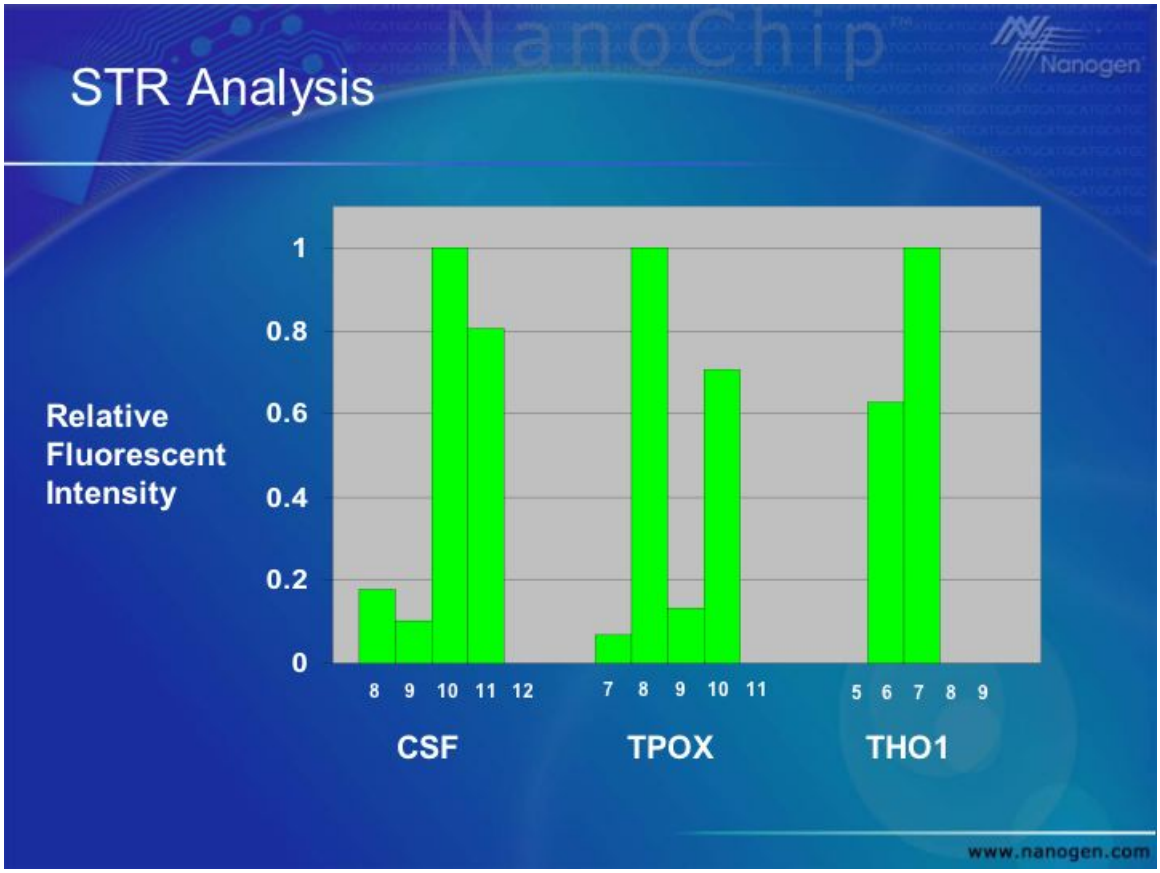


Figure 10b – Graph of data collected from above experiment. CSF alleles – 10/11, TPOX alleles – 8/10, and TH01 alleles – 6/7. These samples had previously been genotyped by The Bode Technology Group and the results are concordant.

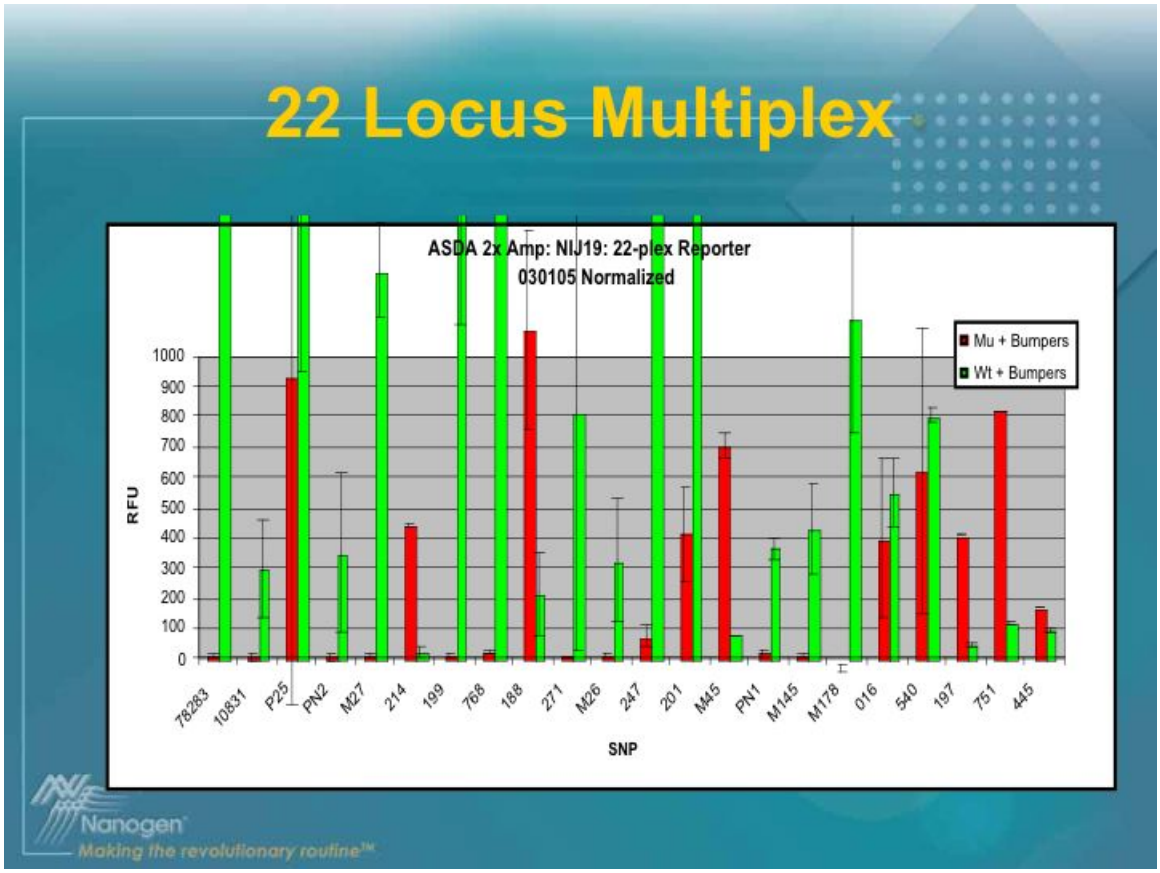


Figure 11 – Discrimination results from a simultaneous amplification of 22 loci. Results are concordant with tests from individual PCR amplification of the same loci.

Figure 12. The Nanochip® Molecular Biology Workstation. The NanoChip System is a fully integrated system capable of electronically loading samples onto a microarray and interrogating samples using the built in fluorescent reader. The system has the capacity to analyze up to 4 arrays in a single run. The 100-site electronic microarray is contained within a plastic cartridge. A permlayer, into which streptavidin has been embedded, is molded on top of the microarray. The insert shows the cartridge with 100 array sites or electrodes.

Figure 13. The Nanochip® 100-site electronic microarray is contained within a plastic cartridge with fluidic ports.

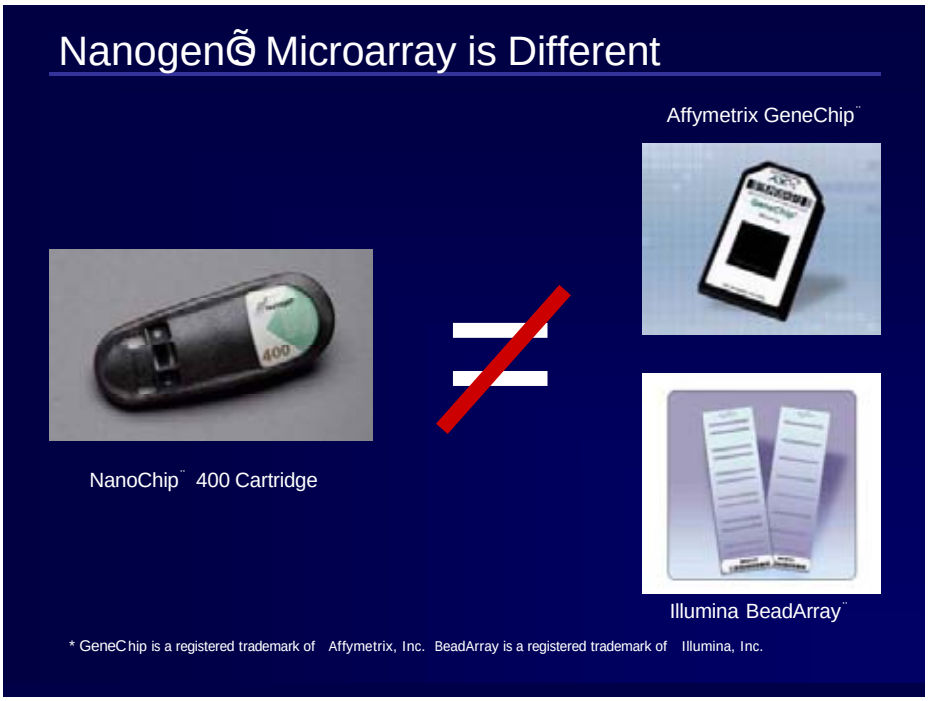


Figure 14a – A comparison of Nanogen's current chip with some other commercially available chips.

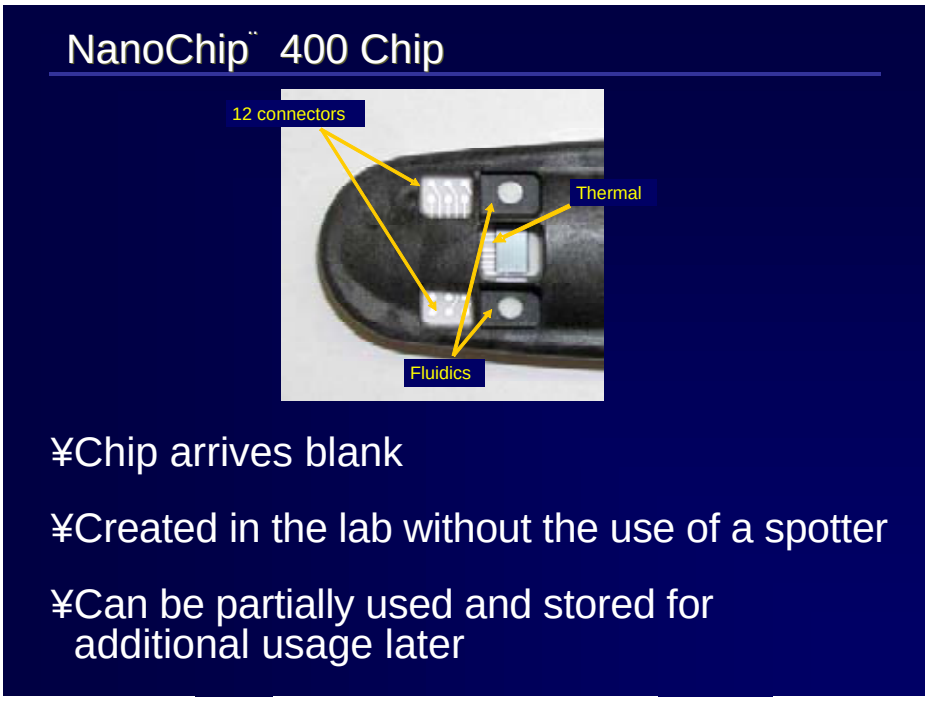


Figure 14b – A view of the "hard" instrument interfaces of the chip. Note that the on-board switching permits control through only 12 connectors. The optical window is on the opposite side.

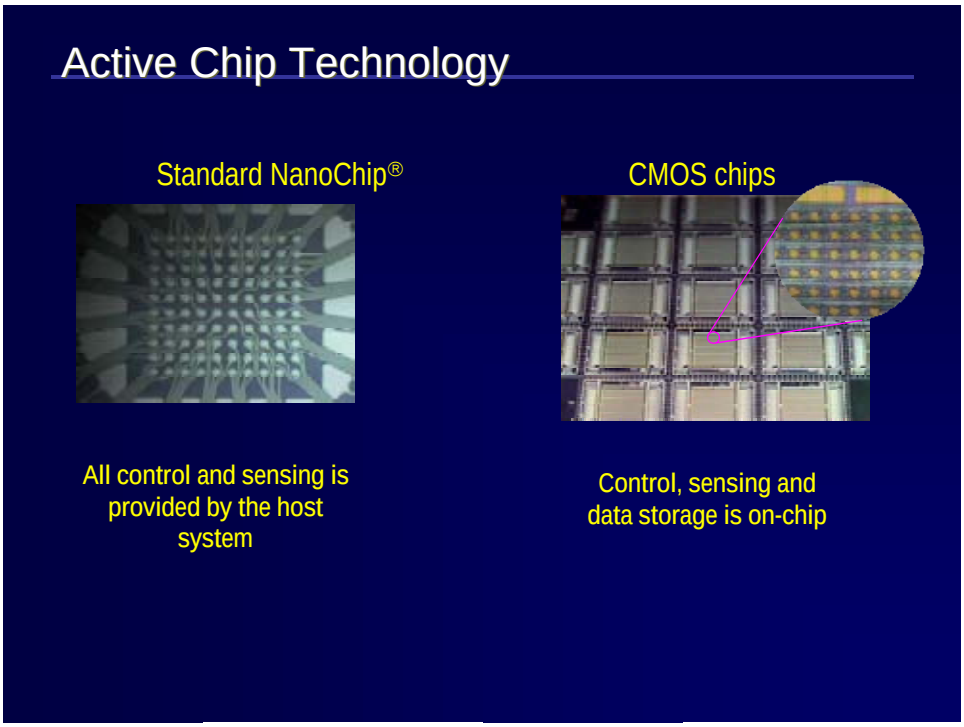
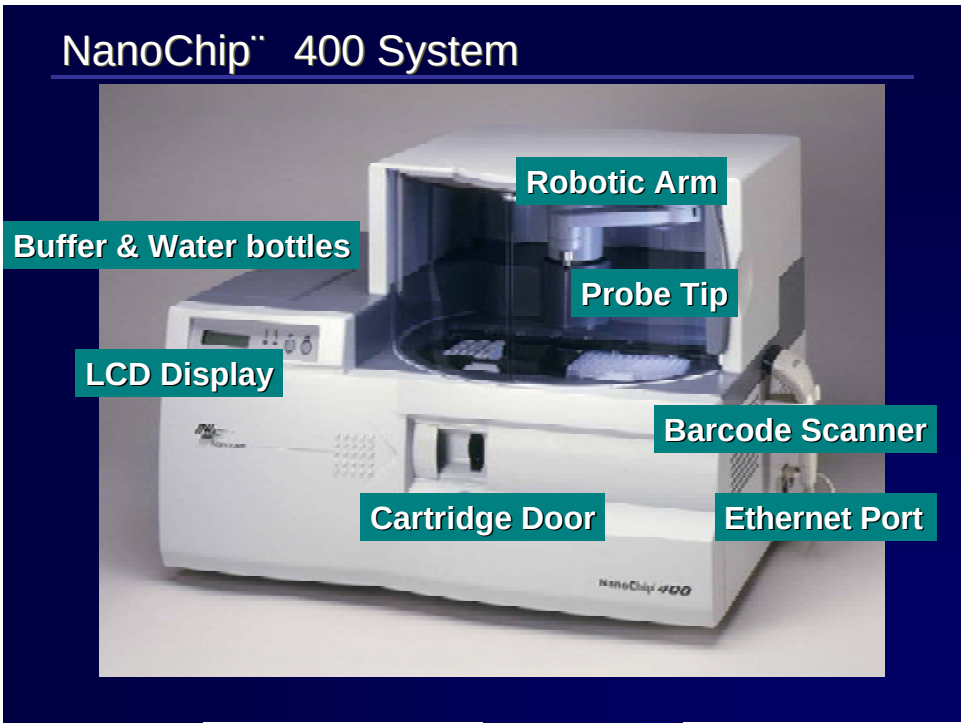


Figure 14c – The old chip format required an individual wire for each pad. The CMOS NanoChip 400 has on-board switching.



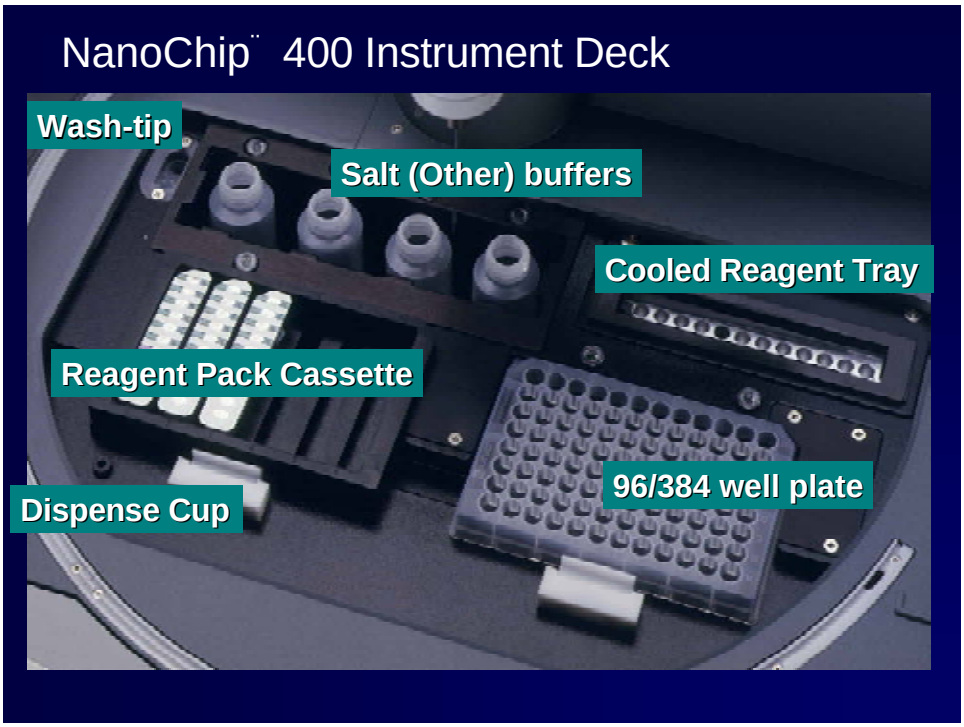
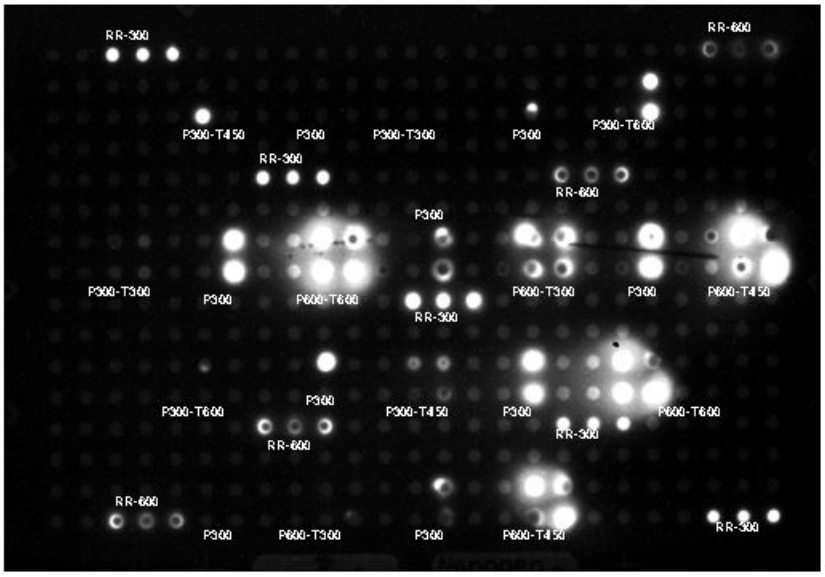


Figure 15 - NanoChip® 400 instrument.



P25 a-SDA using NGI (01-03+0625); Imaged on NIJ#3 for Green; Cart#C08094F0070; 8/18/04

Primers: 300 and 600 nA 60s;
gDNA: 300, 450 and 600 nA 120 s;
RR: 300 and 600 nA 60s

Figure 16a – Image of experimental and control pads for amplification of P25. Experiment was done on an NC400.

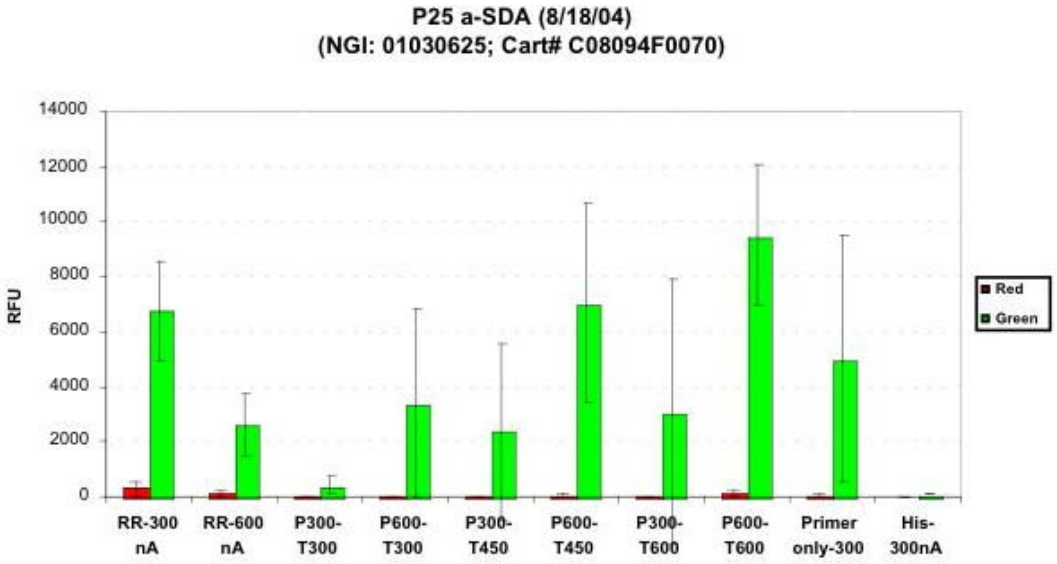


Figure 16b - Results from comparison of different currents used for addressing of primers and target.

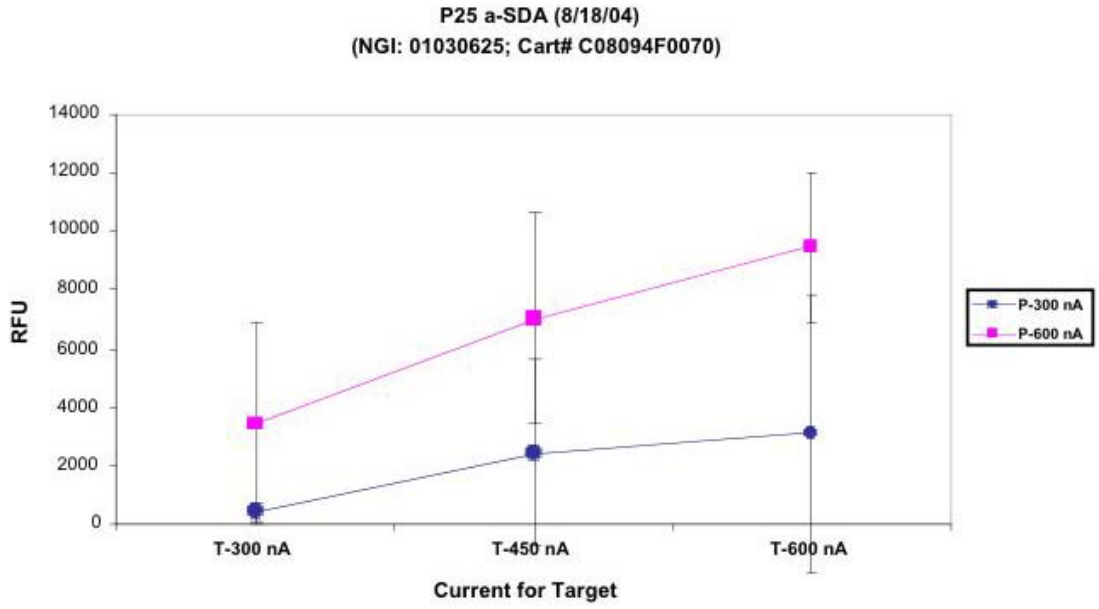


Figure 16c – Graph of data from (b).

Figure 17. Nanogen's portable electronic microarray system with the laptop used to operate the instrument and perform data storage and processing. A port for the 400-site cartridge is labeled separately (not to scale with the instrument).

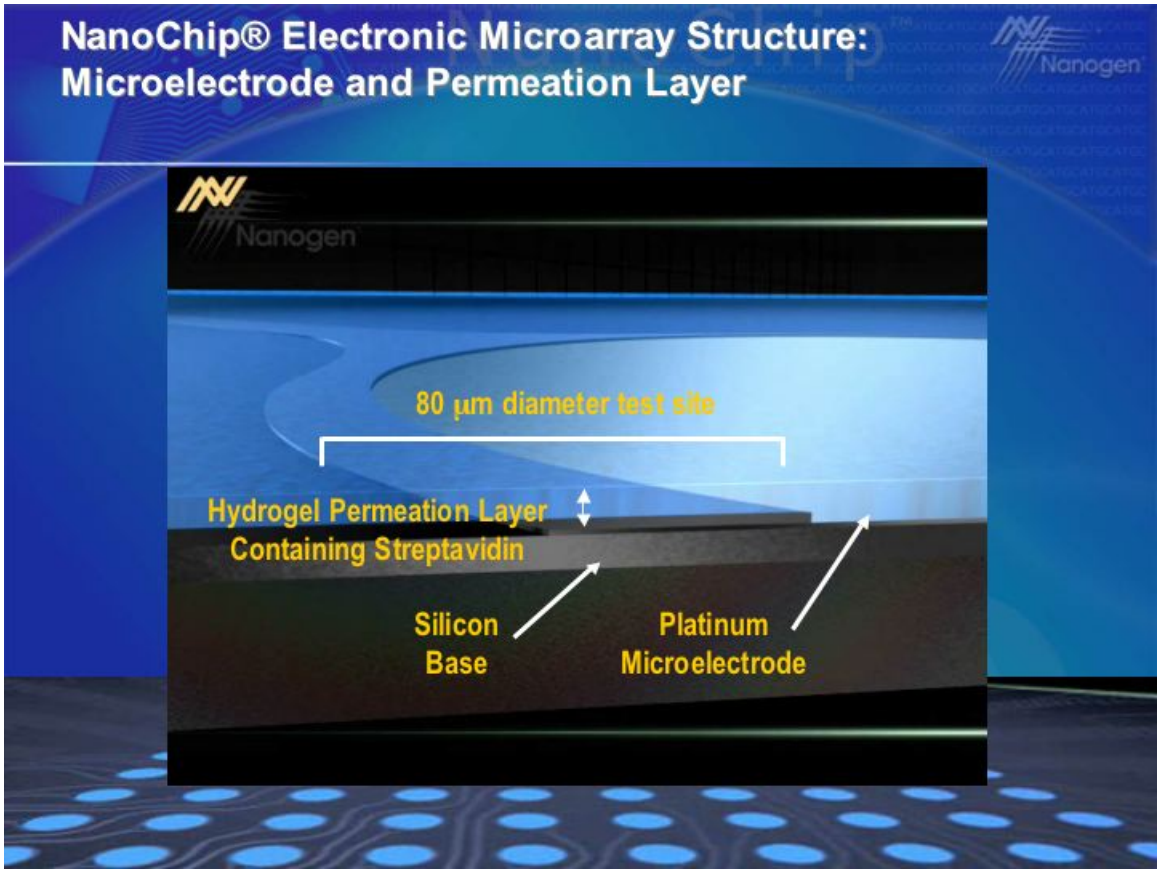


Figure 18 – Cartoon of the cross section of an electronic pad showing the distribution of the permeation layer

13. List of Tables

22-plex aSDA			
SNP	**Sequence Description**	**Oligos**	**Reference**
247	Mitochondrial, HVII, nucleotide 247	Exhibit 1	Budowle, B
P25	Y-chromosome SNP, 3 copies a,b,c	Exhibit 2	Hammer, M
M27	Y SNP	Exhibit 3	Hammer, M
DYS199	Y SNP	Exhibit 4	Hammer, M
DYS188	Y SNP	Exhibit 5	Hammer, M
DYS271	Y SNP	Exhibit 6	Hammer, M
M26	Y SNP	Exhibit 7	Hammer, M
M45	Y SNP	Exhibit 8	Hammer, M
PN1	Y SNP	Exhibit 9	Hammer, M
M145	Y SNP	Exhibit 10	Hammer, M
M178	Y SNP	Exhibit 11	Hammer, M
SRY10831	Y SNP	Exhibit 12	Hammer, M
PN2	Y SNP	Exhibit 13	Hammer, M
TSC0683201	Autosomal SNP	Exhibit 14	Gill, P
TSC0078283	Autosomal SNP	Exhibit 15	Gill, P
TSC0131214	Autosomal SNP	Exhibit 16	Gill, P
TSC0421768	Autosomal SNP	Exhibit 17	Gill, P
TSC1342445	Autosomal SNP	Exhibit 18	Gill, P
TSC0252540	Autosomal SNP	Exhibit 19	Gill, P
TSC0709016	Autosomal SNP	Exhibit 20	Gill, P
TSC0154197	Autosomal SNP	Exhibit 21	Gill, P
TSC0478751	Autosomal SNP	Exhibit 22	Gill, P

Table 1 – A list of the SNP loci used in 22-locus multiplex panel. Sequences of target, amplicon, amplification oligos and analytical oligos are located in the Exhibit indicated.

Exhibit 1

HVII (189-200, 247) We detect sense
Updated 4Oct04 MH

247

Ratio references:

```
     5′-bio-CTTGTAGGACATAATAATAACAATTGAATGTCTGCACAGC-3′                    247 ref-wt

     5′-bio-CTTGTAGGACATAATAATAACAATTAAATGTCTGCACAGC-3′                    247 ref-mut
5′-bio-TAATGCTTGTAGGACATAAT-3′ 247 N1

       3′-GAGTTACAAGCCTGAGTCTATTGTTAACTTACAGA-5′                  247 dsc-wt1 (Tm=38.7)
 5′-bio-****CTTGTAGGACATAATAATAACAATTGAATGTCTGCACAGC***-3′

       3′-ACAGTTCGCTATATGACGATTGTTAATTTACAGAC-5′                  247 dsc-mut5 (Tm=37.0)
 5′-bio-****CTTGTAGGACATAATAATAACAATTAAATGTCTGCACAGC***-3′
```

189

```
       3'-GAGTTACAAGCCTGAGTCTGTCCGCTTGTA-5'                                  189 dsc-wt1 (Tm=38.1)
       3'-GAGTTACAAGCCTGAGTCTGTCCGCTTGTAT-5'                                 189 dsc-wt2 (Tm=39.0)
              5'-***TCAATATTACAGGCGAACATACTTACTAAAGTGTGTTAATTAATT***-3'

       3'-ACAGTTCGCTATATGACGTGTCCGCTCGT-5'                                   189 dsc-mut3 (Tm=40.2)
              5'-***TCAATATTACAGGCGAGCATACTTACTAAAGTGTGTTAATTAATT***-3'
```

195 (overlap with 198)

```
            3'-GAGTTACAAGCCTGAGTCCGCTCGTATGAAT-5'                                195 Dsc-wt1c (Tm=36.6)
           3'-GAGTTACAAGCCTGAGTCCGCTTGTATGAAT-5'                                 195 Dsc-wt1t (Tm=38.1)
               5'-***TCAATATTACAGGCGAACATACTTACTAAAGTGTGTTAATTAATT***-3'

            3'-ACAGTTCGCTATATGACGCGCTCGTATGGAT-5'                                195 Dsc-mut2c (Tm=39.4)
           3'-ACAGTTCGCTATATGACGCCGCTTGTATGGAT-5'                                195 Dsc-mut2t (Tm=40.8)
               5'-***TCAATATTACAGGCGAACATACCTACTAAAGTGTGTTAATTAATT***-3'
```

198 (overlap with 200)

```
                    3'-GAGTTACAAGCCTGAGTCTTATGACTTCACACAATTAA-5'                  198 Dsc-wt3c(Tm=42.0)
                    3'-GAGTTACAAGCCTGAGTCTTATGATTTCACACAATTAA-5'                  198 Dsc-wt3t(Tm=39.2)
               5'-***TCAATATTACAGGCGAACATACTTACTAAAGTGTGTTAATTAATT***-3'

                   3'-ACAGTTCGCTATATGACGTTTATAACTTCACACAATTA-5'                   198 Dsc-mut4c(Tm=38.3)
                   3'-ACAGTTCGCTATATGACGTTTATAATTTCACACAATTA-5'                   198 Dsc-mut4t(Tm=35.3)
               5'-***TCAATATTACAGGCGAACATACTTATTAAAGTGTGTTAATTAATT***-3'
```

200 (overlap with 198)

```
            3'-GAGTTACAAGCCTGAGTCTTTATCATTTCACACAATTAA-5'                         200 Dsc-wt6c(Tm=39.2)
            3'-GAGTTACAAGCCTGAGTCTTTATTATTTCACACAATTAA-5'                         200 Dsc-wt6t(Tm=38.6)
      5'-***TCAATATTACAGGCGAACATACTTACTAAAGTGTGTTAATTAATT***-3'

             3'-ACAGTTCGCTATATGACGTTTATCACTTCACACAATTA-5'                         200 Dsc-mut4c(Tm=40.6)
            3'-ACAGTTCGCTATATGACGTTTATTACTTCACACAATTA-5'                          200 Dsc-mut4t(Tm=38.3)
       5'-***TCAATATTACAGGCGAACATACTTACTGAAGTGTGTTAATTAATT***-3'
```

189-200 References

```
5'-BIO-TCAATATTACAGGCGAACATACTTACTAAAGTGTGTTAATTAATT-3'   REF 189-200 ATCA
5'-BIO-TCAATATTACAGGCGAACATACTTACTGAAGTGTGTTAATTAATT-3'   REF 189-200 ATCG
5'-BIO-TCAATATTACAGGCGAACATACTTATTAAAGTGTGTTAATTAATT-3'   REF 189-200 ATTA
5'-BIO-TCAATATTACAGGCGAACATACTTATTGAAGTGTGTTAATTAATT-3'   REF 189-200 ATTG
5'-BIO-TCAATATTACAGGCGAACATACCTACTAAAGTGTGTTAATTAATT-3'   REF 189-200 ACCA
5'-BIO-TCAATATTACAGGCGAACATACCTACTGAAGTGTGTTAATTAATT-3'   REF 189-200 ACCG
5'-BIO-TCAATATTACAGGCGAACATACCTATTAAAGTGTGTTAATTAATT-3'   REF 189-200 ACTA
5'-BIO-TCAATATTACAGGCGAACATACCTATTGAAGTGTGTTAATTAATT-3'   REF 189-200 ACTG
5'-BIO-TCAATATTACAGGCGAGCATACTTACTAAAGTGTGTTAATTAATT-3'   REF 189-200 GTCA
5'-BIO-TCAATATTACAGGCGAGCATACTTACTGAAGTGTGTTAATTAATT-3'   REF 189-200 GTCG
5'-BIO-TCAATATTACAGGCGAGCATACTTATTAAAGTGTGTTAATTAATT-3'   REF 189-200 GTTA
5'-BIO-TCAATATTACAGGCGAGCATACTTATTGAAGTGTGTTAATTAATT-3'   REF 189-200 GTTG
5'-BIO-TCAATATTACAGGCGAGCATACCTACTAAAGTGTGTTAATTAATT-3'   REF 189-200 GCCA
5'-BIO-TCAATATTACAGGCGAGCATACCTACTGAAGTGTGTTAATTAATT-3'   REF 189-200 GCCG
5'-BIO-TCAATATTACAGGCGAGCATACCTATTAAAGTGTGTTAATTAATT-3'   REF 189-200 GCTA
5'-BIO-TCAATATTACAGGCGAGCATACCTATTGAAGTGTGTTAATTAATT-3'   REF 189-200 GCTG
```

Exhibit 2

P25 (C-A)
Report anti-sense

```
       5'-3'
Sense  TACACAAAACCAGGATTCATGTGCGATAAAAAACATATTTCTCAATGTTTCTAAGAGTATGATTGAGACAGGTAAATTTGCCCAGCATATGAATAATTTGACAGTCT
Anti   ATGTGTTTTGGTCCTAAGTACACGCTATTTTTTGTATAAAGAGTTACAAAGATTCTCATACTAACTCTGTCCATTTAAACGGGTCGTATACTTATTAAACTGTCAGA

                                                                    P25bumpers1  5'-ATGCCTACAAAATGACAC-3'
                                                                    P25primer s2 (sense primer) 5'-bio-TTTATATAT
       CTTCTAGGCCAAAGCCTAGAATGAAATTGTGCCATACGTGGAACAGACACGTAAGCCATGTATAACACCTTCTTTGGCTATGCCTACAAAATGACACTTTTATATAT
       GAAGATCCGGTTTCGGATCTTACTTTAACACGGTATGCACCTTGTCTGTGCATTCGGTACATATTGTGGAAGAAACCGATACGGATGTTTTACTGTGAAAATATATA
       TCCTGGACCATCACCCTCAGCAAGTGAATTATC-3'

                                                  A
       TCCTGGACCATCACCTGGGTAAAGTGAATTATCTGCCTGAAACCTGCCTGCAAATAGAATTGTGTCTCGTATCTCGGTCCATCATGTATGTGATGTGACAACCTTAT
       AGGACCTGGTAGTGGACCCATTTCACTTAATAGACGGACTTTGGACGGACGTTTATCTTAACACAGAGCATAGAGCCAGGTAGTACATACACTACACTGTTGGAATA
                                                  3'-TATCTTAACACAGAGCATAGAGCCAGGT-bio-5' P25 NP1 (nested primer)

                                             5'-CTGCCTGAAAATAGCAGTATATCGCTTGACA-3' P25-mut disc2 (Tm=33.2)
                                           5'-CCTGCCTGCAAATACTGAGTCCGAACATTGAG-3' P25-wt  disc2 (Tm=42.4)

       ACTGCCTTGGACAAGCACTTACAGTACATTGTGACACATAAGTGTGCAATGCACCCAGGTGATGTGATTCTCCTTCCTGGGATCTGCCAACAGGAAGCATTGTAATG
       TGACGGAACCTGTTCGTGAATGTCATGTAACACTGTGTATTCACACGTTACGTGGGTCCACTACACTAAGAGGAAGGACCCTAGACGGTTGTCCTTCGTAACATTAC
                3'-TTCGTGAACGACTCCTAACACTGTGTATTCACACGT-BIO-5' P25primer as6 (anti-sense primer)

       TATCACTTGGCTCAACACCTAAGTTGTGTTTCTTTTTTGCTTTAGCCTTTGTCTTGATTACAGGGAGATTGTGACATATTGTTGGGTCCAGCACCAATGTGTGATCA
       ATAGTGAACCGAGTTGTGGATTCAACACAAAGAAAAAACGAAATCGGAAACAGAACTAATGTCCCTCTAACACTGTATAACAACCCAGGTCGTGGTTACACACTAGT
                                                                                  3'-CTCTAACACTGTATAACAACC-5' P25bumperas1
```

Ratio references

5'-bio-CAT GAT GGA CCG AGA TAC GAG ACA CAA TTC TAT TT**G** CAG GCA GGT TTC AGG-3'	P25 refwt
5'-bio-CAT GAT GGA CCG AGA TAC GAG ACA CAA TTC TAT TTT CAG GCA GGT TTC AGG-3'	P25 refmut

Exhibit 3

M27 C->G
We report anti-sense

```
ACAAGCAAGAGAGGAGAACATAATGGAGCTCTGAAAGATTTATAGATACTCTCATTCCTTTAGCATGTCACATTTCTGGACAAACCATGTACTGGGAGTA
TGTTCGTTCTCTCCTCTTGTATTACCTCGAGACTTTCTAAATATCTATGAGAGTAAGGAAATCGTACAGTGTAAAGACCTGTTTGGTACATGACCCTCAT


GGGGATGACTGAACGGAAGTCAAAGTTATAGTTACTGGAAATACAAACTGTGGCAGTAGAAAACCCTAGGCACAAGGGAAGTAAAATATTAACCACTCCA
CCCCTACTGACTTGCCTTCAGTTTCAATATCAATGACCTTTATGTTTGACACCGTCATCTTTTGGGATCCGTGTTCCCTTCATTTTATAATTGGTGAGGT

GGCTGGAGTGCAGTGGCGCAATCTGGGCTCACAGCAAGCTCTGCCTCCTGGGTTCACACCATTCTCCTGCCTCAGGCTCCCGAGTAGCAGGGAGTACAGG
CCGACCTCACGTCACCGCGTTAGACCCGAGTGTCGTTCGAGACGGAGGACCCAAGTGTGGTAAGAGGACGGAGTCCGAGGGCTCATCGTCCCTCATGTCC

CACCCGCCACCAGGCCTGGCTAGTTTTTTTTGTATTTTTTAGTAGAGATGGGGTTTTACTGTGTTAGCCAGTATGGCCTCGATTTCCTGACCTCGTGATC
GTGGGCGGTGGTCCGGACCGATCAAAAAAAACATAAAAAATCATCTCTACCCCAAAATGACACAATCGGTCATACCGGAGCTAAAGGACTGGAGCACTAG


                                          M27-s2                5'-BIO-TCACAATGGCATCTACCACTCCTCAGCC
        M27 bumper s2     5'-ATTACAGGAGTGAGCCACCA-3'
CGCCCACGTCAGCCTCCTAAAGTGTGGGGATTACAGGAGTGAGCCACCATGCCCAGCTGAAACAATAGTTCTTCACAATGGCATCTACCACTATGTCCAC
GCGGGTGCAGTCGGAGGATTTCACACCCCTAATGTCCTCACTCGGTGGTACGGGTCGACTTTGTTATCAAGAAGTGTTACCGTAGATGGTGATACAGGTG
ATTTGCA-3'    M27-s2
                                                                    Wt RR3'-TGGTGATACAGGTG-

 ATTT       CTGTCCTGAACCTCGATTCCTATAGGTTGATGTGTTGAGAACCAGACAATACGAAATAGAAGACAAATCATGAGCTTACAGAACCTGAAA
 TAAACGTGGAGACAGGACTTGGAGCTAAGGATATCCAACTACACAACTCTTGGTCTGTTATGCTTTATCTTCTGTTTAGTACTCGAATGTCTTGGACTTT
                    3'-AGCTAAGGATATCCAACTACACAACTC-bio-5'   M27-NP2
                                              3'-TCTTGGTCTGTTCGACTCCATCTTCTGTTTAGTACTCGAA-bio-5'   M27-as2
-TAAACGTGGAGACAGGACTTGGAGCTAAGGATATC Wt RR
   TTGCACCTCTGTCCCTGAGTCCGAACATTGAG-3'    DSC4-WT      45.0C
   5'-CACCTGTGTCCTGAGCAGTATATCGCTTGACA-3'     DSC3-MUT     44.0C

CTTTTTACACTGGGCAGTGTGGTAGACAGAACAGCAGTGGCTGCCCAAAGATGATCATGTTTTAAGTCCTGACATCTGTGAATTATCATATTGGGAAAAG
GAAAAATGTGACCCGTCACACCATCTGTCTTGTCGTCACCGACGGGTTTCTACTAGTACAAAATTCAGGACTGTAGACACTTAATAGTATAACCCTTTTC
                                                  3'-GTACAAAATTCAGGACTGTAGACA-5'   M27 bumper as2
```

Exhibit 4

DYS199 (AC007034)
We report sense

DYS199S1JFa/D199sda2 anchored AMPLICONS

Exhibit 5

Dys 188

We report anti-sense

```
GAGAAGGCATGGTGTGCAAGGACCTGATTTGCTGTTGGAGGGTGACAATGGAGACAGCAGATAATGTCTGTTTTCAGGACAGCTGAAGCAGGTCAGCCCCATCCTGA
CTCTTCCGTACCACACGTTCCTGGACTAAACGACAACCTCCCACTGTTACCTCTGTCGTCTATTACAGACAAAAGTCCTGTCGACTTCGTCCAGTCGGGGTAGGACT

                                                    188 s-YH Bbv      5'-BIO-TTCATCTCTATATATGAGGGCAGAGCCT
                                                                 DYS188S1  5'-TTCATCTCTATATATGAGGGCAGAGCCT
5'-AGGAATGCCAGCTGTAGGCCCA-3' 188 bumper s1-YH
AGGAATGCCAGCTGTAGGCCCAAAGATCCAAAGGTGTCTCCCAAGGCCACCTGTCACTAGACTGTGATCAGCATTTGCCTTCATCTCTATATATGAGGGCAGAGATT
TCCTTACGGTCGACATCCGGGTTTCTAGGTTTCCACAGAGGGTTCCGGTGGACAGTGATCTGACACTAGTCGTAAACGGAAGTAGAGATATATACTCCCGTCTCTAA

CAGCATGATAATCCAGT-3' 188 s-YH Bbv
CAGCATGATAATCCAG-3'           T
TAGAATGATAATCCAGTTGGCTGAAATTTCCATTTTCCATATGCAGATAGATGGAAATACATGCATATCTATTCACTTTCCACATGCAGATAGATGGAAATACATGC
ATCTTACTATTAGGTCAACCGACTTTAAAGGTAAAAGGTATACGTCTATCTACCTTTATGTACGTATAGATAAGTGAAAGGTGTACGTCTATCTACCTTTATGTACG
          Wt RR ACCGACTTTAAAGGTAAAAGGTATACGTCTATCTACCTT-5'
                        3'-TAAAAGGTATACGTCTATCTACC-BIO-5' 188 N1
                            3'-AGGTATACGTCTATCTACC-BIO-5' 188 NP-YH
                                                  3'-TATGTACGTATAGCGACTCCAAAGGTGTACGTCTATCTACCTT-BIO-5'
                                                                                  188 as1-YH

ATATCTATTATTAGATAGAAGGGAGAATTTGTTAATGAGTACAGACAAGAGAAAGTGGNGTAAATTTGTCTGTGGGAAATATGTTCATGGAGCGAATATCCAACAAT
TATAGATAATAATCTATCTTCCCTCTTAAACAATTACTCATGTCTGTTCTCTTTCACCNCATTTAAACAGACACCCTTTATACAAGTACCTCGCTTATAGGTTGTTA
            3'-ATCTTCCCTCTTAAACAATTACTC-5' 188 bumper as1-YH
```

We report antisense

```
antisense amplicon:
                  34.7 5'-AAATTTCTATTTTCCAGCAGTATATCGCTTGACA-3'188 MUT DISC11
               38.4 C     5'-AATTTCCATTTTCCATTATTTCTGAGTCCGAACATTGAC-3' 188 WT DISC7
3'-AGCCCTACTATTAGGTCAACCGACTTTAAAGGTAAAAGGTATACGTCTATCTACCTTTATGTAGGGCTCGATAAGTGAAAGGTGTACGTCTATCTA-5'
```

Exhibit 6

DYS271(AC011302)

We report anti-sense

```
1-sense  5′-GAACTCCAATATTAGAGAAAGTTATTAGTAAAACAGTTGGAGGCACTGGTCAGAATGAAGTGAATGGCACACAGGACAAGTCCAGACCCAGGAAGGTCCA
antisense 3′-CTTGAGGTTATAATCTCTTTCAATAATCATTTTGTCAACCTCCGTGACCAGTCTTACTTCACTTACCGTGTGTCCTGTTCAGGTCTGGGTCCTTCCAGGT

           DYS271b1
        5′-GTAACATGGGAGAAGAACGG-3′  5′-bio-AATTCAGGGCTCCCTTGGGCTCCCCTCAGCAAAAATGTAGG-3′ DYS271s1JFa
                                    5′-bio-AATTCAGGGCTCCCTTGGGCTCCCCTCAGCAAAAATGTAGGTT-3′ 271 s-YH Bbv
101        GTAACATGGGAGAAGAACGGAAGGAGTTCTAAAATTCAGGGCTCCCTTGGGCTCCCCTGTTTAAAAATGTAGGTTTTATTATTATATTTCATTGTTAACA
           CATTGTACCCTCTTCTTGCCTTCCTCAAGATTTTAAGTCCCGAGGGAACCCGAGGGGACAAATTTTTACATCCAAAATAATAATATAAAGTAACAATTGT
                                                                                                  wt RR ATTGT
                 G
201        AAAGTCCATGAGATCTGTGGAGGATAAAGGGGGAGCTGTATTTTCCATTAAAAAAAAAAAAAAAAAAGAAAACAGTTTCCAGATTAGGTGGACCTAGCCTT
           TTTCAGGTACTCTAGACACCTCCTATTTCCCCCTCGACATAAAAGGTAATTTTTTTTTTTTTTTCTTTTGTCAAAGGTCTAATCCACCTGGATCGGAA
           TTTCAGGTACTCTAGACACGGGCTCTTTCCCCCT-5′ wt RR
                           3′-TCCTATTTCCCCCTCGACATA-BIO-5′ 271 NP2-YH
                           271 AS2-YH        3′-ATAAAAGGTAATTTCGACTCCTTTTTTTCTTTTGTCAAAGGTCTA-BIO-5′
                           271 as-YH Bbv     3′-ATAAAAGGTAATTTTTCGACTCCTTTTTCTTTTGTCAAAGGTCTA-BIO-5′
                                                                                  3′-TCCACCTGGATCGGAA

301        CAGTGCAAGTAAAAAGTGCTCTCTGAAGAACAAAGGAAGTAGAGACAGT-3′
           GTCACGTTCATTTTTCACGAGAGACTTCTTGTTTCCTTCATCTCTGTCA-5′
           GTCA-5′ 271 bumper as1-YH
```

Antisense AMPLICONS:

```
                                      5'-CAAAAGTCCGTGAGCAGTATATCGCTTGACA-3' 271 mut-dsc 4 (Tm=37.5)
                                    5'-ACAAAAGTCCATGACTGAGTCCGAACATTGAG-3' 271 WT disc 1 (Tm=37.4)
3′-AGCCCTTTTTACATCCAAAATAATAATATAAAGTAACAATTGTTTTCAGGTACTCTAGACACGGGCTCTTTCCCCCTCGACATAAAAGGTAA-5′
                                                                                          |
                                                                                        biotin
```

Exhibit 7

Exhibit 8

M45 G-A

```
                                                                                5'-BIO-CAGTA
                    M45 bumps(-88/20) 5'-GCTGGCAAGACACTTCTGAG-3'
CTGGTTTAGGAAATCACTCCTGTATGCTAGCAGGAATGTTGCTGGCAAGACACTTCTGAGCATCGGGGTGTGGACTTTACGAACCAACCTTTTAACAGTA
GACCAAATCCTTTAGTGAGGACATACGATCGTCCTTACAACGACCGTTCTGTGAAGACTCGTAGCCCCACACCTGAAATGCTTGGTTGGAAAATTGTCAT


ACTCTAGGAGAGAGCCTCAGCAAAATTGGCA-3' M45(-18/35)S4
ACTCTAGGAGAGAGGATATCAAAAATTGGCAGTGAAAAATTATAGATAGGCAAAAAGCTCCTTCTGAGGTCCAGGCCAGGAGATAGTAGGATTTAAGAAA
TGAGATCCTCTCTCCTATAGTTTTTAACCGTCACTTTTTAATATCTATCCGTTTTTCGAGGAAGACTCCAGGTCCGGTCCTCTATCATCCTAAATTCTTT
                                                    (20/32)AS5   3'-CAGGTCCCGACTCCTATCATCCTAAATTCTTT-Bio-5'
                                                3'-CGTTTTTCGAGGAAGACTCCAGGTCG-BIO5' M45N3


CAAACAAACAAAAACAACCACAAATGACCTTTGGTGCCACTGTCACAACTGTTGCTCATCAGAGTAGGAGAGTTGTAGCAAAGGCATTAAAGAAGGACAA
GTTTGTTTGTTTTTGTTGGTGTTTACTGGAAACCACGGTGACAGTGTTGACAACGAGTAGTCTCATCCTCTCAACATCGTTTCCGTAATTTCTTCCTGTT
                  3'-TACTGGAAACCACGGTGAC-5'M45 bumpas(75/19)


GCAGCTGAAGAGCCTGAATCCTTGTGTTGTAAGCTATTTTGGTTTCCTTTCAAGAAAGGGCTGTGGTCTGTGGAAGGTGTCAGGAACATATTTTCACGGT
CGTCGACTTCTCGGACTTAGGAACACAACATTCGATAAAACCAAAGGAAAGTTCTTTCCCGACACCAGACACCTTCCACAGTCCTTGTATAAAAGTGCCA

We report antisense


              5'-ATAGATAGGCAAAAAGAAACTGAGTCCGAACATTGAG-3' M45 dsc2 wt (37.6C)
3'-AGCCCCACTTTTTAATATCTATCCGTTTTTCGAGGGGCTCTCCAGGTCCGGTCCTCTATC-bio5'(2/36)as1    WT

             5'-TATAGATAAGCAAAAAGAAAGCAGTATATCGCTTGACA-3' M45 dsc1 mut (36 C)
3'-AGCCCCACTTTTTAATATCTATTCGTTTTTCGAGGGGCTCTCCAGGTCCGGTCCTCTATC-bio5'(2/36)as1    MUT
```

References

```
3'-TCACTTTTTAATATCTATCCGTTTTTCGAGGAAGACTCCA-bio5'  WT M45 ref C

3'-TCACTTTTTAATATCTATTCGTTTTTCGAGGAAGACTCCA-bio5'  MuT M45 ref T
```

Exhibit 9

PN1 (AC010137)
We report anti-sense

We report anti-sense

Exhibit 10

M145 (G-A)

Report anti-sense

Anchored Amplicon (104 bp)

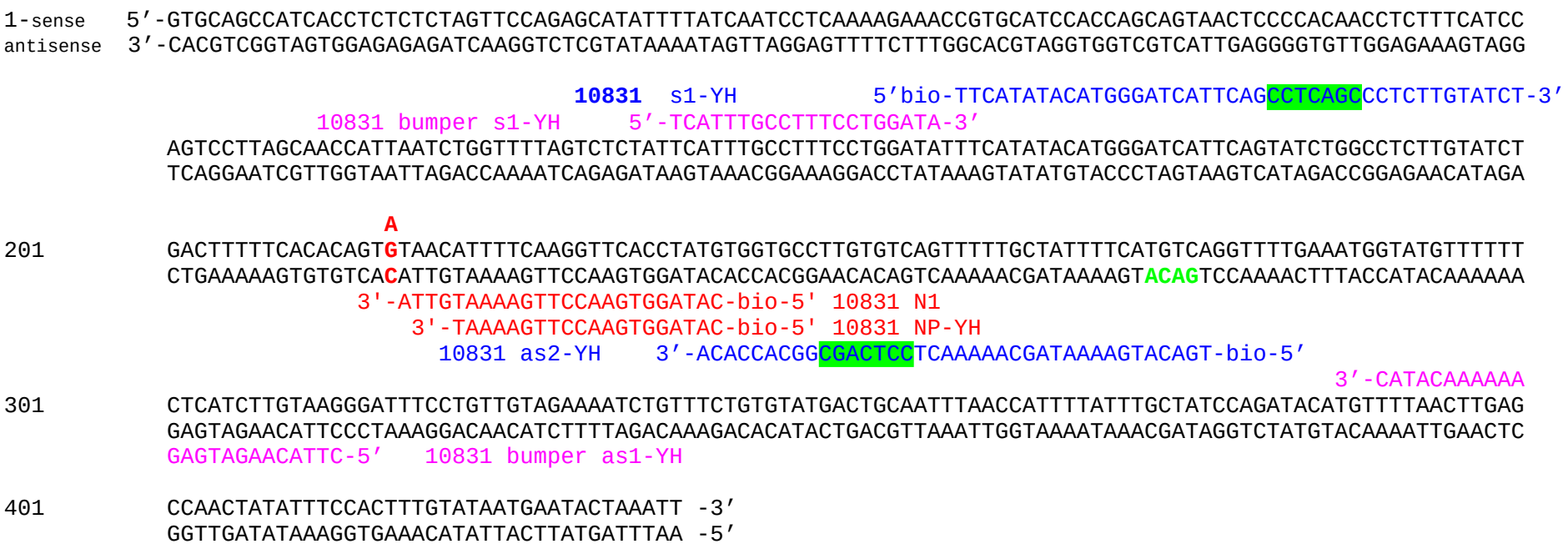
Exhibit 11

M178 C->T

```
        AGGGGAAACCAGGTAGCTGGATCCCCTATCCTCATTCTGCAGCTGGATTTGTCCAGGAAAAAGAGAAGTATTAAGCCTAAAGAGCAGTCAGAGTAGAATG
        TCCCCTTTGGTCCATCGACCTAGGGGATAGGAGTAAGACGTCGACCTAAACAGGTCCTTTTTCTCTTCATAATTCGGATTTCTCGTCAGTCTCATCTTAC

        CTGAATTTTCAGAAGTTTTATATTAACATAATCATTCATCTTTTTTGTCCTGATAATTACTCAGGAGGAAACTGAGAGGGCATGGTCCCTTTCTATGGAT
        GACTTAAAAGTCTTCAAAATATAATTGTATTAGTAAGTAGAAAAAACAGGACTATTAATGAGTCCTCCTTTGACTCTCCCGTACCAGGGAAAGATACCTA

        AGCAATACTCAGTGTCCCAAT-3' M178 BUMPS (M178 PCR)
                                          5'-BIO-CAGGCAGAGACTCCGAAACCTCAGCTGGATTAGTTG-3' M178 s2 Bbv
                                                                                          T (mutant)
        AGCAATACTCAGTGTCCCAATTTTCCTTTGGGACACTGGGACACAGGCAGAGACTCCGAAAGTCTGCATGGATTAGTTGTTCATTCACCACAGCTCCTTA
        TCGTTATGAGTCACAGGGTTAAAAGGAAACCCTGTGACCCTGTGTCCGTCTCTGAGGCTTTCAGACGTACCTAATCAACAAGTAAGTGGTGTCGAGGAAT
                                                                                          3'-CGAGGAAT

        GTGTGCCAGGAGAACTATATATGGCCTTTGGTTTCATTCAGGGACAGGGAAACTTGAACCCATGCCTATTCATTCTCATTAAAGTAGCAGAAGTCATGTT
        CACACGGTCCTCTTGATATATACCGGAAACCAAAGTAAGTCCCTGTCCCTTTGAACTTGGGTACGGATAAGTAAGAGTAATTTCATCGTCTTCAGTACAA
    3'-ACACGGTCCTCTTGATATATAC-BIO-5' M178 NP-YH
                                    3'-AAGTAAGTCCCTCGACTCCTGAACTTGGGTACGGATAAG-bio-5' M178 as1-YH Bbv
                                                                                    3'-GTCTTCAGTACAA

        AGAGACAGTATTGCTGCATTCAGTACTCCTGCCTTTAACGCTTCTGACGCTTCCTGAAAGCAGCCCCAGCTCTCCATATGGCAAAACAAAGGCAACCTTA
        TCTCTGTCATAACGACGTAAGTCATGAGGACGGAAATTGCGAAGACTGCGAAGGACTTTCGTCGGGGTCGAGAGGTATACCGTTTTGTTTCCGTTGGAAT
        TCTCTGTCATAACGAC-5' M178 BUMPAS (M178 PCR bio)

        TGCAAAGCCTTCTCAGGGAACCCTCAGAAAGGTTTAAACTTAGGTTCACAGTTTTTAGAGAATAATGTCCTCATTGCTCCCTCTGGCACTAGCAGTTTGT
        ACGTTTCGGAAGAGTCCCTTGGGAGTCTTTCCAAATTTGAATCCAAGTGTCAAAAATCTCTTATTACAGGAGTAACGAGGGAGACCGTGATCGTCAAACA

        ACCAGGAGATCTGTTGGCTACTGTTACCCTAGGGTATGGCAATGGTATGTAGGCAATGAAAAATCTTACAGTACTTATTATGGAAAACCAACTTTTTTAT
        TGGTCCTCTAGACAACCGATGACAATGGGATCCCATACCGTTACCATACATCCGTTACTTTTTAGAATGTCATGAATAATACCTTTTGGTTGAAAAAATA


        5'-bio-TTC TCC TGG CAC ACT AAG GAG CTG TGG TGA ATG AAC AAC TAA TCC AT-3'     M178 ref-wt2
        5'-bio-TTC TCC TGG CAC ACT AAG GAG CTA TGG TGA ATG AAC AAC TAA TCC AT-3'     M178 ref-mut2
        5'-bio-TTC TCC TGG CAC ACT AAG GAG CTG TGG TGA ATG AAC-3'                    M178 ref-wt
        5'-bio-TTC TCC TGG CAC ACT AAG GAG CTA TGG TGA ATG AAC-3'                    M178 ref-mut

We report antisense:
                                    5'-CACCATAGCTCCTATAGCAGTATATCGCTTGACA-3' 38.6C 178 Mut 2
                                  5'-TCACCACAGCTCCTCTGAGTCCGAACATTGAG-3'   46.3C M178 Dsc-WT4
3'CTCTGAGGCTTTCAGACGTACCTAATCAACAAGTAAGTGGTGTCGAGGAATCACACGGTCCTCTTGATATATACCGGAAACCAAAGTAAGTCCCTGT-5' WT
```

Exhibit 12

SRY1532 (aka 10831; L08063)
We report anti-sense



Y10831s1JFb/Y10831s2JF anchored AMPLICONS:

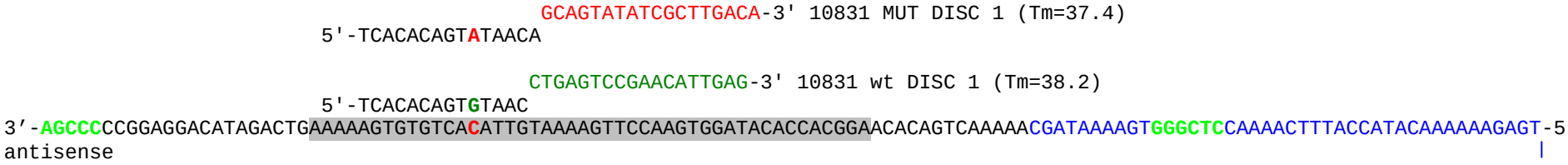


Exhibit 13

PN2 (AC010137) C→ T

We report anti-sense

```
1-sense    5′-GTACATCCATGAACCCTGAGGGCTGCATCATCCATAGCTTGAGGTCCCCGGTGAGTCCCTTGATGCAAATGAGAAAGAACTTCTCTCATGAGGGTTTTGG
antisense  3′-CATGTAGGTACTTGGGACTCCCGACGTAGTAGGTATCGAACTCCAGGGGCCACTCAGGGAACTACGTTTACTCTTTCTTGAAGAGAGTACTCCCAAAACC

           PN2bumps
         5′-ATAACATTTCTCTTTGGTCTG-3′                    PN2 sbub Bbv  5′-bio-TTAGGTTTTGGATCTTCATGCTGCCTCAGCGAGCATTA
                                                PN2 s1-YH Bbv  5′-bio-TTCTCTTAGGTTTTGGATCTTCACCTCAGCTAAAGGAGCATT-3′
                                                   PN2 s2-YH Bbv           5′-bio-TTGGATCTTCATGCTGGTTCCTCAGCCATTA
101        ATAACATTTCTCTTTGGTCTGGTAACACCCATAAAGGTTGTCTAATAGTGGCTTGAGTTCTCTTAGGTTTTGGATCTTCATGCTGGTTAAAGGAGCATTA
           TATTGTAAAGAGAAACCAGACCATTGTGGGTATTTCCAACAGATTATCACCGAACTCAAGAGAATCCAAAACCTAGAAGTACGACCAATTTCCTCGTAAT

           ATAAA-3′PN2bubs2T
           ATAAAACTAA-3′ PN2 s2-YH Bbv
201        ATAAAACTAATGCCTTCTCCTCCTAGGGGCACCTCTCTTAAGGAAAAGATGGCTGGAGCTGATTCTCCTGAGGTAGAGGGGAATCGGGAGTTCTGAATAC
           TATTTTGATTACGGAAGAGGAGGATCCCCGTGGAGAGAATTCCTTTTCTACCGACCTCGACTAAGAGGACTCCATCTCCCCTTAGCCCTCAAGACTTATG
                                          3′-ATTCCTTTTCTACGACTCCCGACTAAGAGGACTCCATCT-bio-5′ PN2 as1-YH Bbv
                                                      PN2 as2-YH Bbv      3′-ATCTCCCCTTACGACTCCAGACTTATG
                              PN2 as3-YH Bbv                  3′-CTCGACTAAGACGACTCCATCTCCCCTTAGCCCTCAAG-bio-5′
                   3′-AGGATCCCCGTGGAGAGAAT-bio-5′ PN2 N1

301        CGTTTTTTACATTGCTCTATCTCACGTTGAAGTCCTTTCAGAGAAGCGTACTTAGGCTGGTAGTGAAGAGGCTCTTGGGATGATTACCAAGAGGCAGGGG
           GCAAAAAATGTAACGAGATAGAGTGCAACTTCAGGAAAGTCTCTTCGCATGAATCCGACCATCACTTCTCCGAGAACCCTACTAATGGTTCTCCGTCCCC
           GCAAAAAATGTA-BIO-5′ PN2 as2-YH Bbv
                      3′-AGATCGAGTGCAACTTCAGGA-5′ PN2BUMPAS

401        tacaaggaggtgggacaatgtgagtaggagaagcat-3′
           atgttcctccaccctgttacactcatcctcttcgta-5′
```

Exhibit 14

TSC 0683201
We report anti-sense

Exhibit 15

TSC 0078283 Assembled ds DNA (updated 9Aug04)
Report antisense
Sense primers are above ds DNA; antisense primers are below.

```
5′-TCCATTTGTCTCATGTGAACTAGCGATACAGTGAAGAGATCAGATACATCCTTAACATTCCCAAAGCTCAATGAAATAGTAGGTATATGGTAAATTAGAA
3′-AGGTAAACAGAGTACACTTGATCGCTATGTCACTTCTCTAGTCTATGTAGGAATTGTAAGGGTTTCGAGTTACTTTATCATCCATATACCATTTAATCTT

CTAATAACTTATAGTCAAGTTCTGAATTGGATGGACTGAGGTCAAACTCATACAAGGTATTGGAAGAAGGAAGACTTAAACTGGTTGGTAACCACCAGTG
GATTATTGAATATCAGTTCAAGACTTAACCTACCTGACTCCAGTTTGAGTATGTTCCATAACCTTCTTCCTTCTGAATTTGACCAACCATTGGTGGTCAC

                                    5′-TTCATGGATGTGATAAGAACC                    5′-GACTCTCTGGATGGGC
                                      sense bumper 2 (49.4)                      sense bumper 1 (50.3)
                                                                                                    CC-
GCCATTCTGCGAGTAAGTAGCCTTGCGTGGCAAAAGAGCTTCATGGATGTGATAAGAACCTTGAAATGGGGAGATTGGCTTGGACTCTCTGGATGGGCCC
CGGTAAGACGCTCATTCATCGGAACGCACCGTTTTCTCGAAGTACCTACACTATTCTTGGAACTTTACCCCTCTAACCGAACCTGAGAGACCTACCCGGG

   TGTAATCACAAGCATCCTcctcAGcGGACTCGAGAG   sense primer 1 (65.8, 50.2, 50.4)
   TGTAATCACAAGCATCCTcAgcAGTGGACTCGAGAG   sense primer 2 (64.8, 43.7, 53.8)
   TGTAATCACAAGCATCCTcAgcAGTGGACTCGAGAGAC sense primer 3 (65.5, 43.7, 56.3)
-AATGTAATCACAAGCATCCTcAgcAGTGGACTCGAGAGAC sense primer 4 (66.3, 50.0, 56.3)
                                    C (derived/mutant)
 AATGTAATCACAAGCATCCTTAAAAGTGGACTCGAGAGACAGAAGCATCAGTGTCAGAGTGGCATGACATGAGAAAGACTCAACCACCCTTTGCTGGCTT
 TTACATTAGTGTTCGTAGGAATTTTCACCTGAGCTCTCTGTCTTCGTAGTCACAGTCTCACCGTACTGTACTCTTTCTGAGTTGGTGGGAAACGACCGAA
                                    CGTAGTCACAGTCTCACCGT-5′ NP (57.3; only 1 designed)
                              Antisense primer 1 (65, 46, 48)        TACTCTTTCTGAGcgacTccGAAACGACCGAA-5′
                              Antisense primer 3 (66, 46, 52.6)   CTGTACTCTTTCTGAGcgacTccGAAACGACCGAA-5′
                                                  Antisense primer 2 (65.1, 44.9, 49.7)   GACCGAA-
                                                                      antisense primer x CCGAA-

TGAAGATGGAGGAGGAGTCCACAAGCCAAATAATGTGGGCAGCTTCTAGAAGCTGAATAAAACAAGAAAATGGATTCTGCCTTAGAGTCTCTGGAAAGGA
ACTTCTACCTCCTCCTCAGGTGTTCGGTTTATTACACCCGTCGAAGATCTTCGACTTATTTTGTTCTTTTACCTAAGACGGAATCTCAGAGACCTTTCCT
ACTTCcgaCTCCTCCTCAGGTGTTC (2)
ACTTCTACCgaCTCCTCAGGTGTTCGG-5′(65.6, 45.3, 50.2) STRONG 2nd (not ordered)
 CTTCTACCTCCTCCTCAG-5′              CACCCGTCGAAGATCTT-5′
 antisense bumper 1 (50.1)         antisense bumper 2 (50.0)
```

Exhibit 16

Ss100540 (TSC 0131214) W(297, A/T)

We report anti-sense

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CTGTTAAAACGTTCTACTCTGGCAAGATGTGGTGGGCTTGGAGCTTAGAAAGGAAACTGGGGTTATGCTAGTGCTCTGCAAGGGCCCTGCCCAGATAAAA      100
GACAATTTTGCAAGATGAGACCGTTCTACACCACCCGAACCTCGAATCTTTCCTTTGACCCCAATACGATCACGAGACGTTCCCGGGACGGGTCTATTTT

                                                                                214 bumper s1     5’-TTTGGAGGGAA
TTGTTGGTCTTGTCTGTAATTAGTATTCCAAAGGTTCAGGTGGTGTTGCATCTCCATATCAATGTTCAGCTAGTAGGTAAAGGGACATATTTGGAGGGAA      200
AACAACCAGAACAGACATTAATCATAAGGTTTCCAAGTCCACCACAACGTAGAGGTATAGTTACAAGTCGATCATCCATTTCCCTGTATAAACCTCCCTT

CTAACTCTG-3’
          5’-bio-ACTATTATGCTCTTCACTTTCCTCAGCTTATTCTCTCAAG-3’ 214 s1Bbv
                                                                                T
CTAACTCTGGCCTAGCTACTATTATGCTCTTCACTTTTTCTTTTTTATTCTCTCAAGCCCCAGCATCATGCAGTCCTCAGTTGGGTGCTTACGTGCACTT      300
GATTGAGACCGGATCGATGATAATACGAGAAGTGAAAAAGAAAAAATAAGAGAGTTCGGGGTCGTAGTACGTCAGGAGTCAACCCACGAATGCACGTGAA

CTCTTCCTTGCAGCAGTCCTCGAGGCAGGGCAGATTCCTCTTTAAGGTGCTCGCATGTTACCCCTGGGTTCTGCTGACCCAACATTAGCTACAAAGTGGGA      400
GAGAAGGAACGTCGTCAGGAGCTCCGTCCCGTCTAAGGAGAAATTCCACGAGCGTACAATGGGGACCCAAGACGACTGGGTTGTAATCGATGTTTCACCCT
3’-AAGGAACGTCGTCAGGAGCTCCGTCC-bio-5’ 214 NP1
                                    214 as3Bbv            3’-GACCCAAGACCGACTCCTTGTAATCGATGTTTCACCCT-bio-5’

CAGTCCCACAGTGCTCCCTCTTCCTTGTTGTGGGGAAGACCGCAAAAAGACCTCTTTTGCTTTAGTGACTTTGTTCCTACTCAATACAGGAAGTGTTGACC      500
GTCAGGGTGTCACGAGGGAGAAGGAACAACACCCCTTCTGGCGTTTTTCTGGAGAAAACGAAATCACTGAAACAAGGATGAGTTATGTCCTTCACAACTGG
       3’-CACGAGGGAGAAGGAACAAC-5’ 214 bumper as1

AGCAAAAATAATAATTACAAAGGCTAAGTTCTGATTTATTGTTCCTGACTCCCGTGTGATTAACTGGAAACCCTGTTGTTCTCCTTTTTAATCCTAAAGT      600
TCGTTTTTATTATTAATGTTTCCGATTCAAGACTAAATAACAAGGACTGAGGGCACACTAATTGACCTTTGGGACAACAAGAGGAAAAATTAGGATTTCA

TAGAGTTTGCCAAACCTACTCCTCCTTTAATACCACCCCTGCTAAAAGGGAGATCTCCAATCCCTTTCTTAAGAGGCCCCTTTCCTGTTGTAAGCTAACC      700
ATCTCAAACGGTTTGGATGAGGAGGAAATTATGGTGGGGACGATTTTCCCTCTAGAGGTTAGGGAAAGAATTCTCCGGGGAAAGGACAACATTCGATTGG

AGAGCAGACACTGGGCCTCCTTTCTCAGAAGGGCACTGCGCATCTG
TCTCGTCTGTGACCCGGAGGAAAGAGTCTTCCCGTGACGCGTAGAC
```

Exhibit 17

TSC 0421768
Report antisense

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5′-GTGGTGCAATCTCGGCTCACCGCAACCTCCACCTCCCAGGTTCAAGAGATTCTCCTGCCTCCGCCTCCCGAGTAGCTGGGACTACAGGCGCTTGCCACCA
3′-CACCACGTTAGAGCCGAGTGGCGTTGGAGGTGGAGGGTCCAAGTTCTCTAAGAGGACGGAGGCGGAGGGCTCATCGACCCTGATGTCCGCGAACGGTGGT

TGCCCGGCTAATTTTTTGGGGTTTTGTTCAGCAGAGACAGGGTTTCACTCTGTTAGCCAGGATGGTCTCGATCTCCTGACCTCATGATCCACCTGCCTCG
ACGGGCCGATTAAAAAACCCCAAAACAAGTCGTCTCTGTCCCAAAGTGAGACAATCGGTCCTACCAGAGCTAGAGGACTGGAGTACTAGGTGGACGGAGC

                                                                    sense primer 1 TGCTACAGCGA-
                                                                    sense primer 2   CTACAGCGA-
                                                                    sense primer 3 TGCTACAGCGA-
                                  bumper sense 1 (49.6, none) TCAACATCAACGTCAACAG (51.4C for PCR)
                                  bumper sense 2 (55.5, none) TCAACATCAACGTCAACAGCAC (56.9C for PCR)
GCCTCCCAAAGTACTGGGATTACAGGCGTGAGCCACTGCACCTGGCGAAAATTGATTAATTTTCAACATCAACGTCAACAGCACAACTCTGCTACAGCGA
CGGAGGGTTTCATGACCCTAATGTCCGCACTCGGTGACGTGGACCGCTTTTAACTAATTAAAAGTTGTAGTTGCAGTTGTCGTGTTGAGACGATGTCGCT

AGAGCctCAGCAAATGAAGCATCAATAATT sense primer 1 (65.1, 52.1, 50.4, moderate, primer dimer)
AGAGCctCAGCAAATGAAGCATCAATAATT sense primer 2 (63.2, 45.9, 50.4, very weak, primer dimer)
AGAGCctCAGCAAATGAAGCATCAATA    sense primer 3 (65.2, 52.1, 48.5, moderate)

                                    G (mutant)
AGAGCTACAGCAAATGAAGCATCAATAATTTCAATGCGTTCACAATGCAAGAGGCATCACTAAGCCAAATGAGTGAGAAAACACCCTAATGCATTAGAGT
TCTCGATGTCGTTTACTTCGTAGTTATTAAAGTTACGCAAGTGTTACGTTCTCCGTAGTGATTCGGTTTACTCACTCTTTTGTGGGATTACGTAATCTCA
                  CGTAGTTATTAAAGTTACGCAAGTGTTACGTTCTCCGTAGTGATTCGGT-5′ wt RR1 (40.82%, moderate)
                             Antisense primer 1  (65.5, 52.7, 50.4)   CTCACTCTTTTGTGcGAcTcCGTAATCTCA-
                             Antisense primer 3  (65.0, 52.7, 47.3)     CACTCTTTTGTGcGAcTcCGTAATCTCA-
                             Antisense primer 4  (64.2, 51.2, 47.7)                 GATTACGTAATCTCA-
                     (59.8C for PCR)    3′-TTACGTTCTCCGTAGTGATTCGGT nested primer 1 (59.2, moderate)

                  CGTAGTTATTAAAGTTACGCAAGTGTTACGTTCTCCGTAcgacTTCGGT-5′ wt RR2 (42.86%, moderate)
  Antisense primer 2 (66.0, 50.0, 52.8)    TTACGTTCTCCGTAcgacTcCGGTTTACTCACTCTTTTG (moderate)
    AS2, NP2, RR2 are a set             GTGTTACGTTCTCCGTAcgacTTCGGT nested primer 2 (63.9, 58.4, moderate)

 TCTGAGGAGGAAGAGGTCTCTGGGATAGGATGGCCTAGGAAGGCTTATGGACTCATTGTCCTCAAATAAATCTCCAAAGATGAATAAAAATTCAGATAGC
 AGACTCCTCCTTCTCCAGAGACCCTATCCTACCGGATCCTTCCGAATACCTGAGTAACAGGAGTTTATTTAGAGGTTTCTACTTATTTTTAAGTCTATCG
-AGACTCCTCC Antisense primer 1 and 3 (weak)
-cGACTCCTCCTTCTCCAGAGACC Antisense primer 4 (moderate)
                           CCTACCGGATCCTTC  antisense bumper 1 (46.4, none)
                      CTATCCTACCGGATCCTTCC antisense bumper 2 (53.1, moderate; add 5′ G=strong)
```

Exhibit 18

TSC 1342445

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5′-AGTCCTCATGTTCTTGCTCATGCAAGTCCCTCAACACCCTGCCAACCTCTGCCCCCACCACTGTCCCTTTTTAACCTCCAATATATATATACTCAATATT
3′-TCAGGAGTACAAGAACGAGTACGTTCAGGGAGTTGTGGGACGGTTGGAGACGGGGGTGGTGACAGGGAAAAATTGGAGGTTATATATATATGAGTTATAA

TTTATTACAGAGAAAGCAAATATTTGATGTTCTTTTAAGTTATGCATTTAAGGAACATACTAAATCTCTCTTATGGTCACTCTCTCTCTCTCTCTCTCTC
AAATAATGTCTCTTTCGTTTATAAACTACAAGAAAATTCAATACGTAAATTCCTTGTATGATTTAGAGAGAATACCAGTGAGAGAGAGAGAGAGAGAG

                                                                                    445srs3 Bio5′-TCAGAA
                                                                                      445srs2 Bio5′-AGAA
                                                                                     445srs1 Bio5′-CAGAA
                                                                              445srs4 Bio5′-GTAATAGCCATTCAGAA
                                  (55.6C for PCR) Bumper 445 s – RS   5′-TAACCAGGGCTTGCGTAATAG-3′
TCACTATCACACATTCTCTCTCTCTTTCTCTCTCTCTCTGTCTCTCTCTGCTCTGTCCACTTTAGGCCTTAACCAGGGCTTGCGTAATAGCCATTCAGAA
AGTGATAGTGTGTAAGAGAGAGAGAAAGAGAGAGAGAGACAGAGAGAGACGAGACAGGTGAAATCCGGAATTGGTCCCGAACGCATTATCGGTAAGTCTT

CTAACTAGTCTGGGAAACTCCTCAGCCCCTCGGTGCCA-3′ 445srs3
CTAACTAGTCTGGGAAACTCCTCAGCCCCTCGGTGCCAGC-3′
CTAACTAGTCTGGGAAACTCCTCAGCCCCTCGGTGCCAG-3′
CTAACTACCTCAGCAAACTCTCTTTTCCC-3′
                                             A (mutant, sense/plus strand)
CTAACTAGTCTGGGAAACTCTCTTTTCCCTCGGTGCCAGCCTCCTGCATGGGCCTGTCTCCCTGTCCCCCATGCTGTGGTGCCTCCAGCACGTTTGCCTT
GATTGATCAGACCCTTTGAGAGAAAAGGGAGCCACGGTCGGAGGACGTACCCGGACAGAGGGACAGGGGGTACGACACCACGGAGGTCGTGCAAACGGAA
                    GGAGTCGGGGAGCCACGGTCGGAGGACGTACCCGGACAGAGGGACAGGGGGT-5′ refAwt
                                             T (mutant, antisense strand)
                                                  3′-GGACAGAGGGACAGGGGGTAC-5′ Bio 445 NAP 1 (62.8C for PCR;
                                                                                        104 nts plus biotin)
                                                   3′-GACAGAGGGACAGGGGGT-5′ Bio 445 NAP 2

CCTGGCAGCATTTTCTATTCAGCATTCTCCCCACCCTGTCAAAACTTACTGCTGCTGTGCTTTAATCCCTCAAGGTGTTGTTCTTTGTTTATCTTATCCA
GGACCGTCGTAAAAGATAAGTCGTAAGAGGGGTGGGACAGTTTTGAATGACGACGACACGAAATTAGGGAGTTCCACAACAAGAAACAAATAGAATAGGT
    3′-CGTAAAAGATAAGCGACTCCAGGGGTGGGACAGTTTTGAATGAC-5′ Bio 445 asrs1  3′-CCACAACAAGAAACAAATAGAAT-5′ Bumper
     3′-GTAAAAGATAAGCGACTCCAGGGGTGGGACAGTTTTGAATGAC-5′ Bio 445 asrs2                                  asrs1
      3′-TAAAAGATAAGCGACTCCAGGGGTGGGACAGTTTTGAATGAC-5′ Bio 445 asrs3
                                                                  3′-GAGTTCCACAACAAGAAACAAATAG-5′Bump asrs2
        3′-AAAGATAAGTCGTAACGACTCCGGGACAGTTTTGAATGACGACGAC-5′ Bio asrs4
         3′-AAGATAAGTCGTAACGACTCCGGGACAGTTTTGAATGACGACGAC-5′ Bio asrs5
          3′-AGATAAGTCGTAACGACTCCGGGACAGTTTTGAATGACGAGCAC-5′ Bio asrs6

TAAATTCATGTGATAGGATGTAGAATCCATGACTGCTTCATATAGATAAACTATTTGTATTTTGGCAAGAGTTTATTAAATTTTACTCTCAAACATGGAT
ATTTAAGTACACTATCCTACATCTTAGGTACTGACGAAGTATATCTATTTGATAAACATAAAACCGTTCTCAAATAATTTAAAATGAGAGTTTGTACCTA
```

Exhibit 19

TSC 0252540

Report antisense strand (i.e. discriminators are complementary to antisense)

5′-TTCGAGTCGAACACGACGAGTCTAAAAGGCTGTGGGGCATCCAGATGGAGCTATCTGGGCTGGAGAGACAAACATGGAAGCCAGCAGCAAGGAGTTGGAT
AAGCTCAGCTTGTGCTGCTCAGATTTTCCGACACCCCGTAGGTCTACCTCGATAGACCCGACCTCTCTGTTTGTACCTTCGGTCGTCGTTCCTCAACCTA
5′-GCTATTTAAAACCACAGAACAACAAGGAAGGCTTTACAAATGCTGTGGCCACATGGCAGAAGAGGTCAATCCCCGGGAAGCATGGCAAGGCTGTGGCCGG
CGATAAATTTTGGTGTCTTGTTGTTCCTTCCGAAATGTTTACGACACCGGTGTACCGTCTTCTCCAGTTAGGGGCCCTTCGTACCGTTCCGACACCGGCC

59.8C for PCR Bumper sense 1 (57.5, weak) GATGCT-
53.4C for PCR Bumper sense 2 (50.1, weak) CT-
AGGGCTGGGGCAGGGCCAAGCCGCGGTGGCGCCTCCCCTCCTGCTTCCCTAAAAGAGGGCAAGATGGGGCAATGTGCAGCCTGGCCTCCTCTGAGATGCT
TCCCGACCCCGTCCCGGTTCGGCGCCACCGCGGAGGGGAGGACGAAGGGATTTTCTCCCGTTCTACCCCGTTACACGTCGGACCGGAGGAGACTCTACGA

CCATTCTCTAGGTCCATCCCctCAgCGGAAACTGCTGGGTC (69.5, 54.5, 57.9, strong)
ATTCTCTAGGTCCATCCCctCAgCGGAAACTGCTGG (67.3, 50.5, 52.7, strong)
Italics are part of 2nd structure CTCTAGGTCCAT*CCCAGC*cTcaGcAACT*GCTGGG*TCTG (69.3, 54.4, 58.2, strong)
AGCGGCCCATTCTCTAGGTCCAcCtCAGCATGGGAAACT (strong)
CCCATTCTCTAGGTCCAcCtCAGCATGGGAAACT (strong)
GCCGTCCGATCAGCGGCCCATTCTCTAGGTCCATCCCAGCAT (very strong)
TCCGATCAGCGGCCCATTCcCTcaGcCCATCCCAGCA (73.4, 62.3, 53.4, moderate) sense primer 1
GATCAGCGGCCCATTCcCTcaGcCCATCCCAGC (70.8, 56.4, 51.3, moderate) sense primer 2
ATCAGCGGCCCATTCcCTcaGcCCATCCCAG (69.7, 55.5, 46.6, moderate) sense primer 3
CAGCGGCCCATTCcCTcaGcCCATCCCAGC (70.9, 53.6, 51.3, moderate) sense primer 4
CAGCGGCCCATTCcCTcaGcCCATCCCA (69.6, 53.6, 44.2, moderate) **sense primer 5**
CAGCGGCCCATTCTCTAGGTCCATCCCA (67.4C for PCR, none) sense PCR primer 1
CAGCGGCCCATTCTCTAGGTCCATC (63.7C for PCR, none) sense PCR primer 2
(54.2C for PCR w/o Y) GAAACTGCTGGGTCTGY frwrd “assay” primers on website
-GTCCCAGGCCTG bumper sense 1 and 2 **T** (mutant)
GTCCCAGGCCTGCCGTCCGATCAGCGGCCCATTCTCTAGGTCCATCCCAGCATGGGAAACTGCTGGGTCTG**C**ACCCCCACCTTCTCCCAGCCCTGGCTCC
CAGGGTCCGGACGGCAGGCTAGTCGCCGGGTAAGAGATCCAGGTAGGGTCGTACCCTTTGACGACCCAGACGTGGGGGTGGAAGAGGGTCGGGACCGAGG
68.2C for PCR Nested primer 1 (66.9, none) TGGGGGTGGAAGAGGGTCGGGA-5′
62.8C for PCR **Nested primer 2** (**60.3**, none) GGGGGTGGAAGAGGGTCG-5′
AGGTAGGGTCGTACCCTTTGACGACCCAGAC**G**TGGGGGTGGAAGAGGGTCG RR wt (strong)
97 bp from bumper sense 1 to NP2 AGGTAGGGTCGTACCCTTTGACGACCCAGAC**A**TGGGGGTGGAAGAGGGTCG RR mut
CTCCTGTGGctgAGGTCATTTGTAGTCTAAAAGGC (plus strand extending to 5′ end of antisense primer 1)
CTCCTGTGGGGCAGGTCATTTGTAGTCTAAAAGGCTGGGGTGAACTGTCCACAGCCAGGGTCAAGAGCTGCTCTGTAGAAAGCTAGCATGTGTGGCCTTG
GAGGACACCCCGTCCAGTAAACATCAGATTTTCCGACCCCACTTGACAGGTGTCGGTCCCAGTTCTCGACGAGACATCTTTCGATCGTACACACCGGAAC
GAGGACACCCCGTCCAGTAA-5′ antisense PCR primer 1 (60.7C for PCR, strong; reverse “assay” primer on website)
CCCCGTCCAGTAAACATCAGATTTTCCG-5′ antisense PCR primer 2 (61.8C for PCR, none)
GAGGACACCgacTCCAGTAAACATCAGATTTTCCG (63.1, 49.8, 42.1, weak) **antisense primer 1** (strong w/1 or 2 3′ Gs)

Exhibit 20

TSC 0709016

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5′-CCAATATAACTTTAATAATAGGCGGCAGGAGGATTTGGTCCTTTGGCTGGGGTTTGCCATCCTTTGGTATAGATTGTATAGAAAGACTGAAAACTCTATT
3′-GGTTATATTGAAATTATTATCCGCCGTCCTCCTAAACCAGGAAACCGACCCCAAACGGTAGGAAACCATATCTAACATATCTTTCTGACTTTTGAGATAA


TAATCAGTTAATTAACTCATATGAATTTGTTAAAAGATGAGAAAGCATCACAAAATGTGTGCCTCAATAACATAATGACTAGGACTCTTCTCTGGAGTTT
ATTAGTCAATTAATTGAGTATACTTAAACAATTTTCTACTCTTTCGTAGTGTTTTACACACGGAGTTATTGTATTACTGATCCTGAGAAGAGACCTCAAA


                                                                    016 bumps 5′- TATGACCCAGCTCACCTT-
AAAAGGAATTCTCTGGCAGTGTCTTCAGGCCCTATGCCCTGGTGGTCACCCTGAGAAGACGTCTTCAGGGCAGGGCTGTGTCTATGACCCAGCTCACCTT
TTTTCCTTAAGAGACCGTCACAGAAGTCCGGGATACGGGACCACCAGTGGGACTCTTCTGCAGAAGTCCCGTCCCGACACAGATACTGGGTCGAGTGGAA

GCTCC – 3′ (016 bumps)
5′-                          CCTCAGCGGATCCACACAGGGAATGACAGGGAACCA
5′-BioCTCCAACCAAGCTCCATTCCTAGTGTCCTCAGCATCCACACAGG-3′ 016s13
    5′-BioAACCAAGCTCCATTCCTAGTGTCCTCAGCATCCACACAGGGAA-3′ 016s12
5′-BioCTCCAACCAAGCTCCATTCCTAGTGTCCTCAGCATCCACACAGGGAATG-3′ 016s11   T(mutant)

GCTCCACTCCAACCAAGCTCCATTCCTAGTGTGCTGAGGATCCACACAGGGAATGACAGGGAACCACTAATATCACAAAAGGCCTAGGCCTCTGGGCTGC
CGAGGTGAGGTTGGTTCGAGGTAAGGATCACACGACTCCTAGGTGTGTCCCTTACTGTCCCTTGGTGATTATAGTGTTTTCCGGATCCGGAGACCCGACG
                              ??BbvC1B site                                  016nap1-3′-GGAGACCCGACG
                                                                              016nap2-3′-GAGACCCGACG

CTGCATGGGTGACTTTCCAAGATCAGTCGAGTGAACAAGGAGTTGGTCTTAAAAGATGTACAGCAAACCCTTGAAGCTTTGCTGAAAAAACAACTCACAA
GACGTACCCACTGAAAGGTTCTAGTCAGCTCACTTGTTCCTCAACCAGAATTTTCTACATGTCGTTTGGGAACTTCGAAACGACTTTTTTGTTGAGTGTT
GACGTAC-5′Bio                                  016 bumpas2 3′-CGTTTGGGAACTTCGAAACGACTT 3′-GTTGAGTGTT
GACGTA-5′Bio

             AAGGTTCTAGTCAGCGACTCCGTTCCTCAACCAGAATTTTCTACAT-Bio-5′ 016as4
              AGGTTCTAGTCAGCGACTCCGTTCCTCAACCAGAATTTTCTACATG-Bio-5′ 016as5
               GGTTCTAGTCAGCGACTCCGTTCCTCAACCAGAATTTTCTACATG-Bio-5′ 016as6


AAAGCAGATTAACAGGAGAAAAGATGTGATATGGATTCAGTTAATGAGAACTCAGGGAAGTGACCAGCAGTTAATTGTTTTCTTCTTTGGTAGGTCTGGA
TTTCGTCTAATTGTCCTCTTTTCTACACTATACCTAAGTCAATTACTCTTGAGTCCCTTCACTGGTCGTCAATTAACAAAAGAAGAAACCATCCAGACCT
TTTCGTCTAATTG -5′016 bumpas1             3′-CTCTTGAGTCCCTTCACTGGTCG-5′ 016 bumpas3

CTTTAGGCAGACAAGGGAACTTTAGAGAATCTAATGCTTTGGGAGAGACAGTATGGGGGAGATCAGGGAGACCTTGAGGCTCCCTTTTCAGTTCAGCATG
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Exhibit 21

TSC 0154197 Y(C/T) Ss1314263
We report anti-sense

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AGACCAGTCAGGAAGAGGCAGACCCTAGCTCCCGGCCTCTCTGGAAAGCCTGGGGGTCCACAGGAAACCTGCCAAACTGCCTCCTTGCCCTGTGGGGGCA       100
TCTGGTCAGTCCTTCTCCGTCTGGGATCGAGGGCCGGAGAGACCTTTCGGACCCCCAGGTGTCCTTTGGACGGTTTGACGGAGGAACGGGACACCCCCGT


GCAACTGTCCCTGTCCTGTCGGCGCTGCTGCATCCTGACCACCCCTCGGCACAGTAAACACACGGCTTGGCGGGGCTTTCCCACATGGGCAGGAAGGAGC       200
CGTTGACAGGGACAGGACAGCCGCGACGACGTAGGACTGGTGGGGAGCCGTGTCATTTGTGTGCCGAACCGCCCCGAAAGGGTGTACCCGTCCTTCCTCG

TAAGACCCTCAGCTTACTTGGTGCTGCTACAGTCAACCTCGGCCACCTTCACTGTGGGCAGCGTCTGCGAAGCCACCGGCTCAATGGTGAGCGTGTTGTC       300
ATTCTGGGAGTCGAATGAACCACGACGATGTCAGTTGGAGCCGGTGGAAGTGACACCCGTCGCAGACGCTTCGGTGGCCGAGTTACCACTCGCACAACAG

                                        5′-AGGCAGGCAGGGTGGTAAGG-3′ 197 bumper s1
                                                                  5′-bio-GGGTGTGTCCCCCAGC
                                                                       5′-bio-TGTCCCCCAGC
CTCCACGGCGGCCCGTACCAGCACCGAGAGCTGTGGCAGACCAGGGAGGCAGGCAGGGTGGTAAGGGGCTGGGCCCTGGCGGAGGGGTGTGTCCCCCAGC       400
GAGGTGCCGCCGGGCATGGTCGTGGCTCTCGACACCGTCTGGTCCCTCCGTCCGTCCCACCATTCCCCGACCCGGGACCGCCTCCCCACACAGGGGGTCG


TGGCCTCAGCCCTGCCATGC-3′ 197 Bbv s1
TGGCCCCGCCTCAGCCATGCCTCACC-3′ 197 Bbv s2
    5′-CTGAGTCCGAACATTGAGCTCCTGCATCG-3′ 197 Dsc-wt5 (36.5)
   5′-CTGAGTCCGAACATTGAGCCTCCTGCATCG-3′ 197 Dsc-wt4 (41.8)
5′-CTGAGTCCGAACATTGAGTCACCTCCTGCATCG-3′ 197 Dsc-wt3 (49.3)
 5′-CTGAGTCCGAACATTGAGCACCTCCTGCATCG-3′ 197 Dsc-wt2 (47.3)
  5′-CTGAGTCCGAACATTGAGACCTCCTGCATCG-3′ 197 Dsc-wt1 (44.8)
    5′-GCAGTATATCGCTTGACACTCCTGCATTG-3′ 197 Dsc-mut4 (32.5)
 5′-GCAGTATATCGCTTGACACACCTCCTGCATTG-3′ 197 Dsc-mut3 (44.3)
  5′-GCAGTATATCGCTTGACAACCTCCTGCATTG-3′ 197 Dsc-mut2 (41.4)
  5′-GCAGTATATCGCTTGACATCCTCCTGCATTG-3′ 197 Dsc-mut1 (38.1)
                                  5′-TGAAGTCCAGGCAGGGGCCCACGTCCTCC-3′ 197 stab
                                   T
TGGCCCCGCCCCTG                  CGTGAAGTCCAGGCAGGGGCCCACGTCCTCCCGGTACACCCTTTCCAGGAAGGGGCAGGTCTTGTC       500
ACCGGGGCGGGGACGGTACGGAGTGGAGGACGTAGCACTTCAGGTCCGTCCCCGGGTGCAGGAGGGCCATGTGGGAAAGGTCCTTCCCCGTCCAGAACAG
                                  3′-TTCAGGTCCGTCCCCGGGTGCA-bio-5′ 197 NP1
                                       3′-TCCGTCCCCGGGTGCAGGAG-bio-5′ 197 NP2
                                                                3′-GGAAAGGTCCTTCGACTCCCAGAACAG

CAGGGTGGGGGATTCCCTCCAGGCCTGGAACTCTGCAAACAGGATTGTGTCCACCTGTGGGGGAGGGCAAGCGAGGGTGGGGGCGGTCAGGGTACTGGGGC      601
GTCCCACCCCCTAAGGGAGGTCCGGACCTTGAGACGTTTGTCCTAACACAGGTGGACACCCCCTCCCGTTCGCTCCCACCCCCGCCAGTCCCATGACCCCG
GTCCCACCCCCTAA-bio-5′ 197 Bbv as1
                 3′-TCCGGACCTTGACGACTCCGTCCTAACACAGGTGGACAC-bio-5′ 197 Bbv as2
                       3′-CTTGAGACGTTTCGACTCCCACAGGTGGACACCCCCT-bio-5′ 197 Bbv as3
                                                          197 bumper as1   3′-CCGCCAGTCCCATGACCCCG-5′
CAGCATAGAAGATGCAGGCTGAGCTGAAGGGGAGCGAGGGAGGACGTGGGGCAGGCCCAGCAAGCCAGCAGGTCGATCCCCAGCAGCCGTGCAGGGAAGCC      701
GTCGTATCTTCTACGTCCGACTCGACTTCCCCTCGCTCCCTCCTGCACCCCGTCCGGGTCGTTCGGTCGTCCAGCTAGGGGTCGTCGGCACGTCCCTTCGG
```

Exhibit 22

TSC 0478751
Report sense (i.e. discriminators' sequences are antisense strand)
5'-CAAAAGGAGGAACAGGCTCATGGTTCCTGATTCTAGTCTTTCCCTCACTGTCCTACAAAGAGGTACCTTCCATGTGACCCCAAGGCAAAGTTCAGTAGCA
3'-GTTTTCCTCCTTGTCCGAGTACCAAGGACTAAGATCAGAAAGGGAGTGACAGGATGTTTCTCCATGGAAGGTACACTGGGGTTCCGTTTCAAGTCATCGT

GTTAGTGAGCTTCCTTTGTTCTGCTCTTTCTTCCATTCCATTTCTTCTTTTTCTACAAATGCTGCCAAAACCAGACTATTAGAGCATCCTCATTAGCACC
CAATCACTCGAAGGAAACAAGACGAGAAAGAAGGTAAGGTAAAGAAGAAAAAGATGTTTACGACGGTTTTGGTCTGATAATCTCGTAGGAGTAATCGTGG

Sense primer 2 (67.2, 52.0, 49.9) GCCAA-
Sense bumper 1 GCTGCATGGGTTTCCAG (52.9, weak, no)
GAGAGAATGGAATGTTCTCGCCAGGGATGCGCTCACTGATGCTGCATGGGTTTCCAGTCACTGCAACTCTGGTGAGGTGCTGGGTTCTGTGCAGAGCCAA
CTCTCTTACCTTACAAGAGCGGTCCCTACGCGAGTGACTACGACGTACCCAAAGGTCAGTGACGTTGAGACCACTCCACGACCCAAGACACGTCTCGGTT

Sense primer 1 GTGGTCATCCGGGCCtCAGcAGCAGCAATTCTT (68.1, 52.8, 49.8, none)
Nonamplifying primer 1 (not nested) G*g*CtCAGcAGCAGCAATTCTT (58.5*, 52.0, none; 59.5 for PCR)
(use with sense 1; *once antisense amplicon binds to NP1, it will extend 3 more nts and have this Tm with NP1, penultimate 5' nt of NP1 disrupts Bbv)
-GGAAAATGGTGTCCTcagcACTCTGAGCCA (67.2, 52.0, 49.9, moderate) **sense primer 2**
Nonamplifying primer 2 (nested) GCCCCAGAAGCAGCAATTCTT (58.3, weak; 59.4 for PCR)
Wt RR1 (55.6% GC, moderate) 5'-G*g*CtCAGcAGCAGCAATTCTT**T**TTCCCATACTCTCgct*A*AGGCCC
(use with sense 1 + antisense 1)
Wt RR2 (53.3% GC, moderate) 5'-GCCCCAGAAGCAGCAATTCTT**T**TTCCCATACTCTCgct*A*AGGCCC
(use with antisense 1)
Wt RR3 (51.1% GC, moderate) 5'-GCCCCAGAAGCAGCAATTCTT**T**TTCCCATACTCTCAGAAAGGCCC
(lowercase in primers do not match genomic; underlined nts in RRs or NP match to BbvC1 B site in primers except italicized nts are designed so as not to have complete BbvC1 B site)
A (mutant)
GGAAAATGGTGTCCTTCCAACTCTGAGCCACATGTGGTCATCCGGGCCCCAGAAGCAGCAATTCTT**T**TTCCCATACTCTCAGAAAGGCCCTTTCAGGAGT
CCTTTTACCACAGGAAGGTTGAGACTCGGTGTACACCAGTAGGCCCGGGGTCTTCGTCGTTAAGAAAAAGGGTATGAGAGTCTTTCCGGGAAAGTCCTCA
Antisense primer 1 (67.3, 52.5, 50.0, moderate) AAGGGTATGAGAGcgacTCCGGGAAAGTCCTCA-
Antisense primer 2 (68.6, 52.7, 55.0, moderate) GAGTCTTTCCGGGcgAcTCCTCA-
Antisense primer 3 (70.4, 52.7, 63.2, moderate) AAGGGTATGAGAGTCTTTCCGGGcgAcTCCTCA-
Antisense primer 4 (68.2, 52.7, 52.2, moderate) GTCTTTCCGGGcgAcTCCTCA-

CCCATCTGCTTCTGAATCCAAAACCTTTTTTCAAGGATAATTAAAATTAGTCCAAAGATGCTTGGGGATGGTTGATGCCCTTGCATCTTCCTCTATTTTC
GGGTAGACGAAGACTTAGGTTTTGGAAAAAAGTTCCTATTAATTTTAATCAGGTTTCTACGAACCCCTACCAACTACGGGAACGTAGAAGGAGATAAAAG
-GG **Antisense primer 1**
-GGGTAGACGAAGAC Antisense **primers 2, 3, 4**
antisense bumper 1 GTTCCTATTAATTTTAATCAGGTTTCTAC (51.5, weak, no; 52.1 for PCR)

Exhibit 23

Table 1. SDA primers

SNP	*Forward AP*	*Reverse AP*	*NAP*
247	5'-Bio-CAA TAT TAC AGG CGA ACA TAC CCT CAG CAG TGT GTT AAT TAA T-3'	5'-Bio-TTG TTA TGA TGT CTG TGT GGA CCT CAG CTG TGC AGA CA-3'	5'-Bio-TAATGCTTGTAGGACATAAT-3'
P25	5'-Bio-TTT ATA TAT TCC TGG ACC ATC ACC CTC AGC AAG TGA ATT ATC-3'	5'-Bio-TGC ACA CTT ATG TGT ACA AAT CCT CAG CAA GTG CTT-3'	5'-Bio-TGG ACC GAG ATA CGA GAC ACA ATT CTA T-3'
M27	5'-Bio-TCA CAA TGG CAT CTA CCA CTC CTC AGC CAT TTG CA-3'	5'-Bio-AAG CTC ATG ATT TGT CTT CTA CCT CAG CTT GTC TGG TTC T-3'	5-Bio-CTC AAC ACA TCA ACC TAT AGG AAT CGA-3'
199	5'-Bio-ATG TGG CCA AGT TTT ATC TGC TGC CTC AGC TTT CAA ATA GGT-3'	5'-Bio-AAT TTA AGG GCA TCT TTC ATC CTC AGC ACC AGC TCT TC-3'	5'-Bio-CTG ACA ATG GGT CAC CTC T-3'
188	5'-Bio-TTC ATC TCT ATA TAT GAG GGC AGA GCC TCA GCA TGA TAA TCC AG-3'	5'-Bio-TTC CAT CTA TCT GCA TGT GGA AAC CTC AGC GAT ATG CAT GTA T-3'	5'-Bio-CCA TCT ATC TGC ATA TGG AAA AT-3'
271	5'-Bio-AAT TCA GGG CTC CCT TGG GCT CCC CTC AGC AAA AAT GTA GG-3'	5'-Bio-ATC TGG AAA CTG TTT TCT TTT TTT CCT CAG CTT TAA TGG AAA ATA-3'	5'-Bio-ATA CAG CTC CCC CTT TAT CCT-3'
TSC 0078283	5'-Bio-TGT AAT CAC AAG CAT CCT CCT CAG CGG ACT CGA GAG-3'	5'-Bio-CTT GTG GAC TCC TCC TCA GCC TTC AAA GCC AG-3'	5'-Bio-TGC CAC TCT GAC ACT GAT GC-3'
M26	5'-Bio-TTA CAA TTT TTT TAA ACC AAC CTC AGC TTT TTT TCT GAA-3'	5'-Bio-TAG AAG TTA GTT ATA TGT TCA CCT CAG CAC ATG ACG AAA-3'	5'-Bio-AAT TTC ATA GGC CAT TCA GTG-3'
TSC 0156245	5'-Bio-CTG TTC TTT CCA TCT CCC CAT CCT CAG CTC CTG CCT TG-3'	5'-Bio-GGC AAA AGC CTG AAC ATC CCC TCA GCT ATT TCG AGC A-3'	5'-Bio-GTT GGG AGG GAC AGG CCA TG-3'
M45	5'-Bio-CAG TAA CTC TAG GAG AGA GCC TCA GCA AAA TTG GCA GTG-3'	5'-Bio-TGT TTT TGT TTG TTT GTT TCC CTC AGC CTA CTA TCT CC-3'	5'-GCT GGA CCT CAG AAG GAG CTT TTT GC-3'
PN1	5'-Bio-GAT ATG TTT TCC TGC ATT TGT CCT CAG CCA AGG GTC TT-3'	5'-Bio-TTG ATG CTT GTC TTA TTA TAC CTC AGC AAG ATC TCC AAA G-3'	5'-Bio-AGA AGT ATC TCT TCC CCT TTA A-3'
M145	5'-Bio-TAG CGG CAT ACT TGC CTC CCC TCA GCT CCT AGA CAC CA-3'	5'-Bio-TCC CAC GTG GCC GCT AGG TTC CCT CAG CTC CTT TTT GGA TC-3'	5'-Bio-TTC TTG ATT AGG CTA AGG CT-3'
M178	5'-Bio-CAG GCA GAG ACT CCG AAA CCT CAG CTG GAT TAG TTG-3'	5'-Bio-GAA TAG GCA TGG GTT CAA GTC CTC AGC TCC CTG AAT GAA-3'	5'-Bio-CAT ATA TAG TTC TCC TGG CAC A-3'
10831	5'-Bio-TTC ATA TAC ATG GGA TCA TTC AGC CTC AGC CCT CTT GTA TCT-3'	5'-Bio-TGA CAT GAA AAT AGC AAA AAC TCC TCA GCG GCA CCA CA-3'	5'-Bio-CAT AGG TGA ACC TTG AAA ATG TTA-3'
PN2	5'-Bio-TTG GAT CTT CAT GCT GGT TCC TCA GCC ATT AAT AAA ACT AA-3'	5'-Bio-GAA CTC CCG ATT CCC CTC TAC CTC AGC AGA ATC AGC TC-3'	5'-Bio-TAA GAG AGG TGC CCC TAG GA-3'
TSC 0131214	5'-Bio-ACT ATT ATG CTC TTC ACT TTC CTC AGC TTA TTC TCT CAA G-3'	5'-Bio-TCC CAC TTT GTA GCT AAT GTT CCT CAG CCA GAA CCC AG-3'	5'-Bio-CCT GCC TCG AGG ACT GCT GCA AGG AA-3'
TSC0421768	5'-Bio-TGC TAC AGC GAA GAG CCT CAG CAA ATG AAG CAT CAA TA-3'	5'-Bio-CCT CCT CAG AAC TCT AAT GCC TCA GCG TGT TTT CTC ACT C-3'	5'-Bio-TGGCTTAGTGATGCCTCTTGCATT-3'
TSC 0709016	5'-Bio-CTC CAA CCA AGC TCC ATT CCT AGT GTC CTC AGC ATC CAC ACA GG-3'	5'-Bio-GTA CAT CTT TTA AGA CCA ACT CCT TGC CTC AGC GAC TGA TCT TGG-3'	5'-Bio-CAT GCA GGC AGC CCA GAG G-3'
TSC 0252540	5'-Bio-CAG CGG CCC ATT CCC TCA GCC CAT CCC A -3'	5'-Bio- GCC TTT TAG ACT ACA AAT GAC CTC AGC CAC AGG AG -3'	5'-Bio-AGG GCT GGG AGA AGG TGG GGG T -3'
TSC 1342445	5'-Bio-TCA GAA CTA ACT AGT CTG GGA AAC TCC TCA GCC CCT CGG TGC CA -3'	5'-Bio-CAG TAA GTT TTG ACA GGG TGG GGA CCT CAG CGA ATA GAA AAT -3'	5'-Bio-CAT GGG GGA CAG GGA GAC AGG -3'

Exhibit 24

Table 2. Discriminators

SNP	*Wild-Type Discriminator*	*Mutant Discriminator*	*Stabilizer*
247	5'-AGA CAT TCA ATT GTT ATC TGA GTC CGA ACA TTG AG-3'	5'-CAG ACA TTT AAT TGT TAG CAG TAT ATC GCT TGA CA -3'	N/A
P25	5'- CTG CCT GCA AAT ACT GAG TCC GAA CAT TGA G-3'	5'-CCT GCC TGA AAA TAG CAG TAT ATC GCT TGA CA-3'	N/A
M27	5'-TTG CAC CTC TGT CCC TGA GTC CGA ACA TTG AG-3'	5'-CAC CTG TGT CCT GAG CAG TAT ATC GCT TGA CA-3'	N/A
199	5'-TCC TAA TT**G** TCA GTC TCT GAG TCC GAA CAT TGA G-3'	5'- CC TAA TT**A** TCA GTC CGC AGT ATA TCG CTT GAC A -3'	N/A
188	5'-AAT TTC CAT TTT CCA TTA TTT CTG AGT CCG AAC ATT GAC -3'	5'-AAA TTT CTA TTT TCC AGC AGT ATA TCG CTT GAC A 3'	N/A
271	5'-ACA AAA GTC C**A**T GA C TGA GTC CGA ACA TTG AG -3'	5'- CAA AAG TCC **G**TG ATG CAG TAT ATC GCT TGA CA -3'	N/A
TSC 0078283	5'-ACT CGA GAG ACA GAT CTG AGT CCG AAC ATT GAG-3'	5'-GAG ACA CAA GCA TCA TTG CAG TAT ATC GCT TGA CA-3'	N/A
M26	5'-AAT TAT CGC AGA GAA CTG AGT CCG AAC ATT GAG -3'	5'-GAA TTA TCA CAG AGA GCA GTA TAT CGC TTG ACA -3'	N/A
TSC 0156245	5'-CTG AGT CCG AAC ATT GAG TGG CTC CCA GCC C-3'	5'-GCA GTA TAT CGC TTG ACA GGC TCC CAG CTC-3'	5'-AGC CTG CAC ATG GCC TGT CCC TCC CAA C-3'
M45	5'- ATA GAT AGG CAA AAA GAA ACT GAG TCC GAA CAT TGA G-3'	5'- TAT AGA TAA GCA AAA AGA AAG CAG TAT ATC GCT TGA CA-3'	N/A
PN1	5'-GAG AGC CTT TTG TCC TGA GTC CGA ACA TTG AG-3'	5'-AGA GCT TTT TGT CGC AGT ATA TCG CTT GAC A-3'	N/A
M145	5'-AGA AAG GCG AGA GCT GAG TCC GAA CAT TGA G-3'	5'-AAA GAA AGG CAA GAG GCA GTA TAT CGC TTG ACA-3'	N/A
M178	5'-TCA CCA CAG CTC CTC TGA GTC CGA ACA TTG AG -3'	5'-CAC CAT AGC TCC TAT AGC AGT ATA TCG CTT GAC A-3'	N/A
10831	5'-TCA CAC AGT GTA ACC TGA GTC CGA ACA TTG AG -3'	5'-TCA CAC AGT ATA ACA GCA GTA TAT CGC TTG ACA-3'	N/A
PN2	5'-AAA CTA ATG CCT TCT CCT CAC TGA GTC CGA ACA TTG AG-3'	5'-ACT AAT GCT TTC TCC TCA GCA GTA TAT CGC TTG ACA-3'	N/A
TSC 0131214	5'-ACG TGC ACT TCT CCT GAG TCC GAA CAT TGA G-3'	5'-CGT GCT CTT CTC GCA GTA TAT CGC TTG ACA-3'	N/A
TSC0421768	5'-CAA TGC GTT CAC ATC TGA GTC CGA ACA TTG AG-3'	5'-CAA TGG GTT CAC AAG CAG TAT ATC GCT TGA CA-3'	N/A
TSC 0709016	5'- AAC CAC TAA TAT CAC CTG AGT CCG AAC ATT GAG -3'	5'- AAC CAC TTA TAT CAC GCA GTA TAT CGC TTG ACA -3'	N/A
TSC 0252540	5'-GGT CTG **C**AC CCC TCT GAG TCC GAA CAT TGA G -3'	5'- GGT CTG **T**AC CCC CTT GCA GTA TAT CGC TTG ACA -3'	N/A
TSC 1342445	5'- GCC TCC TGC ATG GTC TGA GTC CGA ACA TTG AG -3'	5'- GCC TCC AGC ATG GGG CAG TAT ATC GCT TGA CA -3'	N/A

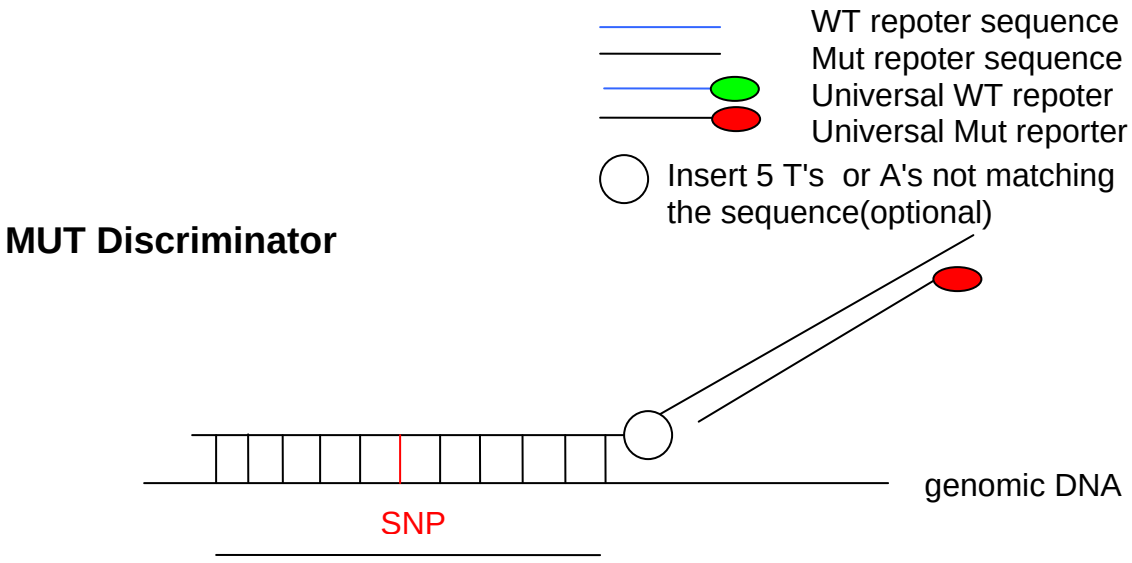
Exhibit 25

Table 3. Ratio references

SNP	*Wild-Type RR*	*Mutant RR*
247	5’-bio-CTT GTA GGA CAT AAT AAT AAC AAT T**G**A ATG TCT GCA CAG C-3’	5’-bio-CTT GTA GGA CAT AAT AAT AAC AAT T**A**A ATG TCT GCA CAG C-3’
P25	5’-bio- CAT GAT GGA CCG AGA TAC GAG ACA CAA TTC TAT TTG CAG GCA GGT TTC AGG -3’	5’-bio- CAT GAT GGA CCG AGA TAC GAG ACA CAA TTC TAT TTT CAG GCA GGT TTC AGG -3’
M27	5’-bio-CTA TAG GAA TCG AGG TTC AGG ACA GAG GTG CAA ATG TGG ACA TAG TGG T-3’	5’-bio-CTA TAG GAA TCG AGG TTC AGG ACA CAG GTG CAA ATG TGG ACA TAG TGG T-3’
199	5’-bio- GAC CTG ACA ATG GGT CAC CTC TGG GAC TGA **C**AA TTA GGA AGA GC -3’	5’-bio- GAC CTG ACA ATG GGT CAC CTC TGG GAC TGA **T**AA TTA GGA AGA GC -3’
188	5’-bio-TTC CAT CTA TCT GCA TAT GGA AAA TGG AAA TTT CAG CCA-3’	5’-bio-TTC CAT CTA TCT GCA TAT GGA AAA TAG AAA TTT CAG CCA-3’
271	5’-bio-TCC CCC TTT CTC GGG CAC AGA TCT CAT GGA CTT TTG TTA-3’	5’-bio-TCC CCC TTT CTC GGG CAC AGA TCT CAC GGA CTT TTG TTA-3’
TSC 0078283	5’-bio-TGC CAC TCT GAC ACT GAT GCT TCT GTC TCT CGA GTC CAC TTT TAA GGA TGC-3’	5’-bio-TGC CAC TCT GAC ACT GAT GCT TGT GTC TCT CGA GTC CAC TTT TAA GGA TGC-3’
M26	5’-bio- TCA TAG GCC ATT CAG TGT TCT CTG CGA TAA TTC TAA TTC AGA AAA AAA T -3’	5’-bio- TCA TAG GCC ATT CAG TGT TCT CTG TGA TAA TTC TAA TTC AGA AAA AAA T -3’
TSC 0156245	5’-bio-GAG GGA CAG GCC ATG TGC AGG CTG GGC TGG GAG CCA AGG CAG GAG GGT G-3’	5’-bio-GAG GGA CAG GCC ATG TGC AGG CTG AGC TGG GAG CCA AGG CAG GAG GGT G-3’
M45	5’-bio-ACC TCA GAA GGA GCT TTT TGC CTA TCT ATA ATT TTT CAC T -3’	5’-bio-ACC TCA GAA GGA GCT TTT TGC TTA TCT ATA ATT TTT CAC T -3’
PN1	5’-bio-AGA AGT ATC TCT TCC CCT TTA AGA CAA AAG GCT CTC CCT CTC AAG-3’	5’-bio-AGA AGT ATC TCT TCC CCT TTA AGA CAA AAA GCT CTC CCT CTC AAG-3’
M145	5’-bio-TTG ATT AGG CTA AGG CTG GCT CTC GCC TTT CTT TCT GGT GTC TAG GAA A-3’	5’-bio-TTG ATT AGG CTA AGG CTG GCT CTT GCC TTT CTT TCT GGT GTC TAG GAA A-3’
M178	5’-bio-TTC TCC TGG CAC ACT AAG GAG CTG TGG TGA ATG AAC -3’	5’-bio- TTC TCC TGG CAC ACT AAG GAG CTA TGG TGA ATG AAC -3’
10831	5’-bio-AGG CAC CAC ATA GGT GAA CCT TGA AAA TGT TA**C** ACT GTG TGA AAA A-3’	5’-bio-AGG CAC CAC ATA GGT GAA CCT TGA AAA TGT TA**T** ACT GTG TGA AAA A-3’
PN2	5’-bio-AGA GAG GTG CCC CTA GGA GGA GGA GGC ATT AGT TTT-3’	5’-bio-AGA GAG GTG CCC CTA GGA GGA GGA **A**GC ATT AGT TTT-3’
TSC 0131214	5’-bio-GAG GAC TGC TGC AAG GAA GAG AAG TGC ACG TAA GCA CCC AAC TGA GGA C-3’	5’-bio- GAG GAC TGC TGC AAG GAA GAG AAG AGC ACG TAA GCA CCC AAC TGA GGA C-3’
TSC0421768	5’-bio-TGG CTT AGT GAT GCC TCT TGC ATT GTG AAC GCA TTG AAA TTA TTG ATG C-3’	5’-bio-TGG CTT AGT GAT GCC TCT TGC ATT GTG AAC CCA TTG AAA TTA TTG ATG C-3’
TSC 0709016	5’-bio-GCA GCC CAG AGG CCT AGG CCT TTT GTG ATA TAA GTG GTT CCC TGT CAT TCC CTG TGT G -3’	5’-bio- GCA GCC CAG AGG CCT AGG CCT TTT GTG ATA TAT GTG GTT CCC TGT CAT TCC CTG TGT G -3’
TSC 0252540	5’-bio- GCT GGG AGA AGG TGG GGG T**G**C AGA CCC AGC AGT TTC CCA TGC TGG GAT GGA -3’	5’-bio- GCT GGG AGA AGG TGG GGG T**A**C AGA CCC AGC AGT TTC CCA TGC TGG GAT GGA -3’
TSC 1342445	5’-bio- TGG GGG ACA GGG AGA CAG GCC CAT GCA GGA GGC TGG CAC CGA GGG GCT GAG G -3’	5’-bio- TGG GGG ACA GGG AGA CAG GCC CAT GCT GGA GGC TGG CAC CGA GGG GCT GAG G -3’

Appendix I

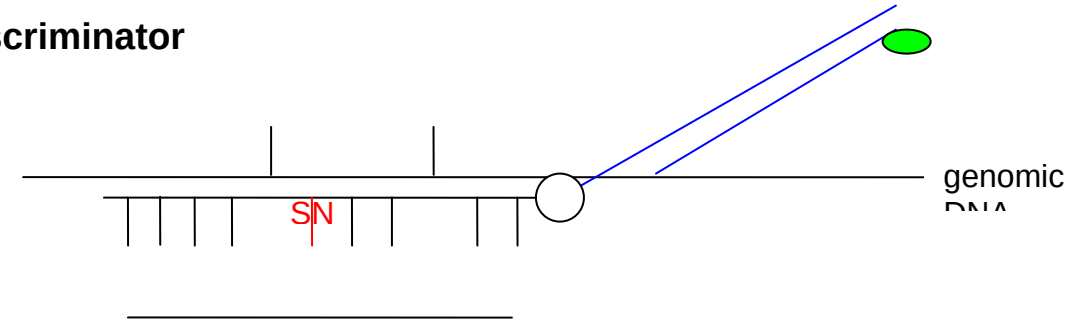
Discriminator design



MUT Discriminator

40 ºC (SNP does not have to be in middle; right end can be shorter)

WT Discriminator



40 ºC (SNP does not have to be in middle, right end can be shorter)

Guidelines for Discriminator Design:

- Perform Tm calculations, e.g., using the IDT oligo analyzer. http://207.32.43.70/biotools/oligocalc/oligocalc.asp
- Set the discriminator concentration to 100 nM (concentration used in reporter mix).
- Use 50 mM Salt (default) for calculations (Low Salt Buffer)
- Stability of Discriminator/genomic DNA complex should be around 40ºC (for example 36-38ºC)
- Try to design WT and Mut discriminator as close in Tm as possible, by adjusting length
- Try to design discriminator for different SNPs used in the same reporter mix as close as possible in Tm value to receive a common discrimination temperature

Appendix II

Example of a Typical SNP PCR Protocol for Theromocycler

Final volume: 50µl

13 min.- 95C
45sec. -94C
45sec. – 45C For 35 cycles
1 min. -70C
5 min. -70C
Hold at 4C

Reaction	Template NIJ #sample (200 ng/ul)	Bumper primer biotinylated (50 µM each)	Bumper primer (5 µM each)	10 x Buffer II	10 mM NTPs	25 mM $MgCl_2$	Amplitaq Gold (1.25 u/µl)	H_2O	Total
Volume in µl	1	0.5	5	5	1	3	0.25	34.25	50

Example of a Typical STR PCR Potocol for Thermocycler

Final volume: 50µL

14 min.- 95C
30sec. -94C
30sec. -60C For 28 cycles
45sec. -70C
5 min. – 70C
Hold at 4C

Appendix III

Guidelines for Developing Anchored SDA SNP Assays

1. Preparation of Oligonucleotides:

- On-chip primers and nested primers: 10 μM in TE
- Bumpers (not biotinylated): 5 μM in TE.
- Bitinylated bumpers: 50 μM in TE
- Ratio references: 100 nM in TE.
- Discriminators: 10 μM in TE.

2. Testing the Discriminators (100, 250 and 500 nM)

- Dilute the Ratio references (Wt, Mut, and both) at different concentrations (2 and 4 nM) in 50 mM Histidine;
- Address the diluted Ratio references on a H2 cartridge (standard hydrogel) with at least duplicated pads using “Target”;
- Wash the cartridge with HSB (high salt buffer);
- Prepare the Reporter Mix in HSB:

  250 nM Wt Discriminator

  250 nM Mut Discriminator

  500 nM 5’-labeled Wt Universal Reporter

  500 nM 5’-labeled Mut Universal Reporter

- Pipette the Reporter Mix into the cartridge
- Run Reader Protocol (NIJ SNP Analysis)
- Analyze the data with the criteria:

  Temperature around 38-40 C;

Pads with Wt Ratio reference only have “Green” signal;

Pads with Mut Ratio reference only have “Red” signal;

Pads with Wt/Mut Ratio references have “Green/Red” signals and the ratio between them is around 1:1.

- Denature the cartridge with 0.1 N NaOH for 5 min and wash several times
- Repeat the above procedure with new Reporter Mix;
- Select the Discriminators, concentrations and temperature.

3. Testing PCR Amplicons with the Selected Discriminators

- Prepare biotinylated PCR amplicons from NIJ samples (#3, 4, 6, 7, 10, 12, and 19) using the PCR preparation protocol;
- Denature the amplicons;
- Measure the concentrations on Agilent Bioanalyzer, Model 2001;
- Dilute the amplicons at different concentrations (2-10 nM/µL in 50 mM Histidine);
- Address the diluted amplicons with Ratio references on a H2 cartridge (standard hydrogel) with at least duplicated pads using “Target”;
- Denature the cartridge with 0.3 N NaOH for 5 min and wash several time
- Report the cartridge with the selected Reporter Mix;
- Run the same Reader protocol;
- Analyze the data;
- Record the genotypes for the tested NIJ samples.

4. Testing On-Chip Amplification and Designed Primers

- Prepare all possible combinations of primers and nested primers with concentration of 50 nM on-chip primers and 200 nM nested primer;
- Address primers on a H3 cartridge (group L hydrogel) primer with at least duplicated pads using electronic addressing;
- Address NIJ# sample of gDNA (20 ng/µL) using electronic addressing; Leave some primer-only-pads;
- Address the Ratio references on some pads using “Target”;

- Amplify for 60 min at 50ºC;
- Denature the cartridge for 5 min with 0.3 N NaOH. Wash several times;
- Report with the selected Reporter Mix and run with the same Reader protocol;
- Analyze the data and choose the best primer combinations with the criteria:

  No signals on primer-only-pads;

  Giving correct genotypes as PCR amplicons;

  Giving the highest signals;

- Design new primers if none of the combinationsproduce satisfactory results and repeat the procedure.

5. Validate of the Best Primer Combinations

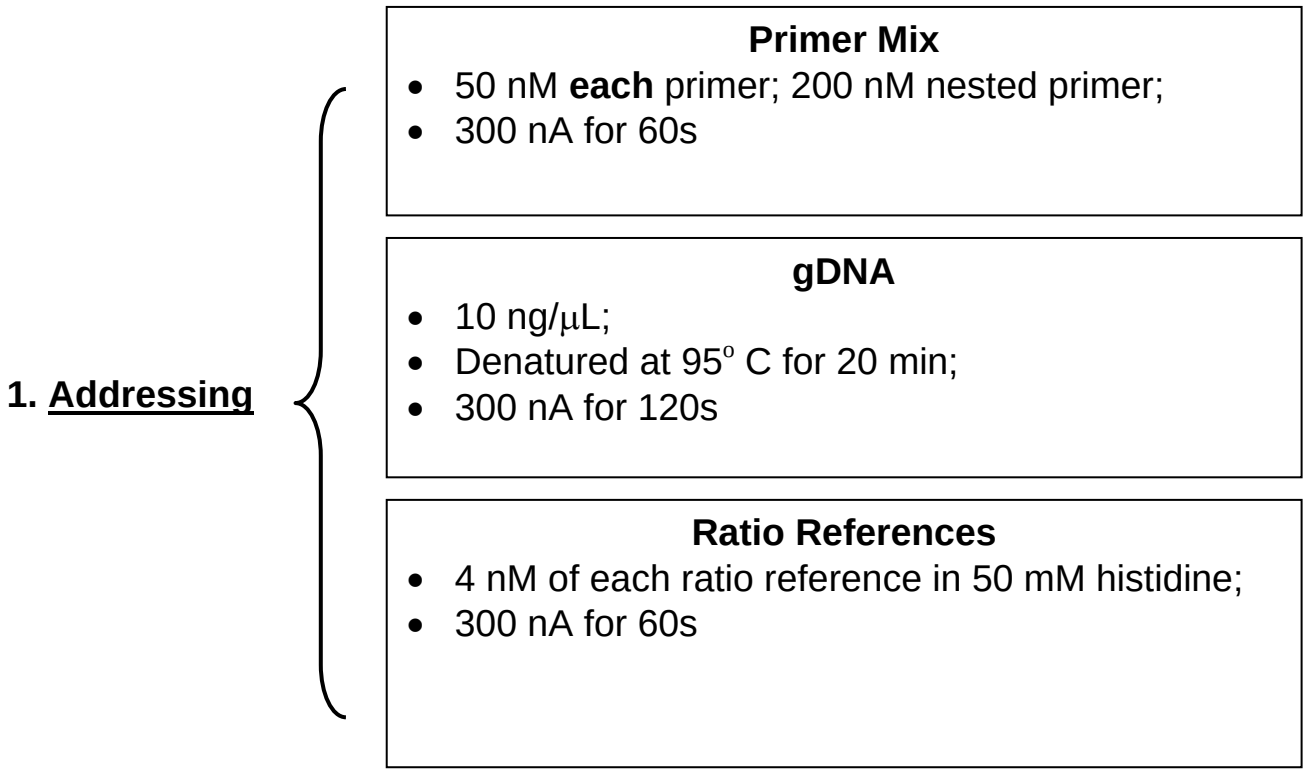
- Test different DNA concentrations (20, 10, 5, 2, 1 ng/µL) to determine the lowest concentration;
- Test different DNA samples (NIJ # genomic DNA samples) to determine if correct genotypes is produced as determined by PCR amplicons;

6. Multiplex different SNPs

- Add the developed new SNP discriminators and reagents to the Multiplex mix and perform testing with multiplexed SNPs in a similar way as with individual SNPs.
- Optimize conditions with respect to all SNPs in the multiplex assay

Appendix IV

Anchored SDA Workflow



2. Amplification

Enzyme Mix (50 uL)

- 28 μL H_2O
- 2.5 μL of $MgCl_2$ (25 mM);
- 2.5 μL of $KiPO_4$ (500 mM);
- 5.0 μL of dNTPs (2.5 mM each);
- 0.5 μL of **each** bumper primer (5 μM);
- 3 μL of Diluent A;
- 2 μL of N. BbvC 1B (100,000 U/mL);
- 6 μL of Bst DNA polymerase (120,000 U/mL);

SDA

- 50 ^{o} C for 45-min;

3. Detection

Denature

- 0.3 N NaOH for 3 min;

Report Mix

- 100 nM each discriminators;
- 250 nM each Univ. Rep.

Image

- 60s for Red
- 240 s for Green;