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# Simple Multiplex Genotyping by Surface-Enhanced Resonance Raman Scattering

Duncan Graham,* Benjamin J. Mallinder, David Whitcombe,† Nigel D. Watson, and W. Ewen Smith

*Department of Pure and Applied Chemistry, University of Strathclyde, Glasgow G1 1XL, U.K.*

**The accurate detection of DNA sequences is essential for a variety of post human genome projects including detection of specific gene variants for medical diagnostics and pharmacogenomics. A specific DNA sequence detection assay based on surface-enhanced resonance Raman scattering (SERRS) and an amplification refractory mutation system (ARMS) is reported. Initially, generation of PCR products was achieved by using specifically designed allele-specific SERRS active primers. Detection by SERRS of the PCR products confirmed the presence of the sequence tested for by the allele-specific oligonucleotides. This lead directly to the multiplex genotyping of human DNA samples for the $\Delta F_{508}$ mutational status of the cystic fibrosis transmembrane conductance regulator gene using SERRS active primers in an ARMS assay. Removal of the unincorporated primers allowed fast and accurate analysis of the three genotypes possible in this system in a multiplex format without any separation of amplicons. The results indicate that SERRS can be used in modern genetic analysis and offers an opportunity for the development of novel assays. This is the first demonstration of the use of SERRS in multiplex genotyping and shows potential advantages over fluorescence as a detection technique with considerable promise for future development.**

The accurate detection of DNA sequences is a necessary part of post human genome molecular biology. The presence of a specific DNA sequence can be used to identify the type of gene under examination and provide diagnostic information useful for identification of disease and for pharmacogenomics.[1−3] Conventional detection involves the use of fluorescent labels covalently attached either to a primer sequence[4] or to dideoxynucleotide triphosphates that are then incorporated into the DNA under scrutiny by PCR.[5] As the fluorophores used tend to have overlapping spectra, which are broad and indistinct, separation by electrophoresis is normally necessary prior to final detection and identification. Separation can be avoided by using fluorophores with different emission profiles[6] or by using time-resolved fluorescence.[7] However, these methods require special fluorophores and more complex equipment that cannot easily resolve mixed fluorophores in varying proportions. Surface-enhanced resonance Raman scattering (SERRS)[8,9] can easily differentiate between mixtures of labels and since both nonfluorophores and fluorophores are effective it can use a much more extensive labeling chemistry.[10,11] This prompted the investigation into using SERRS for multiplex genotyping.

Previous work on DNA and SERS has mainly focused around the detection of unmodified DNA. Kneipp et al.[12−15] detected the presence of nucleic acids by allowing slow adsorption (18 h) onto a metal colloid to produce SERS. Further detection has focused around the use of fluorescent intercalators.[16−19] Unfortunately, this approach suffers from the problem of discrimination between the bound and unbound intercalator. However, VoDinh et al.[20,21] detected cresyl fast violet (CFV)-labeled DNA by SERRS using a solid substrate. This allowed successful detection of DNA although only one label was used and the chemistry of surface adsorption was not discussed.

* Corresponding author: (e-mail) duncan.graham@strath.ac.uk.

† Formerly of Zeneca Diagnostics, Gadbrook Park, Northwich, Cheshire CW9 7RA, U.K., now at DxS Ltd., Manchester Incubator Building, 48 Grafton St., Manchester M13 9XX, U.K.

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Table 1. Sequences Used as Primers in Both Systems Examined

| name | sequence[a] |
|---|---|
| DQA1 | 5′-HEX $(T^*C)_6$ GTG CTG CAG GTG TAA ACT TGT ACC AG |
| DQA2 | 5′-$Rhod_3$ GTG CTG CAG GTG TAA ACT TGT ACC AG |
| Rev110 | (5′-biotin) CAG TCT CCT TCC TCT CCA GGT CCA |
| Rev254 | (5′-biotin) CAC GGA TCC GGT AGC AGC GGT AGA GTT G |
| CFNORM | 5′-HEX $(T^*C)_6$ GTA TCT ATA TTC ATC ATA GGA AAC ACC ACA |
| CFDEL | 5′-$Rhod_3$ GTA TCT ATA TTC ATC ATA GGA AAC ACC ATT |
| CFComB | (5′-biotin) GAC TTC ACT TCT AAT GAT GAT TAT GGG AGA |

[a] HEX = 2,5,2′,4′,5′,7′-hexachloro-6-carboxyfluorescein, Rhod = rhodamine 6G, and T*= 5-(3-aminopropynyl)-2′-deoxyuridine.

Recently we reported the basic methodology for ultrasensitive, reliable detection of modified oligonucleotides[22,23] and the identification of a mixture of oligonucleotides by SERRS.[11] The main problem addressed in the previous study was how to provide the DNA with a suitable chromophore as a label and adsorb the DNA onto the metal surface.

Since SERRS quenches fluorescence, a suitable chromophore was easily provided by addition of a commercially available fluorescent label. However, the problem of surface adsorption was more complex to solve. The metal surface favored in these SERRS studies is that of monodispersed citrate-reduced silver colloid. The colloidal particles have a negatively charged citrate layer on the surface that prevents aggregation and ultimately precipitation.[24] To produce SERRS, the particles must be aggregated in a controlled manner to provide aggregates of a size sufficient to remain in suspension and not precipitate. This process tunes the plasmon frequency to match the excitation frequency used and gives rise to areas of high electric field from the interaction of the surface plasmons in the aggregate. Aggregation is normally achieved by the use of an external charge-modifying agent such as inorganic cations or acid. The advantage of this approach is that aggregate motion creates averaging of the effect, which overcomes the problems with signal variation due to "hot spots" and solid substrates, to enable effective quantitation.

The polyanionic DNA does not adsorb onto the colloidal surface due to electrostatic repulsion. However, the DNA can be modified to contain positively charged moieties that will pull the DNA onto the surface. In addition, to be effective the phosphate backbone must be neutralized. This is done by adding excess spermine. The negative backbone is neutralized, and the remainder of the spermine acts as an effective aggregating agent for the colloid. Thus, neutralization of the backbone allows the positively charged groups to pull the labeled DNA onto the colloid surface. The excess spermine aggregates the colloid and analysis by SERRS produces a spectrum consistent with that of the label. A variety of aggregating agents and DNA backbone neutralizing agents have been tested with these labeled oligonucleotides; however, spermine was found to be the best. This allowed detection down to a level of $8 \times 10^{-16}$ mol.[22]

The results of our initial experiments into the SERRS of DNA indicated that we could obtain a high degree of discrimination and sensitivity by using SERRS as a technique for DNA analysis. The greatest advantage of SERRS appears to be the ability to easily identify the composition of mixtures without separation and has been demonstrated for simple chemical systems. However, the key experiment would be the detection and identification of a specific gene type to demonstrate the first use of SERRS in multiplex genotyping. The achievement of this objective is reported here.

## EXPERIMENTAL SECTION

**Materials.** Spermine tetrahydrochloride (Sigma), trisodium citrate (Fisher), and silver nitrate Aldrich) were of analytical grade. Modified DNA was obtained from the Oswel DNA Unit, University of Southampton, Southampton, U.K.

**PCR Procedures.** The PCR reaction mixture used contained 50 ng of genomic DNA, 200 $\mu$M concentration of each dNTP, 0.5 $\mu$M primer, 1 unit of AmpliTaq DNA Polymerase (Perkin-Elmer), 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 3.5 mM $MgCl_2$, and distilled water to a volume of 100 $\mu$L. The primers DQA1 and CFNORM were HEX labeled and contained the propynyl-modified tail; primers DQA2 and CFDEL were labeled with three rhodamine 6G labels attached to the 5′-terminus via aminohexyl-2′-deoxyribose groups separated by a hexaethylene glycol spacer. The reverse primers Rev110, Rev254, and CFComB (5′-biotin labels when used for capture) were used to control the length of the PCR product. For DNA amplification, the temperature program was initiated at 95 °C for 10 min and subsequent temperature steps were as follows: for the DQA primers, 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 30 s, with 40 cycles, followed by a temperature hold at 72 °C for 7 min; for the CF primers, 94 °C for 30 s, 58 °C for 2 min, and 72 °C for 1 min, with 35 cycles followed by a temperature hold at 72 °C for 7 min. Samples were cooled to 4 °C prior to use. Products were visualized using gel electrophoresis to ensure amplification had occurred. The sequences used as primers are presented in Table 1.

**Biotin/Streptavidin Capture Process.** The biotin-labeled primer was annealed to the DNA in a single step after PCR. The PCR mix was heated to 95 °C for 5 min and the mix allowed to cool. M-280 streptavidin-coated Dynal beads (150 $\mu$L) were prewashed three times with bind and washing (B&W) buffer (150 $\mu$L (10 mM Tris-HCl, pH 7.5; 1 mM EDTA, 1 M NaCl)), prior to addition to the DNA. Incubation time for binding was 30 min. The DNA-coated beads were separated using a magnet, and after a series of washes with B&W (×3), chloroform, and distilled $H_2O$ ( $dH_2O$, ×2), the beads were resuspended in $dH_2O$ (20 $\mu$L). The sDNA was recovered from the Dynal beads using three methods:

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**Method 1.** The Dynal bead/DNA suspension was heated in a 95 °C water bath for 5 min, the Dynal beads were magnetically separated, and free sDNA was removed as supernatant.

**Method 2.** NaOH (20 $\mu$L of 1 M) was added to cleave the target DNA. The supernatant was removed and added to 10 000 MW cutoff Viva-Spin columns. The columns were centrifuged ($\times$ 10 000) and washed with $dH_2O$ (20 $\mu$L) twice. The DNA was removed from the filter by resuspending in $dH_2O$ (20 $\mu$L) and then used for SERRS studies.

**Method 3.** The Dynal bead/DNA suspension was resuspended in a formamide elution buffer (50 $\mu$L of 95% formamide with 10 mM EDTA, pH 8.2). The suspension was heated to 65 °C for 5 min, and the sDNA was removed as the supernatant and examined by SERRS.

**Sample Preparation.** A sample of the purified captured PCR products (20 $\mu$L) was premixed on ice with an aliquot of spermine tetrahydrochloride (20 $\mu$L, $1 \times 10^{-1}$ M) followed by addition of water (500 $\mu$L) and colloid (500 $\mu$L). Blank control spectra were obtained using the same preparation method, replacing the DNA sample with water.

**Colloid Preparation.** The silver colloid was prepared according to a modified Lee and Meisel procedure. A sample of silver nitrate (90 mg) was dissolved in distilled water (500 mL) at 45 °C and heated to near boiling under stirring. A sodium citrate solution was added (10 mL, 1%) and the solution held at boiling for 90 min with continuous stirring.

**Raman Spectra.** SERRS spectra were recorded using a Renishaw 2000 Raman Microprobe instrument and a Renishaw Mark III probe head system. Excitation at 514.5 nm was provided by an Omnichrome (Lambda Photometrics) argon ion laser (25 mW) and a Spectra-Physics model 2020 argon ion laser (100 mW) for the microprobe instrument and a Spectra-Physics model 163 argon ion laser (14 mW) with the Renishaw Mark III. Both instruments gave ~3 mW at the sample. Samples were analyzed either in a plastic microtiter plate using a $\times$10 objective or in a quartz cuvette placed in a Ventacon macrosampler. All scans were collected with the grating centered at 1400 $cm^{-1}$.

## RESULTS AND DISCUSSION

**HLA-DQ System.** Initially we chose to investigate SERRS-active, allele-specific oligonucleotides commonly used to investigate chromosome 6 in the HLA-DQ region. This region encodes for the class II major histocompatability antigens, the human leukocyte antigens, which are concerned with immune response. This system was chosen to obtain PCR products from the modified primers to ensure that they were biologically active and to show that double-stranded DNA could be detected using SERRS.

A forward primer was labeled with a HEX fluorophore that was preceded by a $(T^*C)_6$ tail, where T* was a propargylamino-modified 2′-deoxyuridine. Use of different reverse primers generated two lengths of PCR product. This gave rise to amplicons of 110 and 254 bp that each contained one HEX label. The conditions for amplification had to be modified slightly to allow for the effect of the propargylamines acting as duplex stabilizing agents. A forward primer labeled with three rhodamine 6G fluorophores at the 5′-terminus was also used in the PCR experiments. In SERRS, it is advantageous to use multiple chromophores as an additive effect is observed unlike in fluorescence where self-quenching becomes a problem. Only one length of product was generated with the rhodamine-labeled primer to prove that the alternative labeling (three positively charged labels as opposed to the conventional one) methodology was still biologically compatible.

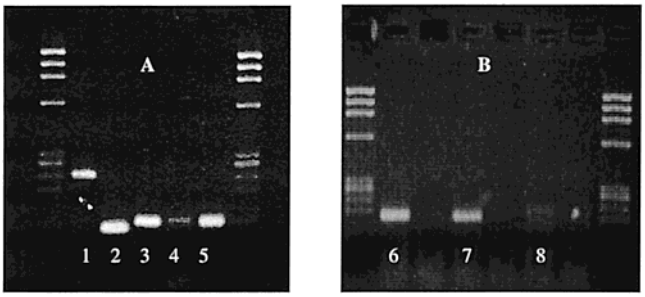


Figure 1. Agarose gel (2.5%) electrophoresis of (A) HLA DQ PCR and (B) ARMS CF PCR products. Lanes: 1, 254-bp product; 2, 110-bp product; 3, 110 bp product with propargylamines; 4, 110-bp product with propargylamine and HEX; 5, 110-bp product with $Rhod_3$. 6, homozygote +/+; 7, heterozygote +/Δ; 8, homozygote Δ/Δ.

Amplification by PCR was successfully completed and generated distinct products as followed by gel electrophoresis.(Figure 1A) A primer containing the propargylamino bases but no label was also used as a control in the PCR reactions. The gel shown displays the 110-bp products as generated by the normal primer, the modified but unlabeled primer, and both the HEX- and rhodamine-labeled primers. All primers gave products, but they display altered electrophoretic mobility. The product generated from the modified, unlabeled primer shows a band that runs slower than expected. This is attributed to the presence of the propargylamines, which produce an area of positive charge as well as increasing the molecular weight. The HEX-labeled version of this primer shows a similar effect. The rhodamine-labeled primer generated a product that did not contain any propargylamines but still produced reduced mobility. This is a known effect with fluorescent labels[25] and is attributed to the increase in mass as well as the change in charge due to the presence of the positively charged rhodamine moieties.

The next step was to detect the amplified products by SERRS. If an attempt were made to examine the PCR mixture directly, then the unincorporated labeled primers would give a positive result. Thus, a method for removal of the excess primers had to be devised. Initially, several commercially available kits for removal of unincorporated primers were tested for their ability to clean up the mixture prior to SERRS. Unfortunately, the sensitivity of SERRS proved that even kits with an efficiency greater than 99% were not rigorous enough in their removal of unincorporated primers and signals could be detected by SERRS in the spiked controls.

To isolate the PCR products, a biotinylated probe was used that would hybridize to the labeled product and could then be immobilized by interaction with streptavidin on a solid surface. This required an extra denaturation followed by hybridization step in the PCR cycle to produce the labeled target sequence hybridized to the biotinylated primer. This complex was then isolated by using streptavidin-coated magnetic Dynal beads followed by washing and denaturation of the duplex to release the single-

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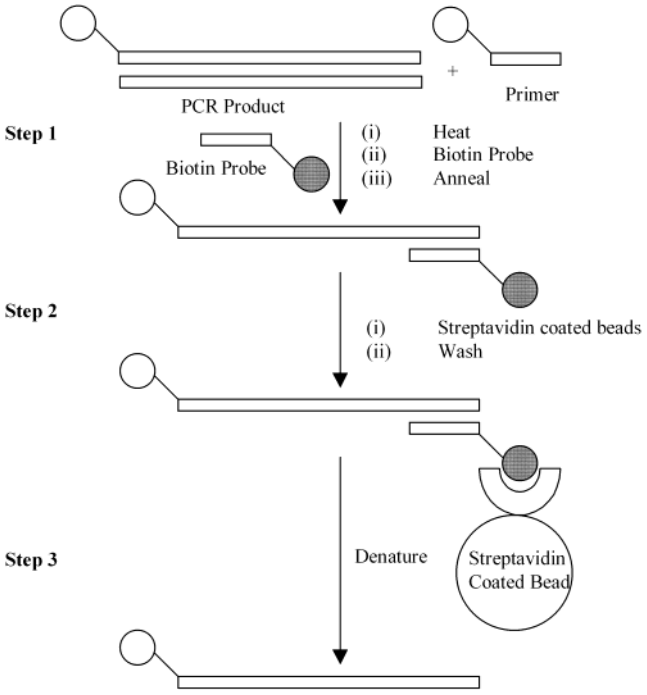


Figure 2. Diagramatic scheme of events used for the separation of the unincorporated primers in the HLA-DQ system. Steps: (1) The PCR mixture is heated to produce sDNA. Introduction of a biotinylated capture probe followed by annealing produces the labeled/biotinylated duplex. (2) Streptavidin-coated magnetic beads introduced and the target complex immobilized onto the beads. Washing removes the excess primers. (3) Denaturation of the immobilized duplex releases the labeled sDNA, which is subsequently detected by SERRS.

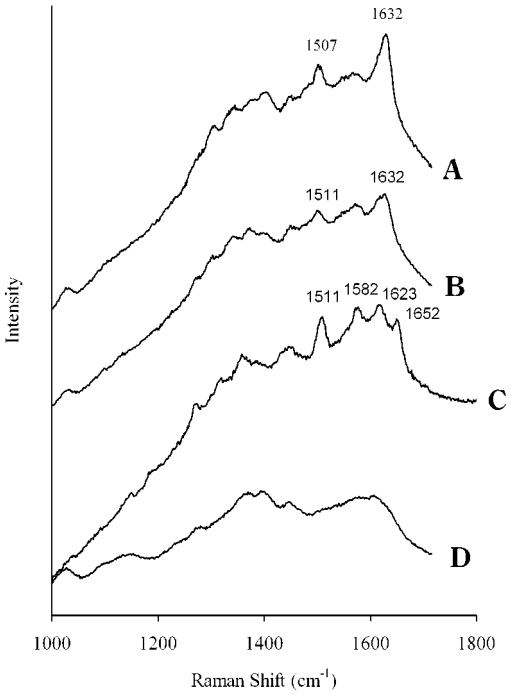


Figure 3. SERRS spectra of the HLA-HQ PCR products isolated by capture on magnetic beads followed by denaturation: (a) 110-bp product HEX; (B) 254-bp product HEX; (C) 110-bp product $Rhod_3$; (D) blank.

stranded labeled product (Figure 2). Washing experiments were conducted with labeled primers and the magnetic beads to determine the extent of nonspecific adsorption. It was found that after two washes no SERRS signals could be detected from the wash. In subsequent experiments, three buffer washes followed by one chloroform and two water washes were used when washing the captured labeled target sequence to ensure complete removal of the unincorporated primer.

Denaturation was achieved by heating to 95 °C and removing the supernatant while the beads were held stationary by a magnet. After the magnetic bead cleanup, the resulting SERRS spectrum was conclusively that of HEX. A similar result was obtained for the rhodamine-labeled primer (Figure 3). The signal output was low, indicating that a large amount of the PCR product was still bound to the magnetic bead. This was due to the small volume used (20 $\mu$L), which cooled from 95 °C rapidly once placed against the magnet, allowing hybridization and hence recapture of the product. Heating the magnet and sample produced only a small improvement in the signal strength, indicating that further work is required to optimize this procedure. The use of sodium hydroxide and formamide buffer as denaturation agents gave no improvement.

An attempt to obtain SERRS directly from the labeled products immobilized on the beads was unsuccessful. Since SERRS can be obtained from labeled DNA immobilized on other surfaces, it was concluded that the beads interfere when attempts to obtain SERRS directly are made.

Thus, we can detect specifically amplified DNA sequences by SERRS. Two lengths of product were successfully detected once the unincorporated primers had been removed by a biotin/streptavidin capture step. This indicated that SERRS could be used to detect PCR amplicons and prompted us to investigate multiplex genotyping by SERRS.

**Cystic Fibrosis Genotyping.** Cystic fibrosis is a widely studied genetically transmitted disease and as such is the ideal system for testing the multiplex genotyping ability of SERRS as disclosed in our previous communication.[26] We chose an amplification refractory mutation system (ARMS) for the cystic fibrosis transmembrane conductance regulator (CFTR) gene $\Delta F_{508}$ mutation as the test system. ARMS had previously been used to demonstrate fluorescence polarization identification of the genotypes present.[27] In that system, the ARMS products were detected by specific hybridization of a labeled probe and polarization measurements were able to distinguish which combination of alleles was present. This method of identification can detect homozygotes easily but becomes more complicated for heterozygotes or multiplexing. We show here that by labeling the ARMS primers with SERRS-active labels we can easily identify the genotypes present whether homozygotes or heterozygotes.

Two suitable ARMS primers were chosen as well as a common primer (Table 1). One ARMS primer tested for the wild-type gene sequence (CFNORM = +) and the other a three-base deletion (CFDEL = Δ) corresponding to the most common mutant found in cystic fibrosis sufferers. CFNORM was labeled at the 5′-terminus with a HEX fluorophore that was preceded by a $(T^*C)_6$ tail, where T* was a propargylamino-modified 2′-deoxyuridine as described previously. The CFDEL primer was labeled with three

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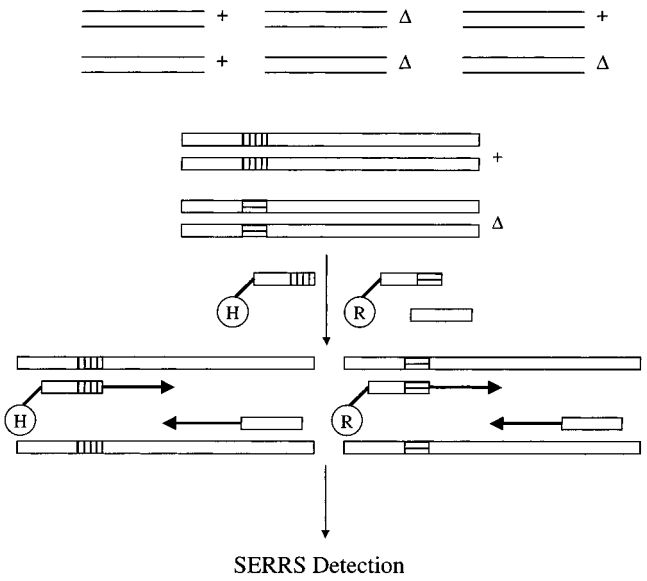


Figure 4. ARMS assay using the two SERRS-active primers and the common primer. The three possible genotypes are shown: +/+, wild-type homozygote; Δ/Δ, deletion mutant homozygote; +/Δ, heterozygote. The situation depicted is that of the heterozygous genotype. (H = HEX, R = rhodamine).

rhodamine fluorophores. A third primer (ComB) was used to control the length of PCR product to 160 bp. The reverse primer (CFComB) was labeled with biotin at the 5′-terminus to allow an optional streptavidin capture step after the ARMS amplicons had been produced. (Biotin is not SERRS active and thus does not produce any conflicting signals if a capture step is not employed.)

The ARMS assay was designed to detect the presence of the three possible variants, two homozygotes +/+ and Δ/Δ, and the heterozygote +/Δ. The three primers were mixed together in the presence of the target DNA and cycled to generate the labeled products (Figure 4). Pretyped DNA (supplied by Astra Zeneca Diagnostics) was used in the assays to allow accurate evaluation of the technique for genotyping. Once generated, the PCR products had to be isolated from the unincorporated labeled primers that would otherwise give rise to false positives.

A number of methods were attempted to remove the unincorporated primers. The biotin/streptavidin method of capture was used as before to provide a method of purification, but the signal-to-noise ratio was poor. To achieve accurate genotyping, the PCR products were isolated by agarose gel electrophoresis (Figure 1B). The homozygote mutant multiplex appeared to give poor PCR amplification; however, it was sufficient for detection by SERRS. The PCR products were then extracted from the gel by a commercially available kit. Once extracted from the gel, the DNA under examination was mixed with spermine to neutralize the phosphate backbone and added to silver colloid. The excess spermine provided the desired aggregation and hence signal intensity. Analysis was carried out using a Renishaw Microprobe and 514.5-nm excitation to produce the spectra shown in 10 s.

The results generated from the assay are shown in Figure 5. Three distinctly different spectra were produced from the assay that corresponded to the three genotypes investigated. The homozygotes both gave the expected single dye spectrum; i.e., +/+ gave the HEX spectrum and Δ/Δ gave rhodamine. The heterozygotic sample gave the mixed-dye spectrum as expected that corresponds to the presence of both dye-labeled primer sequences. The differences in the spectra can be easily differentiated by eye and the genotype of the DNA under examination confirmed without doubt.

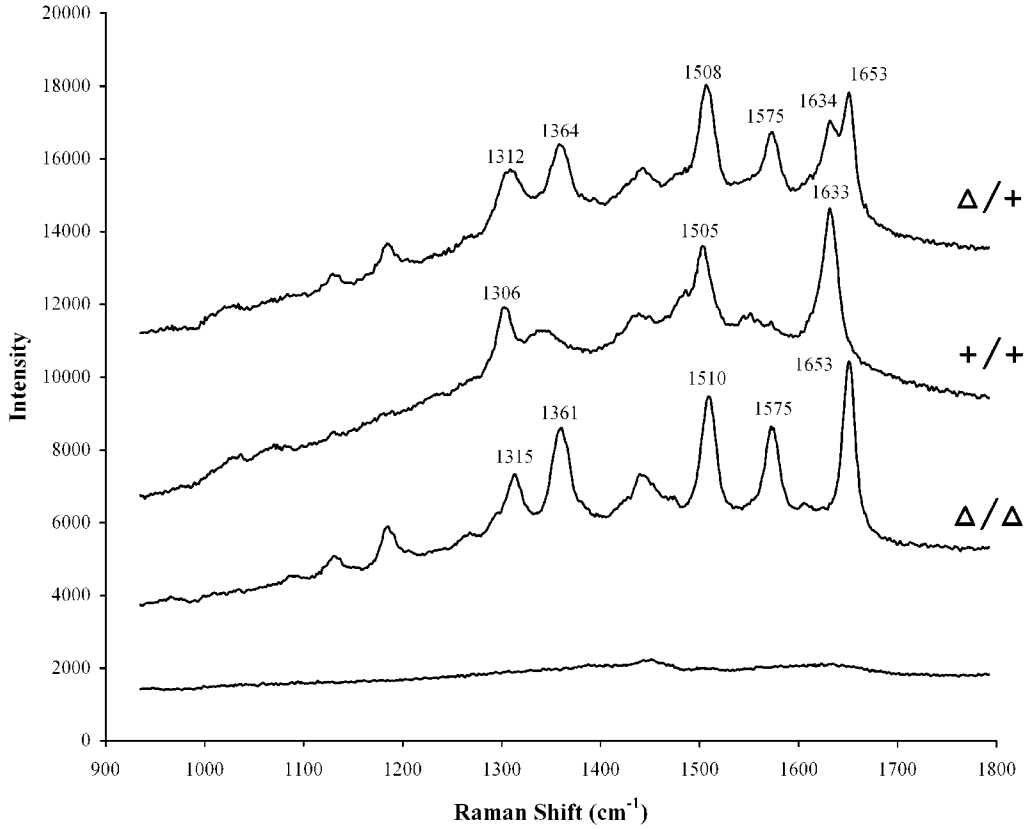


Figure 5. SERRS spectra of the three combinations of genotypes present showing the homozygotes +/+ and Δ/Δ and the heterozygote +/Δ.

In this particular case, we have relied on the well-known specificity of ARMS PCR amplification of the sample DNA. However, the major difference in our study from previous work is the ability to identify the genotype of the DNA without complicated resolution of fluorescence or use of sophisticated primers containing both fluorophores and quenchers. This has been clearly demonstrated, and we were able to identify the genotype of three different DNA samples in a multiplex arrangement without the use of several reactions.

## CONCLUSION

This is the first study of SERRS multiplex genotyping and is intended as an illustration of the scope of the technique for biological analysis. The potential for its use is considerable. There is much greater discrimination between labels than with fluorescence, many more labels can be used, and multiple excitation frequencies can be used to permit identification of a large number of labels in situ without separation. One of the most attractive features of SERRS is the ability to use nonfluorescent as well as the reported fluorescent chromophores. Discrimination between labels then becomes trivial, as almost any class of chromophore can be used as a suitable label to provide a high level of multiplex analysis and opens up a wide range of chemistries available for labeling. In conclusion, this study has shown that SERRS can differentiate between different DNA samples and is a simple and effective technique for identification of the genotype of the DNA in a multiplex system. This demonstration of the use of SERRS in multiplex genotyping shows potential advantages over fluorescence as a detection method with considerable promise for future development.

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