DNA Genotyping from Human FFPE Samples– Reliable and Reproducible

Use this optimized protocol to obtain reliable and reproducible SNP Genotyping data from those difficult human FFPE samples.

- Isolate superior quality DNA from FFPE samples using **RecoverAll™ Total Nucleic Acid Isolation Kit**
- Accurately detect percent functional DNA in samples using **TaqMan® RNase P Detection Reagents (FAM™ Dye)**
- Obtain consistent genotyping results using **TaqMan® SNP Genotyping Assays**

Abstract

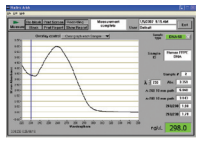
In this study, researchers at Ambion, an Applied Biosystems business, addressed the feasability of obtaining accurate & reproducible SNP genotyping results using FFPE samples (wide variety of tissues and block ages) as viable templates for TaqMan SNP assays. Our data indicates that the probability of successful PCR analysis is greatly reduced when the amount of the functional template in the FFPE DNA sample is less than 5%. The data also demonstrates that the combined use of the RecoverAll™ Total Nucleic Acid Isolation Kit and TaqMan® SNP Genotyping Assays can result in high quality, reproducible, and reliable genotyping data.

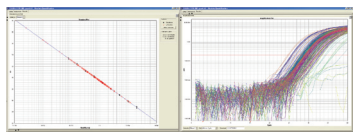
Introduction

The ability to isolate nucleic acids that are suitable for molecular analysis from archived tissue samples enables the retrospective study of diseased tissues at both the genomic and gene expression levels through the use of techniques like single nucleotide polymorphism (SNP) genotyping and Real-Time PCR. While standard methods of preservation which employ formaldehyde are ideal for maintaining tissue structure and preventing putrefaction, this type of preservation makes it difficult to perform molecular analyses on samples.

1 FFPE DNA Isolation

2 Quantification





3 Reaction Setup

4 PCR Amplification & Allelic Discrimination Plate Read

5 Analyze Data & Call Alleles

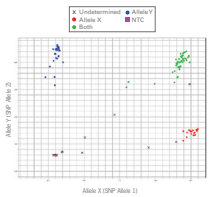


Figure 1. Human FFPE DNA Genotyping Workflow. The successful integration of Ambion and Applied Biosystems products results in reliable SNP data from a wide variety of human FFPE tissues.

Nucleic acids become trapped and modified through protein-protein and protein-nucleic acid cross-links, and other chemical modifications. Furthermore, FFPE DNA is often fragmented to a range of sizes. It can have peak fragment sizes ranging from ~180 base pairs (bp) to sizes that are slightly smaller than those observed with frozen tissue (peak fragment size ~3000 bp).

Both DNA degradation and modification result in decreased template availability that can potentially compromise a SNP genotyping experiment. While A_{260} measurements can measure DNA content, they are not adequate to determine the amount of functional DNA template in a sample. One way to circumvent these problems is to increase input DNA. The TaqMan® RNase P Detection Reagents (FAM™ Dye) provide an accurate method to quantify functional DNA template ensuring that sufficient DNA template is used. Our data shows that if the amount of DNA determined by these two methods differs by more than 20-fold (i.e. less than 5% of the DNA can serve as functional template), PCR analysis is often compromised.

Methods

DNA Isolation

The RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE was used to isolate DNA from 109 human samples from a wide range of tissue types (colon, breast, uterus, kidney, tonsil, skin, muscle, pancreas, prostate, ovary etc) and block ages (1–18 years). A typical DNA isolation requires less than 50 hours: 48 hours for protease digestion and 75 minutes hands-on processing time. Although DNA tends not to fragment as easily as RNA, it appears trapped to a greater degree by the formaldehyde tissue preservation process. Therefore, a longer (2 day) protease digestion treatment is required to release substantial amounts of DNA from these samples (See "Isolating DNA from FFPE-Treated Tissues", right, for more information about this protocol).

DNA Quantification

Both A_{260} and TaqMan® RNase P Detection Reagents (FAM™ Dye) were used to assess the amount of DNA from each FFPE DNA sample (See "Measuring Template Efficiency", page 3, for more information about this protocol). RNase P detection qPCR data (not shown) demonstrates that the RecoverAll™ Total Nucleic Acid Isolation Kit can provide a reproducible source of DNA with ± 0.5 C_t and ± 2% functional template difference between replicate isolations from any one tissue. All assays in this series of experiments were successfully performed with 1 ng (as determined by RNase P detection) of DNA input, which is considered the lower limit of sample required for TaqMan® SNP Genotyping assays. Since the SNP assay requires 1 ng in 2.25 µL, samples that contained a low percentage of functional template were concentrated in a centrifugal evaporator.

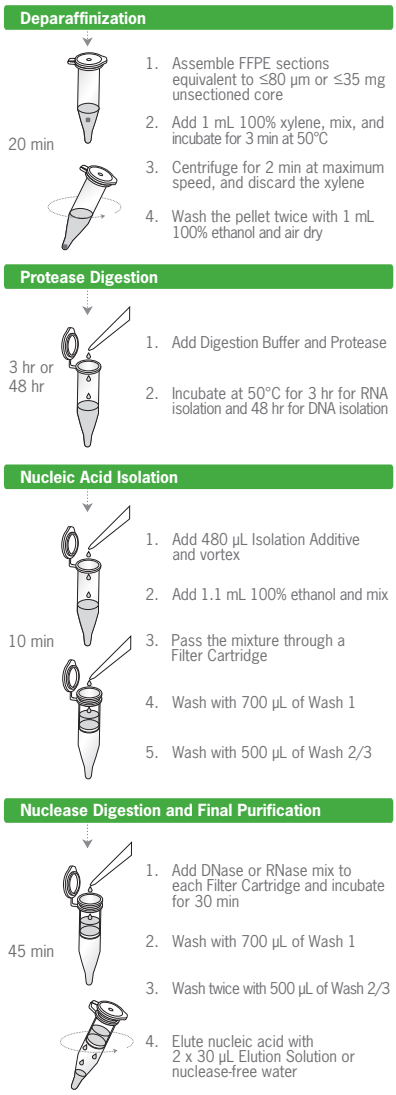
TaqMan® SNP Genotyping Assays

For each SNP assay, 5 µL reactions were performed in duplicate 384-well plates using 1 ng DNA input based on both UV absorbance (A_{260}) and RNase P assays. Amplification was carried out for 40 or 60 cycles in standard mode on a 7900 HT Fast Real-Time PCR System (Applied Biosystems). After amplification, the reactions were evaluated by the Sequence Detection Systems (SDS) 2.2.1 software (Applied Biosystems) using the post-plate read and AutoCalling (quality value threshold: 95) features.

Ten genotyping assays were selected from a preliminary forensic identification panel of 19 small nucleotide polymorphisms (SNPs) having minor allele frequencies of >10% [1], so that all 3 genotypes would be detected in the tested FFPE sample panel. The amplicon lengths of the selected assays ranged from 65–143 bp.

Isolating DNA from FFPE-Treated Tissues

While not as prevalent as in RNA degradation, the degree of fragmentation of DNA that has already occurred in FFPE tissues cannot be reversed. However, Ambion's RecoverAll™ Total Nucleic Acid Isolation Kit yields sufficient DNA for successful SNP genotyping in many FFPE samples. FFPE tissue sections are first deparaffinized using a series of xylene and ethanol washes. The sections are then subjected to a rigorous protease digestion with an incubation time tailored for recovery of RNA or DNA. The nucleic acid is purified using a rapid glass filter methodology that includes an on-filter nuclease treatment. Finally, recovered DNA is eluted into either water or the low salt buffer provided.



Overview of the RecoverAll™ Total Nucleic Acid Isolation Procedure

Measuring Template Efficiency

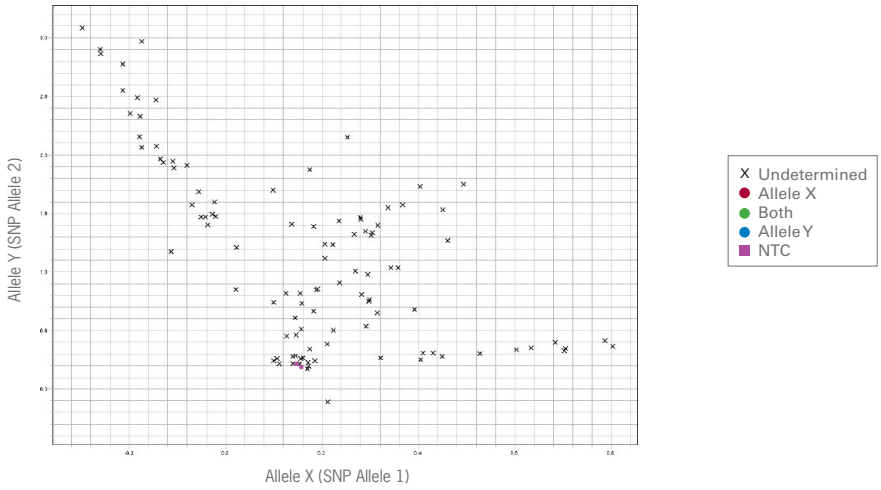
Applied Biosystems recommends quantifying the amount of genomic DNA in samples before using the TaqMan® SNP Genotyping Assays. Prepare a standard curve using the DNA template standards provided in the TaqMan® DNA Template Reagents Kit and the RNase P gene primers and probe provided in the TaqMan® RNase P Detection Reagents Kit. Prepare the PCR Mix using 2X TaqMan® Universal PCR Master Mix, No AmpErase UNG, and 20X RNase P Primer and TaqMan Probe (FAM™ Dye) mix. It is important to perform at least 3 replicates of each standard or sample and to include a no-template control. Combine 10 ng of FFPE DNA and PCR mix in either 96-Well Optical Reaction Plates or 384-Well Clear Optical Reaction Plates (Applied Biosystems). Run the plated reactions on an ABI PRISM® Sequence Detection System or Real-Time PCR System using the following thermal cycling conditions in Standard mode: hold for 10 min at 95°C, and 40 cycles with denaturation for 15 sec at 92°C and anneal/extend for 1 min at 60°C. Finally, use the Sequence Detection Systems software to generate a standard curve to quantify the amount of functional template and, subsequently, calculate the percentage of functional template in each FFPE DNA sample.

For this study, the average yield of DNA/mg tissue, as determined by A_{260} measurements, was 1.26 ± 1.47 µg. This demonstrates the wide variability of yields from FFPE tissue. 10 ng (as quantified by A_{260} measurements) of each sample was then tested with the RNase P assay. The average percentage of functional template is 10.07 ± 7.08%, indicating that A_{260} values are not a reliable gauge of functional templates for FFPE samples.

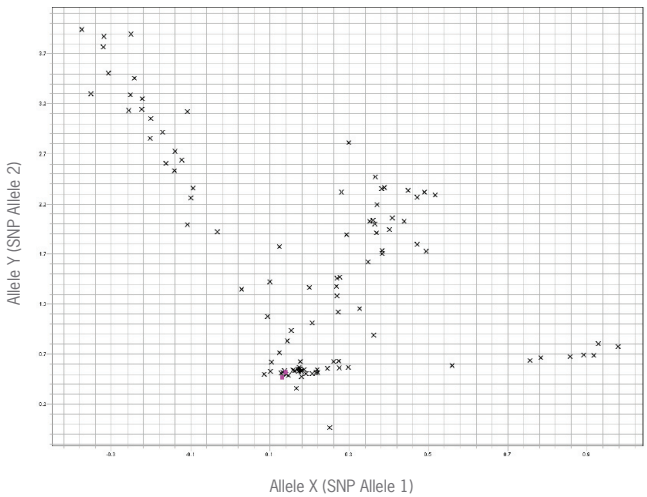
Results

Accurate Quantification of Input DNA and Sufficient Signal Amplification

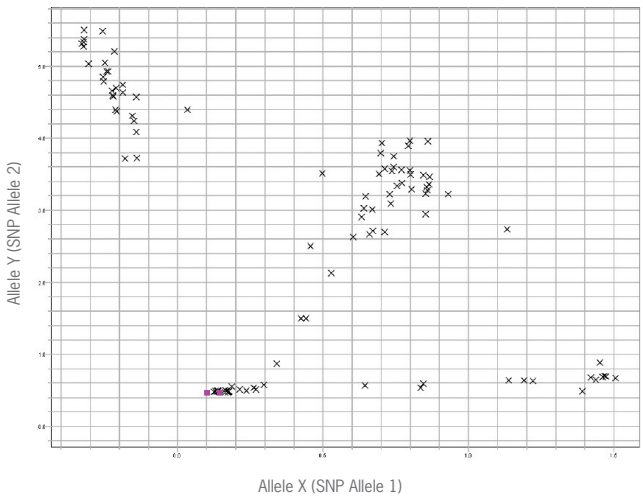
It is critical for the success of SNP genotyping that each sample contains the same concentration of functional DNA template. When comparing human FFPE DNA quantification methods (A_{260} vs. RNase P), the SNP assays based on RNase P quantification produced tighter clustering of genotype groups on allelic discrimination plots after 40 cycles of amplification (Figure 2, compare Panels A and B). Despite quantifying functional DNA using the RNase P assay, late cycle signal detection and a failure to reach the plateau phase were observed in ~50% of samples (Figure 3). Both of these observations suggest that 40 amplification cycles were insufficient and increased the risk of undetermined or inaccurate results when using the AutoCall feature of the Sequence Detection Systems (SDS) software. Since the assay results were acquired from an end-point plate read, it was essential that the signal was in plateau phase of amplification. Therefore allelic discrimination plots of samples amplified for 60 cycles were analyzed to investigate if additional cycles could improve fluorescence signal and data quality. Figure 2C demonstrates that tighter genotype clusters with reduced trailing can be obtained by using 1 ng of DNA (as determined by RNase P detection) and amplifying for at least 50 cycles. In fact, some of the DNA samples that clustered near the no template controls because of low signal can now be genotyped (Figure 2C).



Panel A. 40 cycles, A_{260}-based Sample Quantification



Panel B. 40 cycles, RNase P-based Sample Quantification



Panel C. 60 cycles, RNase P-based Sample Quantification

Figure 2. Allelic Discrimination Plots Comparing FFPE DNA Quantification Methods and Number of Amplification Cycles. 109 human FFPE DNA samples (1 ng each) were amplified for 40 cycles (Panels A, B) or 60 cycles (Panel C) and AutoCalled using Sequence Detection Systems 2.2.1 Software (Applied Biosystems). (A) Sample input based on UV absorbance (A_{260}) quantification. (B), (C) Sample quantification by RNase P detection.

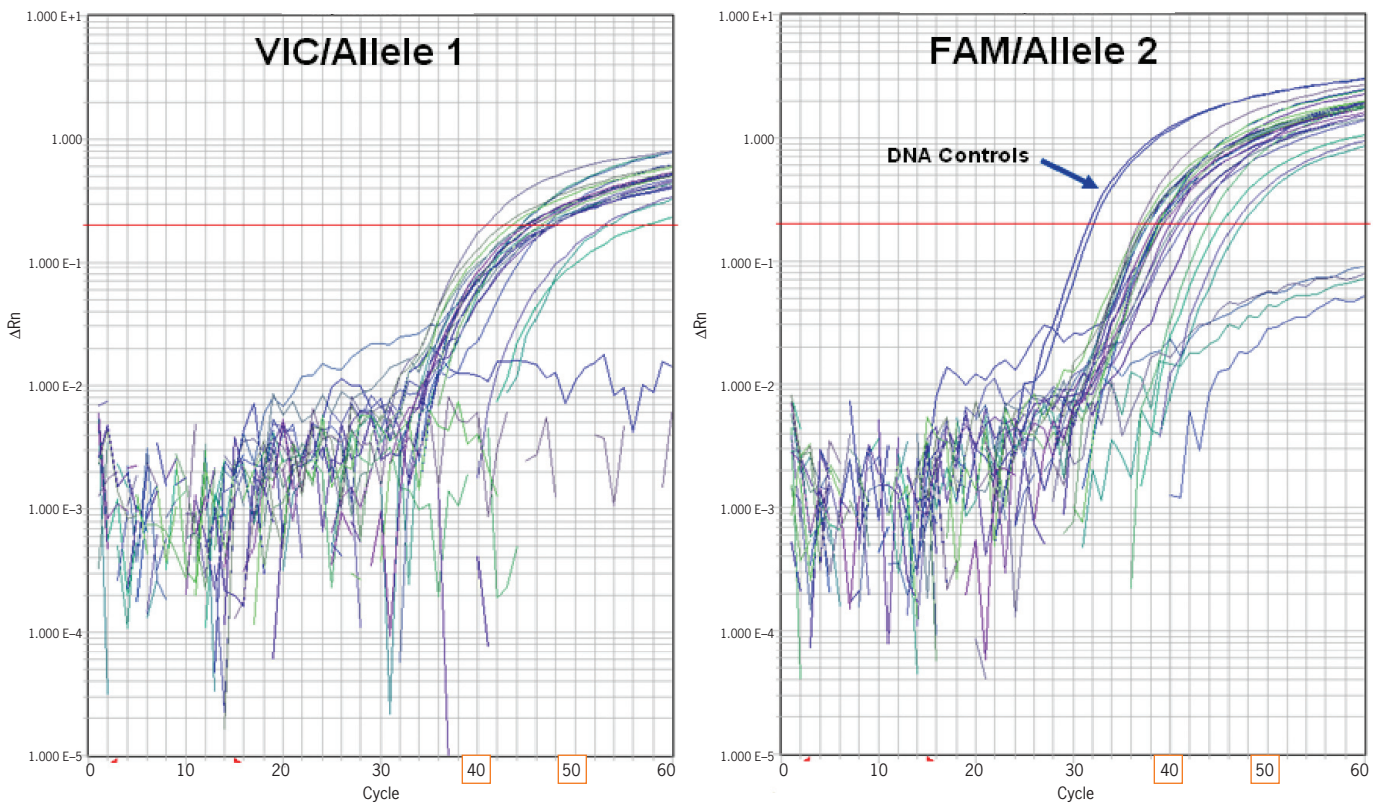


Figure 3. Amplification Plots of RNase P Quantified FFPE DNA after 60 Cycles. Using TaqMan® SNP Assay, 1 ng by RNase P detection of each FFPE DNA sample (n=100) was amplified for 60 cycles. Real Time PCR data indicates that the signal intensities of more than half the FFPE DNA samples that clustered near the no template controls after 40 cycles reached plateau after at least 50 cycles. The genotype of these samples can now be AutoCalled by the SDS Software.

The reproducibility of our recommended SNP genotyping procedure is demonstrated in Figure 4, which shows typical replicate allelic discrimination plots. The reliability of this procedure is indicated by the results for 10 different TaqMan SNP genotyping assays, which are summarized in Figure 5. There is high correlation between observed and expected genotype frequencies, where genotype frequencies are provided for samples containing at least 5% functional template. When samples containing less than 5% functional template are included in the analysis, the percent of samples that can be genotyped decreases. Taken together, these data suggest that to ensure that the AutoCall assigns a genotype to a sample, it is important to use only DNA samples that contain >5% functional template as determined by RNase P detection (Figure 6, Panel B). Otherwise, all samples should be assayed and alleles called manually if the AutoCall feature is unable do so.

Recommendation for TaqMan® SNP Genotyping of Human FFPE DNA

Our data indicates that one can successfully obtain high quality, reproducible, and reliable SNP genotyping data by following these steps:

- Isolate human DNA from FFPE tissue using Ambion's RecoverAll™ Total Nucleic Acid Isolation Kit.
- Perform DNA quantification using A_{260} measurements with Applied Biosystems TaqMan® RNase P Detection Reagents (FAM™ Dye) to determine the percentage of functional template in a DNA sample.
- Amplify 1 ng of functional DNA template (as determined by RNase P detection) for at least 50 cycles on either Applied Biosystems 7900HT PCR System or 9700 GeneAmp® PCR System.
- Collect data by post-plate read on the 7900HT Real-Time PCR System.
- Use the AutoCall feature of the SDS software to accurately and reproducibly call alleles.

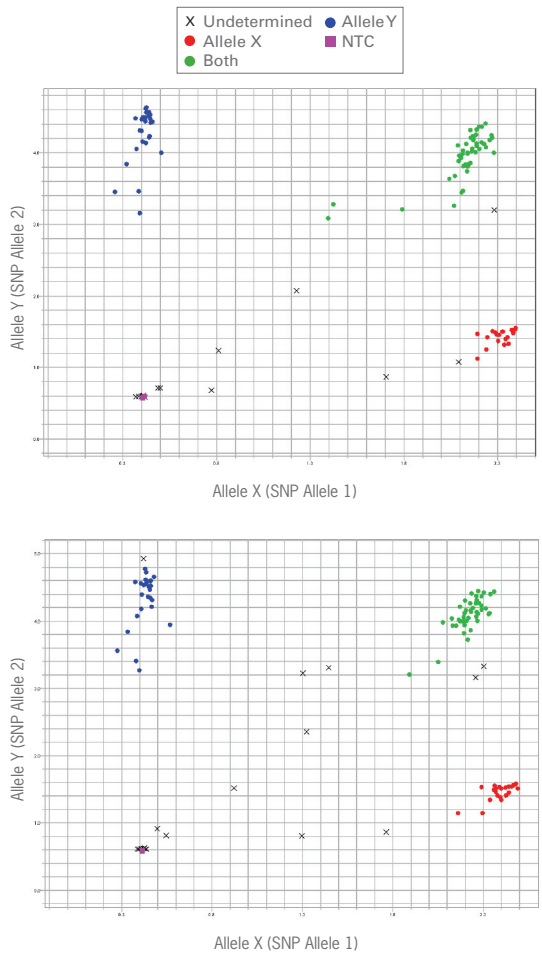


Figure 4. Achieve Highly Reproducible and Reliable Data from TaqMan® SNP Genotyping Assay. 1 ng, as quantified by RNase P detection, of each human FFPE DNA sample (n=105) was amplified in replicate for 60 cycles followed by AutoCalling using SDS 2.2.1 Software. Gene Symbol: GABRA2, Assay ID: C___8263011_10

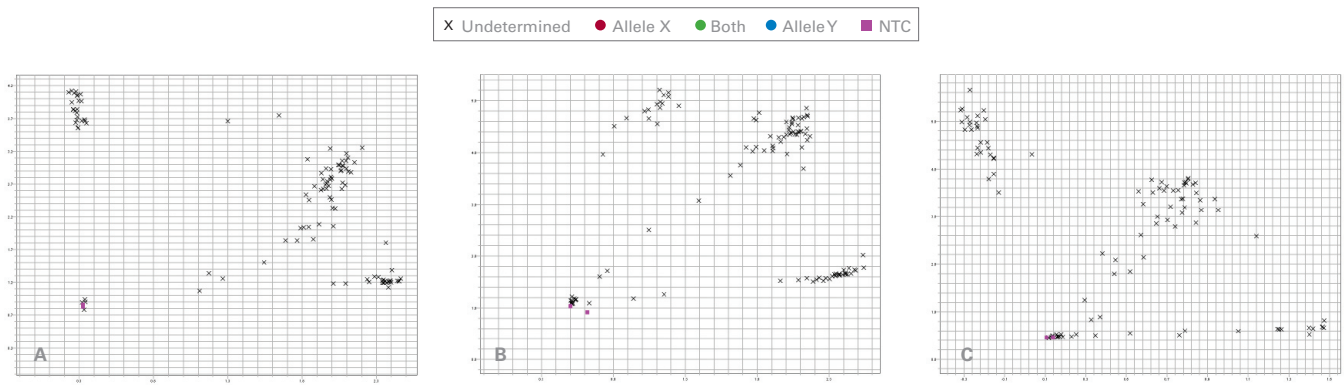
References

1. Kidd K, Andrew J. Pakstis, William C. Speed, Elena L. Grigorenko, Sylvester L.B. Kajuna, Nganyirwa J. Karoma, Selemani Kungulilo, Jong-Jin Kim, Ru-Band Lu Adekunle Odunsi, Friday Okonofua, Josef Parnas, Leslie O. Schulz, Olga V. Zhukova and Judith R (2006) Developing a SNP panel for forensic identification of individuals, *Forensic Sci Int* **164(1):**20–32.

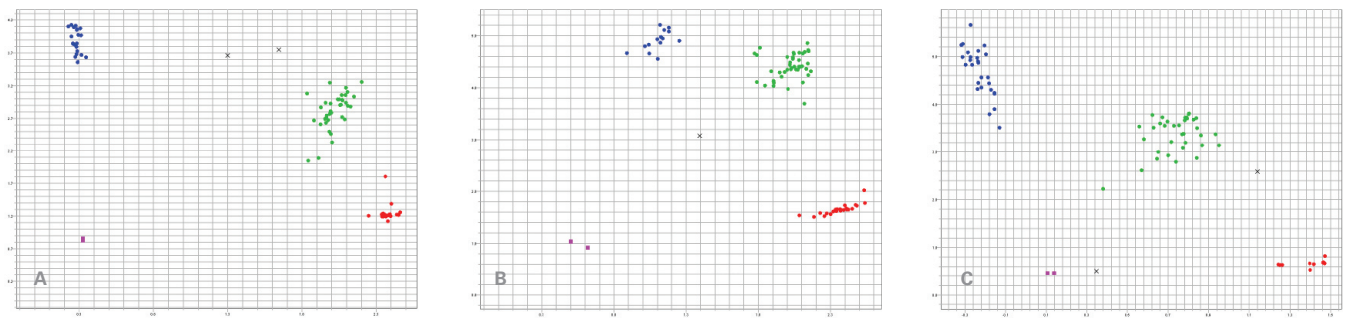
				Observed Genotype Frequencies				Expected Genotype Frequencies				
AB Assay ID	Amplicon Size (bp)	Assay Gene Symbol	dbSNP rs#	A1/A1	A2/A2	A1/A2	Total Samples	A1/A1	A2/A2	A1/A2	% Genotyped	% Genotyped*
C___1256256_1_	143	SASH1	rs2272998	11	27	35	73	11.1	27.1	34.7	99	81
C___8263011_10	132	GABRA2	rs279844	18	22	41	81	18.3	22.3	40.4	99	89
C___2515223_10	114	SYNE1	rs214955	21	18	43	82	22.0	19.0	40.9	99	92
C____411273_10	105	THSD2	rs2503107	24	14	43	81	25.6	15.6	39.9	100	87
C___1880371_10	104	RCHY1	rs13134862	13	31	37	81	12.3	30.3	38.5	100	97
C___7538108_10	81	SORBS1	rs1410059	21	22	37	80	19.5	20.5	40.0	98	92
C___9371416_10	72	HIVEP1	rs132184400	9	37	36	82	8.9	36.9	36.2	100	99
C___7459903_10	70	B4GALT6	rs985492	26	17	39	82	25.2	16.2	40.5	100	98
C___3254784_10	67	HSPA12A	rs740598	27	15	37	79	26.2	14.2	38.6	98	97
C___1619935_1_	65	PHGDHL1	rs1058083	13	29	39	81	13.0	29.0	38.9	99	96

* % Genotyped including samples with ≤ 5 % functional template

Figure 5. Results Summary of TaqMan® SNP Genotyping Assays. Data was tabulated after removing all FFPE DNA samples with a ≤ 5% functional template. The "Expected Genotype Frequencies" (columns 9–11) are based on the gene frequencies of each polymorphism in the sample population. The observed genotype frequencies (columns 5–7) fit the expected genotype frequencies within a 95% confidence interval, using an appropriate chi-square test. Finally, the percent of samples genotyped is shown both before and after removal of the low quality samples (columns 12 and 13).



Set 1. Data Based on All FFPE DNA Samples



Set 2. Data Based on Removal of FFPE DNA Samples Containing ≤ 5% Functional Template

Figure 6. Allelic Discrimination Plots from TaqMan® SNP Genotyping Assays Before and After Removing FFPE DNA Sample with ≤ 5% Functional Template. To graphically illustrate the utility of removing samples that are damaged beyond the 1-in-20 threshold (i.e. 5% or less of the DNA can serve as template), the AutoCall function of SDS 2.2.1 Software was applied to the same data with all samples and after the removal of those below the 5% level. In the first set, no calls are made, while in the second, allele calling works as expected. To generate this data, 1 ng, as quantified by RNase P detection, of each human FFPE DNA sample (n=100+) was amplified for 60 cycles. (A) Gene Symbol: SORBS1, Assay ID: C___7538108_10. (B) Gene Symbol: THSD2, Assay ID: C____411273_10. (C) Gene Symbol: SASH1, Assay ID: C___1256256_1_.

Human FFPE DNA Genotyping Workflow

1 DNA Isolation from FFPE	2 Quantification	3 Reaction Setup	4 PCR Amplification & Allelic Discrimination Plate Read	5 Analyze Data & Call Alleles
PRODUCTS	**PRODUCTS**	**PRODUCTS**	**PRODUCTS**	**PRODUCTS**
• RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE	• TaqMan® RNase P Detection Reagents (FAM™) • TaqMan® DNA Template Reagents	• TaqMan® SNP Genotyping Assays & TaqMan® Universal PCR Master Mix, No AmpErase® UNG	• ABI PRISM® 7900HT Sequence Detection System • Alternatively, perform PCR with the GeneAmp® PCR System 9700 followed by a post-PCR plate read on the 7900HT SDS instrument	• Sequence Detection Systems Software

Green: Ambion kits and reagents.
Blue: Applied Biosystems reagents and instruments.

For more information on these products, contact us or visit www.ambion.com (green listings) and www.appliedbiosystems.com (blue listings).

ORDERING INFORMATION

Description	Quantity	Part Number
Nucleic Isolation		
RecoverAll™ Total Nucleic Acid Isolation Kit	1 Kit	AM1975
Real-Time PCR		
TaqMan® SNP Genotyping Assays (Validated/Coding)	750 rxns	4331183
TaqMan® RNase P Detection Reagents (FAM™)	100 rxns	4316831
TaqMan® DNA Template Reagents	1 Kit	401970
TaqMan® Universal PCR Master Mix (No AmpErase UNG)	1 Kit	4324018
96-Well Optical Reaction Plates	20 Plates	4306737
384-Well Clear Optical Reaction Plates	50 Plates	4309849
Optical Adhesive Covers	100 covers	4311971
7900HT Real-Time PCR System	1 Unit	4329001
9700 GeneAmp® PCR System	1 Unit	N8050200
NanoDrop® Spectrophotometer	1 Unit	ND-1000A

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