**Table 1.** PCR primer design and specificity test for genotyping assay. Four extracts, H23 (CBG Hemp), H25 (CBD Hemp), H35 (CBD Hemp), and NIST 20 (Marijuana), were used to assess the primer specificity. 'P' indicates the presence of the gene, and 'N' indicates the absence of the gene. Red bold font indicates discrepancies between observed and expected results

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Primer Set | Primer Sequences (5’ – 3’) | Annealing  Temp (⁰C) | Tested Extract | | | | |
| Sample | H23 | H25 | H45 | NIST20 |
| THCAS-1064 | F-TTCAATTTTTCATGGTGGAG | 58 | Expected | P | N | N | P |
| R-CATTGCAGTTTCTGGAATT | Observed | P | N | N | P |
| THCAS-366 | F-CCAAGCAACTATTTTATGCTCTA | 58 | Expected | P | N | N | P |
| R-CATCTATTTTGATCGAATGC | Observed | P | N | N | P |
| CBDAS-586 | F-CTTAGTTTGGCKGCTGGGTAT | 58 | Expected | N | P | P | P |
| R-TAGCACTTTTCCATGAACGT | Observed | N | P | P | P |
| THCAS-Like 1 | F1-TTGGGATGTATCTTTTGAT | 58 | Expected | P | N | N | P |
| R1-CCAATTTATATGCTTTTGATTATC | Observed | P | **P** | **P** | P |
| THCAS-Like 2 | F2-AATGTCTCCCACATCCAAG | 63.5 | Expected | P | N | N | P |
| R2-AGCTTCAACCCATGCAGT | Observed | P | **P** | **P** | P |
| THCAS-Like 3 | F3-GTTTACTTCCYGATCAATGA | 53.2 | Expected | P | N | N | P |
| R3-GACATCAACCAGTCTGATT | Observed | P | **P** | **P** | P |
| THCAS-Like 4 | THCAS F4-ATATCCAAGGCACTATTCTATGCT\* | 58 | Expected | P | N | N | P |
| R2-AGCTTCAACCCATGCAGT | Observed | P | N | N | P |
| THCAS-Like 5 | CBDAS+Like F3-ATATCCAAGGCACTATTCTATGCT\* | 58 | Expected | P | N | N | P |
| R3-GACATCAACCAGTCTGATT | Observed | P | N | N | P |

\*Sequences obtained from previous study (see Chapter VI)

**Table 2.** PCR and SBE primers used in SNaPshotTM assay

|  |  |  |  |
| --- | --- | --- | --- |
| Primer | Primer sequence | Primer length (bp) | Final conc. (µM) |
| THCAS-1064&1179 | F-TTCAATTTTTCATGGTGGAG | 20 | 0.05 |
| R-CACATACATCCCAGCTCCTACAT | 23 |
| CBDAS-  586 | F-CTTAGTTTGGCKGCTGGGTAT | 21 | 0.05 |
| R-TAGCACTTTTCCATGAACGT | 20 |
| THCAS-  Like 5 | CBDAS-Like F-ATATCCAAGGCACTATTCTATGCT | 24 | 0.6 |
| R2-GACATCAACCAGTCTGATT | 19 |
| SBE 1064R | acgtcgtgaaagtctgacaaACGTGTTAAAATTTACAACACCA | 43 | 0.3 |
| SBE 1179F | ACTATGTTAAGAAACCAATTCC | 22 | 0.05 |
| SBE 586R | GCCGCGAGGCCATAG | 15 | 0.05 |
| SBE 486R | gaaagtctgacaaGCCATAATTTCGTATCAATG | 33 | 0.4 |

**Table 3.** Interpretation of the SNaPshotTM™ genotypes.Dark gray box indicates the SNP of that marker is not important in that genotype. CBDA marker in genotype 3 can provide CBD information of the marijuana sample. Genotype 5 needs peak height ratio (PHR) to determine if the stain is low or high CBD

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Genotype** | **THCA1064** | **Pseudo486** | **THCA1179** | **CBDA586** | **Identification** |
| **1** | A |  |  |  | Hemp Seed, Cannabis THC%<1% (n=43) |
| **2** | Absent | Usually T |  |  |
| **3** | G | G |  | CBD info. | Marijuana (n=62) |
| **4** | G | G | A/T | G | Hemp Seed (n=1) |
| **5** | G | G/T |  |  | \*PHR (1064G/486G) (n=13) |
| G/A | G/T |  |  |
| G/A | G |  |  |

\*PHR (Peak High Ratio of 1064G/486G) >1= Marijuana; PHR<1= Cannabis THC%<1%

**Table 4.** PCR and Taqman® probes design for TaqManTM real-time PCR SNP genotyping assay

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Primer | Primer sequence | Quencher-reporter | Length (bp) | Tm  (⁰C) | Final conc. (µM) |
| THCAS-1064 | F-TTCAATTTTTCATGGTGGAG | N/A | 20 | 60.5 | 1.8 |
| R-CATTGCAGTTTCTGGAATT | N/A | 19 | 58.3 | 1.8 |
| THCAS-Like 5 | F-ATATCCAAGGCACTATTCTATGCT | N/A | 24 | 60.1 | 0.9 |
| R-GACATCAACCAGTCTGATT | N/A | 19 | 55.3 | 0.9 |
| THCA1064-Probe 1 | GATACAACCATCTTCTACA(G)TGGTGTTG | QSY-ABY | 28 | 66.5 | 0.4 |
| THCA1064-Probe 2 | GATACAACCATCTTCTACA(A)TGGTGTTG | QSY-JUN | 28 | 66.3 | 0.4 |
| Pseudo486-  Probe 1 | GTGGAGGAGGCTATGGA(T)CATT | QSY-FAM | 22 | 65.5 | 0.2 |
| Pseudo486-  Probe 2 | GTGGAGGAGGCTATGGA(G)CATT. | QSY-VIC | 22 | 67.7 | 0.05 |

**Table 5.** Interpretation of the TaqManTM real-time PCR SNP genotyping assay.Dark gray box indicates the SNP of that marker is not important in that genotype

|  |  |  |  |
| --- | --- | --- | --- |
| **Genotype** | **THCA1064** | **Pseudo486** | **Identification** |
| **1** | A/A |  | Cannabis THC%<1% (n=9) |
| **2** | G/A |  | Cannabis THC%<1% (n=2) |
| **3** | G/G |  | Marijuana (n=52), or Hemp Seed (n=3) |
| **4** | Undetermined | T/T | Cannabis THC%<1% (n=36) |
| **5** | Undetermined | T/G | Cannabis THC%<1% (n=1) |
| **6** | Undetermined | Undetermined | Cannabis THC%<1% (n=17\*),  or non-cannabis sample (n=1) |
| **7** | Undetermined | G/G | Marijuana (n=10), or Hemp Seed (n=1) |

\*Include one misclassification bases on the ∆9-THC concentration

**Table 6.** Comparison of SNaPshotTM™ assay and TaqManTM real-time PCR assay

|  |  |  |
| --- | --- | --- |
| **Genotyping assay** | **SNaPshot™** | **Taqman™** |
| Targets | 4 | 2 |
| Instrumentation | PCR, CE | qPCR |
| Time | 3.5-4 hr/96 samples | ~1.5 hr/96 samples |
| Differentiate hemp flower and leaves from marijuana | **Yes** | **Yes** |
| Differentiate marijuana THC>1% from hemp | **Yes** | **Yes** |
| Differentiate hemp seed from marijuana | **Yes** | No |
| Differentiate marijuana THC<1% from hemp | No | No |
| Differentiate non-cannabis plant from cannabis | **Yes** | No |