Development and validation of an Oligonucleotide Probe Hybridization Assay to Subtype Human Immunodeficiency Virus Type 1 (HIV-1) Circulating Recombinant Form CRF02_AG

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Abstract

Objective: To develop and validate an oligonucleotide probe hybridization assay for Human Immunodeficiency Virus Type 1 (HIV-1) Circulating Recombinant Form (CRF) CRF02_AG.

Design: In the p17 coding region of the *gag* gene a CRF02_AG specific signature pattern was observed. Five working probes were designed to discriminate CRF02_AG infections from infections by all other documented subtypes and CRFs in an ELISA format oligonucleotide probe hybridization assay.

Materials and Methods: Nucleic acids were extracted from a panel of HIV-1-positive plasma samples from Cameroon, Bénin, Côte d’Ivoire, Kenya, Zambia, and Belgium, and blood spots from The Gambia. CRF02_AG (n=147) and non-CRF02 (n=100) samples were analyzed to evaluate and validate the oligonucleotide probe hybridization assay.

Results: The CRF02_AG specific oligonucleotide probe hybridization assay has a high sensitivity and specificity implying good Positive and Negative Predictive Values in regions of high and low prevalence. A validation of the assay on West and West Central African samples indicated a sensitivity of 98.4% and a specificity of 96.7%.

Conclusion: The probe assay as a diagnostic tool will allow rapid screening for CRF02_AG. This could be used in tracking the HIV epidemic in terms of documenting the real prevalence of the CRF02_AG strains, and will complement efforts in vaccine development. Moreover, this technology can easily be applied in laboratories in developing countries.
INTRODUCTION

Genetic variation is the hallmark of retroviruses and is also apparent in Human Immunodeficiency Virus (HIV-1). HIV-1 displays important genetic variability, which is driven by a high error rate of the reverse transcriptase, the presence of viral RNA as a dimer allowing for recombination to occur, the high turnover rate of HIV-1 in vivo, and selective immune responses.

By genetic analyses, HIV strains collected from around the world have been shown to have substantial diversity. Representatives of different 'pure' (non-recombinant) subtypes A, B, C, D, F, G, H, J, and K, and 15 Circulating Recombinant Forms (CRF) CRF01 to CRF15 were proposed based on near full-length genome analysis (http://hiv-web.lanl.gov/). The epidemiology of HIV-1 subtypes and CRFs is characterized by their differential distribution and varying significance as a driving cause of the pandemic on regional and global basis. The largest proportion of HIV-1 infections in the year 2000 was due to subtype C strains (47.2%) followed by subtype A and CRF02_AG (27%) and subtype B strains (12.3%)4. Discrimination between subtype A and CRFs comprising fragments of subtype A is hampered by relatively small genetic distances between the parental subtype A and the respective subtype A fragments in CRFs. This often results in low confidence classification when sequences of sub-optimal length are phylogenetically (re)analyzed. In addition, it has not been possible to discriminate subtype A from CRF02_AG by env Heteroduplex Mobility Assay (HMA), and alternate experimental conditions are needed to discriminate between subtype A, CRF01_AE and CRF02_AG, by gag HMA5.

The prototype strain of CRF02_AG, HIV-1 IbNG, was initially reported as a new subtype A isolate from Ibadan, Nigeria6. Analysis of the full-length sequence revealed that HIV-1 IbNG was an A/G recombinant7. Recent and retrospective molecular epidemiology studies indicated that in West and West Central Africa CRF02_AG infections represent 50% – 70% of the circulating strains8-16. In contrast to the high CRF02_AG prevalence in West Africa and West Central Africa countries Cameroon, Gabon and Equatorial Guinea, CRF02_AG infections are scarce in the Republic of Congo17, the Democratic Republic of Congo18, and Eastern and Southern African countries19. Outside Africa, CRF02_AG has been introduced in Europe20-24 and to a minor extend in the US25.

To date, there have been few systematic large-scale attempts to characterize HIV isolates emerging and especially the CRFs from different parts of the world. As such our knowledge about the distribution of HIV
strains in different populations and about changes in that distribution over time, is rather limited. A major
demand in the design and evaluation of efficacious subtype dependent candidate HIV-1 vaccines, is the
development of techniques for large-scale HIV genetic characterization for documenting the true
prevalence rates of HIV subtypes and CRFs in developing countries.

Here we describe the design and potential use of a CRF02_AG specific oligonucleotide probe
hybridization assay for large scale monitoring of the prevalence of CRF02_AG variants.

MATERIALS AND METHODS

Samples
A reference panel of 25 plasmids, containing the complete gag gene of HIV-1 strains belonging to group
M subtypes A-H, CRF01_AE and CRF02_AG, was available. An evaluation panel of plasma samples
(Bénin, n=59; Cameroon, n=53; Kenya, n=50; Zambia, n=10; Belgium, n=80) were selected based on
subtype information obtained in previous studies. A validation panel of plasma samples (Côte
d’Ivoire, n=30; Cameroon, n=60) and dried blood spot samples (The Gambia, n=10) were obtained from
HIV-1 positive individuals of which samples were taken in 2000-2001.

RNA Extractions and RT PCR
RNA extractions were performed as previously described. RT-PCR (Access RT-PCR, Promega, Leiden,
The Netherlands) was performed according to the manufacturer’s recommendations (10 pmol of each
primer, 10 mM dNTP mix, 25 mM MgSO4). The primers and primer position, according to the HIV-1 HXB2
numbering Engine (HIV Sequence Database, http://hiv-web.lanl.gov/), were H1GHMA101 (5’-
TAGTATGGGCAAGCAGGGAG-3’ [HXB2 position 890-909]) and H1Gag1844 (5’-
ACAGCATGCTGTCATCATTTCTTCTAGTG-3’ [HXB2 position 1814-1843]), and the cycle protocol was 45
sec 48°C (cDNA reaction), followed by 2 min at 94°C and 40 cycles for 30, 30, and 90 s at, respectively,
94, 50, and 68°C; and 1 cycle for 7 min at 68°C. Amplified DNA (1 µl) was subjected to a second round
PCR using primers PGF1 [5’-ATAGAKRTAAAAAGACACCAARGAAGC-3’ (HXB2 position 1063-1088)] and
BHGHMA625 [5’-B-CATTCTGCAGCTTCTCATTGAT-3’ (HXB2 position 1402-1424; biotin labeled)].
Cycling conditions were 2 min at 94 °C and 35 cycles of 30, 30, and 60 s at, respectively 94, 52, and 72 °C; and 1 cycle for 7 min at 72 °C. Nested PCR was carried out in a 50µl reaction mixture containing 10pmol of each primer, 20 mM dNTP mix, 25 mM MgSO₄.

Oligonucleotide probe hybridization assay procedure

Streptavidin Coated Microtitre plate (MTP) binding and detection: The oligonucleotide probe hybridization assay was performed on streptavidin coated 96 well MTP. All wash steps were done in a volume of 230µl, and were incubated for 2 min at room temperature (rt), unless mentioned otherwise. All incubations at 37°C were done in an air incubator. All incubations at 65°C were done on a Multi-Blok Heater 2004-ICE (Lab-Line Instruments, Melrose Park, Il).

Tris buffer based binding protocol: The MTP was equilibrated by washing three times with Tris Wash Buffer (100 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween-20). One hundred µl of Tris Binding Buffer (500 mM NaCl; 10 mM Tris-HCl pH 7.5; 1mM EDTA) was added to each well. For each sample, 2 µl of PCR product (~100 - 250ng) was added per well and per probe (combination) to be tested. Allowing for appropriate controls (see below), a maximum of 21 samples could be loaded per MTP. The MTP was incubated for 30 min at 37 °C, followed by 15 min at rt. The MTP was washed twice with Tris Wash Buffer.

Denaturation and hybridization: Bound PCR product was denatured by adding 230 µl 0.15M NaOH and incubation at rt for 10 min. Washes with 0.15M NaOH were repeated 3 times with respective incubation times of 5 min, 2 min, and 2 min. The MTP was then washed 3 times with Tris Neutralization Buffer (100mM Tris-HCl pH 7.5), followed by washing once with Hybridization Buffer (0.6 M NaCl; 20mM Na₂PO₄ pH 7; 1 mM EDTA; 1 x Denhardt's solution [1% Ficoll 400 (F-4375; Sigma-Aldrich, Missouri, USA); 1% polyvinylpyrrolidone 360 (PVP-360; Sigma-Aldrich); 1% BSA (B-4287; Sigma-Aldrich)]. Three µl FITC-labeled probes (1 pmol / µl) were added to 100 µl Hybridization buffer in each well. The MTP was incubated for 2 hr at 65°C on the Multi-Blok Heater. The hybridization/probe solution was immediately discarded after hybridization.
**Stringent washes:** The MTP was washed twice for 5 min with 6XSSC/0.1% SDS buffer at 37 °C in an air incubator, then washed twice for 30 min at 65°C with 1.8XSSC. The wash buffer was immediately discarded after each step.

**Tris buffer based detection:** The MTP was blocked with Antibody Incubation Buffer (100 mM Tris-HCl pH 7.5; 150 mM NaCl; 1% (w/v) blocking reagent (1096176; Roche Diagnostics Belgium) for 10 min at rt. The Antibody Incubation Buffer was then discarded. One hundred µl of diluted antibody (1/2500, Anti-fluorescein-AP [1426346; Roche Diagnostics Belgium]) was transferred in each well. The MTP was incubated for 45 min at 37 °C, followed by 15 min at rt. The MTP was washed 4 times with Tris Wash Buffer. The wash buffer was discarded. (Optionally the Tris Wash Buffer may not be discarded at the last wash, and the MTP can be stored at 4°C overnight). The “hybridized oligonucleotide – antibody complex” bound to MTP, was incubated with 190 µl of the colorimetric substrate Para-nitrophenylphosphate (pNPP) (i.e. one Tris-buffer tablet (T-8790; Sigma) and one pNPP tablet (N-2770; Sigma) dissolved at rt in 20 ml sterile water) in each well. pNPP is hydrolyzed to p-nitrophenol upon addition to the complex, p-nitrophenol is yellow in color and can be detected at 405 nm. At 405 nm both absorbance and scattered light is measured, at 650 nm only scattered light is measured, the latter is subtracted from the former to eliminate error due to scattering. Absorbance readings were measured immediately using an ELISA reader, whereby the kinetics are followed every 5’ over a period of 2 hr. The Kincalc program automatically calculates the OD of each sample’s reaction to the different probe(s).

**Controls on each MTP:** As a positive and negative control for probe reactivity, the probe target fragment of CRF02_AG and non-CRF02 samples, respectively, were processed as indicated above. For each sample reactivity of the PCR product with FITC-labeled PCR primer F-PGF1 was monitored as an indication of quality and quantity of PCR product. Wells, where respectively probe and no PCR product; only PCR product and no probe; no PCR product and no probe were added, scored negative. As a control of binding of PCR product to the MTP, FITC labeled PCR product and no probe were added and NaOH treatment was omitted, which resulted in high signal. As a control of denaturation, FITC labeled PCR product and no probe was added, which resulted in no signal.
Data analysis

Cutoff values (CO) were determined from the 5th percentile of the OD distribution of true positives. Sensitivity = true positives / (true positives + false negatives), with true positives being CRF02_AG samples that reacted positively with one or more of the probes (OD>CO). Specificity = true negatives / (true negatives + false positives), with true negatives being non-CRF02_AG samples that do not react with any of the probes (OD<CO). The Positive Predictive Value = true positives / (true positives + false positives) and indicates the likelihood that a positive test result actually means that a CRF02_AG infection is identified. The Negative Predictive Value = true negatives / (true negatives + false negatives) and indicates the likelihood that a negative test result actually means that a non-CRF02_AG infection is identified.

RESULTS

Identification of a CRF02_AG specific probe target region

Based on sequences representative for all HIV-1 group M subtypes and CRFs, available from the Los Alamos National Laboratory HIV sequence database (HIV Sequence Database, http://hiv-web.lanl.gov/), a near-full length genome alignment was generated and screened for CRF02_AG specific signature patterns. A candidate probe target was identified in the HIV-1 gag p17 coding region. Twenty-one out of 2330 sequences in the 1999 HIV-1 database (http://hiv-web.lanl.gov/) harboring the gag p17 probe target were described as CRF02_AG. Of these, 19/21 and 2/21 respectively matched two different types of the probe target (PAg17α and PAg17β) representative of two distinguished CRF02_AG sub-clades. Primers were designed to amplify a 360 base pair (bp) probe target region by nested PCR. Five working probes (position according to HIV-1 HXB2 numbering Engine (http://hiv-web.lanl.gov/)):

PAg17α1 (5’-CAGGAAGCAGCAGCCAAAATTACCC-3’), PAg17α2 (5’-CAGGAAGCAGCAGCCAAAATTACCC-3’), PAg17α3 (5’-CAGGAAGCAGCAGCCAAAATTACCC-3’) and PAg17α4 (5’-CAGGAAGCAGCAGCCAAAATTACCC-3’) [HXB2 position 1161-1187], and PAg17β (5’-GCACAGGCTGCAGCCAAAATTACCC-3’) [HXB2 position 1163-1187]) were defined.
Optimization of the oligonucleotide probe hybridization assay with reference plasmids

For all samples of the reference panel a positive PCR product was obtained. Using all five probes and the experimental conditions above, all CRF02_AG reference plasmids scored positive, all non-CRF02_AG reference plasmids scored negative. To maximize the number of samples that could be analyzed per plate probe combinations $\text{PAg17}\alpha_1+2$ and $\text{PAg17}\alpha_3+4$ were applied, which was evaluated equally sensitive as using the probes separately (data not shown).

Evaluation of the CRF02_AG specific oligonucleotide probe hybridization assay

Plasma samples were selected from countries with high CRF02_AG prevalence (Bénin, Cameroon) and countries with low CRF02_AG prevalence (Belgium, Kenya, Zambia). The efficiency of PCR amplification for each cohort was as follows: Cameroon (98%; 52/53), Zambia (90%; 9/10), Benin (89%; 51/57), Belgium (85%; 68/80), and Kenya (36%; 18/50). For evaluation of the CRF02_AG oligonucleotide probe hybridization assay a panel of PCR positive CRF02_AG (n=85) and non-CRF02_AG (n=71; 25 subtype A, 8 Subtype B, 9 subtype C, 11 subtype D, 2 Subtype F, 9 Subtype G, 4 Subtype H, 1 CRF01_AE and 2 CRF06_cpx) samples were used (Table 1). As gold standard, phylogenetic classification of the $\text{gag}$ probe target fragment was used\textsuperscript{28}. Comparison of the phylogenetic classification of the panel samples with the probe assay results (OD values) allowed calculations of mean, median, 5th and 95th percentiles. From this we determined cut off values for positivity and negativity. A sample was considered as CRF02_AG when it reacted with an OD>15.0 with either probe or probe combination. A sample was considered as non-CRF02_AG when all probes and probe combinations reacted with an OD<15.0. The OD distribution for the evaluation panel samples is depicted in Fig. 2. The overall sensitivity of the oligonucleotide probe hybridization assay evaluation panel was 97.6% (83/85). A 100% specificity result is documented from the fact that none of the 71 non-CRF02 (Subtype A – H, CRF01_AE and CRF06_cpx) reacted with the CRF02_AG specific probes. Overall, 63.5% (54/85) of the CRF02_AG samples reacted with both $\text{PAg17}\alpha$ probe combinations; 12.9% (11/85) only reacted with probe combination $\text{PAg17}\alpha_3+4$; 1.2% only reacted with probe combination $\text{PAg17}\alpha_1+2$. For West African countries, probe reactivity was only observed with probe combinations $\text{PAg17}\alpha_1+2$ and/or $\text{PAg17}\alpha_3+4$. In Cameroon, 61.7% (29/47) of the CRF02_AG samples reacted with $\text{PAg17}\alpha$ probes, 27.6% (13/47) of samples reacted with probe $\text{PAg17}\beta$. For 3
Cameroonian CRF02_AG samples reactivity to both probe PAg17α combinations and PAg17β was observed. Two Cameroonian CRF02_AG samples did not react with any of the probes.

Validation of the CRF02_AG specific oligonucleotide probe hybridization assay

Samples from Côte d'Ivoire (n=30), Cameroon (n=60), and The Gambia (n=10) had been sent under code for validation of the oligonucleotide probe assay. The efficiency of PCR amplification for each cohort was as follows: Côte d'Ivoire (90%; 27/30), Cameroon (95%; 57/60), and The Gambia (80%; 8/10). Using the oligonucleotide probe hybridization assay, CRF02_AG predictions for the validation panel of the different cohorts were as follows: Côte d'Ivoire (92.6%; 25/27), Cameroon (57.9%; 33/57), and The Gambia (71.4%; 5/7). Subsequently, the code was broken and the assay results were compared with subtype classification of the validation panel that were obtained by HMA and/or sequencing results on gag and/or env gene fragments (Table 2). In case of discrepant results, the probe target fragment was sequenced and analyzed. All but one CRF02_AG samples of Côte d'Ivoire were classified correctly as CRF02_AG infections by the oligonucleotide probe hybridization assay, although the CRF02_AG specific probe target of the false negative sample was conserved. No false positive samples were documented. The samples of The Gambia were correctly identified as CRF02_AG or non-CRF02_AG. For the Cameroonian cohort, one false positive sample was detected. Out of 32 CRF02_AG samples, 9 (28.1%) were reactive PAg17β probe. For 1 Cameroonian CRF02_AG sample reactivity to both probe PAg17α combinations and PAg17β was observed. The OD distribution for the validation panel samples is depicted in Fig. 2. The overall sensitivity for the validation panel was 98.4% (62/63); specificity was 96.3%; Positive Predictive Value was 98.4%; and Negative Predictive Value was 96.3%.

DISCUSSION

The aim of this study was to develop an oligonucleotide probe hybridization assay for identifying HIV-1 CRF02_AG infections. This assay was configured to distinguish CRF02_AG from all other subtypes and CRFs. The close genetic distance between subtype A fragments in CRF02_AG and subtype A hampered differentiation between CRF02_AG and subtype A strains in env Heteroduplex Mobility
Assay\textsuperscript{29}, and the one-tube real-time isothermal amplification subtyping method described by de Baar et al.\textsuperscript{30} The DNA Enzyme Immunoassay (DEIA) genotyping method on the \textit{env} gene developed by Plantier\textsuperscript{31} et al showed sensitivity of 86.6\% (13/15 samples) in identifying CRF02_AG infections. The newly developed oligonucleotide probe hybridization assay shows high signal reaction to probes, differentiating CRF02_AG from other subtypes (A - H) and CRFs (CRF01 and CRF06). The oligonucleotide probe hybridization assay was validated and has a sensitivity of 98.4\%, a specificity of 96.7\%, a Positive Predictive Value of 98.4\%, and a Negative Predictive Value of 96.7\%, which makes the assay very reliable. Evaluating probe target PCR amplification, the sensitivity of PCR amplification of the probe target region much depended on the cohort studied.

Four working probes (PAg17\textalpha{}1 – \textalpha{}4) were sufficient to identify all CRF02_AG in West Africa; in contrast all 5 probes (PAg17\textalpha{}1 – \textalpha{}4 and PAg17\textbeta{}1) are needed to identify CRF02_AG in West Central Africa. This may indicate a further evolution of CRF02_AG in Cameroon or a founder effect by one CRF02_AG variant in West Africa.

The value of the probe assay for determining better estimates of prevalence of CRF02_AG will complement efforts in vaccine development and evaluation. This will provide a rapid and economical tool for the use in large scale screening of this particular subtype at vaccine trial sites especially in West Africa. The high sensitivity and specificity of the test also implies good Positive and Negative Predictive Values in regions of high and low prevalence.

A common drawback of using probes for genotyping in the context of HIV-1 is the huge diversity in place and time. Identification of subtype specific probes allowing to identify a particular subtype by a difference in one point mutation at the probe target region has been evaluated for subtype C with disappointing sensitivity and specificity (results not shown). The CRF02_AG probes were designed based on a distinct signature whereby a deletion pattern as compared to other subtypes and CRFs is conserved. We realize the shortcomings of probe assay in terms of representatativity of the results for the complete genome. Therefore, aging of epidemics and co-circulation of other subtype strains may influence through recombination the representativity of the probe assay result.

The actual future role of the Circulatory Recombinant Forms (CRFs) in the global pandemic must be monitored. The probe assay as a diagnostic tool will allow rapid screening for CRF02_AG. This could be
used in tracking the HIV epidemic in terms of documenting the real prevalence of the CRF02_AG virus infections. This finding might have potential implications on future vaccine, diagnostic, and treatment strategies, because in this time of extensive movement of people between continents, the chances of recombinants to become an epidemic outside Africa increases.

ACKNOWLEDGMENTS

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REFERENCES

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FIGURES AND TABLES

FIGURE 1

CRF02_AG

CRF02.DJ263 (α)  CAGCAG------GCAGCG------GCTGCCA CAGGA---AGCAGT------AGCCAAAATTACCC
CRF02.DJ264 (α)  CAGCAG------GCAGAG------GCTGCCA CAGGA---AGCAGC------AGCCAAAATTACCC
CRF02.IbNG (α)   CAGCAG------ACAGCA------GCTGCCA CAGGA---AGCAGC------AGCCAAAATTACCC
CRF02.MP645 (β)  CAGCAG------ACAGCA------GCTGCCA CAGGA---AGCAGC------AGCCAAAATTACCC

Other CRFs and Subtypes

CRF01.TH.CM240  CAGCAG------GCAGCA------GCTGGCACAGGA AGCAGCAGCAATGTC
CRF03.RU.98001  CAACAG------GCAGCA------ACTGGCACAGGA AGCAGCAGTAAGGTC
CRF04.GR.97PVMY CAGCAG------GCAGCA------GCTGGCAAT---AGCAGCAATGTC
CRF06.BF.BFP90  CATCAG------GCA---------GCTGCCACAGGA AACAGCAGTAATCTC
CRF11.GR.GR17   CAGCAG------GCAGCA------GCTGACTCAGGA AACAGCAACAAGGTC
A.KE.Q23       CAACAG------GCAGCA------GCTGACACAGGA AACAGCAGCCAGGTC
A.SE.SE6594    TCACAG------GCAACA------GCTGACACAGGA AGCAGCAGTAAGGTC
B.US.RF        CAGCAG------GCAGCA------GCTGACACAGGA AACAGCAGCCAGGTC
B.US.SF2       CAACAG------GCAGCA------GCTGACACAGGA AACAGCAGCCAGGTC
C.BR.92BR025   CAGCAG------GCAGCA------GCTGACACAGGA AACAGCAGCCAGGTC
C.BW.96BN01B22 CAGCAG------GCAGCA------GCTGACACAGGA AACAGCAGCCAGGTC
D.CD.ELI       CAGCAG------GCAGCA------GCTGACACAGGA AACAGCAGCCAGGTC
D.CD.NDK       CAGCAG------GCAGCA------GCTGACACAGGA AACAGCAGCCAGGTC
F.BR.BZ162    CAGCAG------GCAGCA------GCTGACACAGGA AACAGCAGCCAGGTC
F.CD.VI174    CAGCAG------GCAGCA------GCTGACACAGGA AACAGCAGCCAGGTC
G.FI.HH8793   CAGCAG------GCAGCA------GCTGACACAGGA AACAGCAGCCAGGTC
G.NG.92NG083  CAGCAG------GCAGCA------GCTGACACAGGA AACAGCAGCCAGGTC
H.BE.VI991    CAGCAG------GCAGCA------GCTGACACAGGA AACAGCAGCCAGGTC
H.BE.VI997    CAGCAG------GCAGCA------GCTGACACAGGA AACAGCAGCCAGGTC
J.SE.SE9173   CAGCAG------GCAGCA------GCTGACACAGGA AACAGCAGCCAGGTC
J.SE.SE9280   CAGCAG------GCAGCA------GCTGACACAGGA AACAGCAGCCAGGTC
K.CD.EQTBT11C CAGCAG------GCAGCA------GCTGACACAGGA AACAGCAGCCAGGTC
K.CM.MP535    CAGCAG------GCAGCA------GCTGACACAGGA AACAGCAGCCAGGTC

15
Legend:

Alignment of the conserved signature specific for CRF02_AG with all other subtypes and CRFs comprising fragments of subtype A. The black box indicates amino acid insertion, deletion within different subtypes and CRFs compared to probes (bold – italic) specific for CRF02_AG.
Legend: Oligonucleotide Probe Hybridization assay results are depicted according to their genetic subtype classification as CRF02_AG or non-CRF02_AG samples. A sample was considered as CRF02_AG when it reacted with an OD>15.0 with either probe or probe combination. Only the highest OD value obtained for with either probe combinations PAg17α1+2, PAg17α3+4, or with probe PAg17β is indicated.
### TABLE 1. Evaluation Panel: Summary of the Probe Assay Results

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<th>B</th>
<th>C</th>
<th>D</th>
<th>F</th>
<th>G</th>
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Legend: The table lists the different cohorts used and the genetic subtype classification of the analyzed samples as compared to the overall oligonucleotide probe hybridization result per subtype.

### TABLE 2. Validation Panel: Summary of the Probe Assay Results

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<td>0/1</td>
</tr>
</tbody>
</table>

Legend: The table lists the different cohorts used and the genetic subtype classification of the analyzed samples as compared to the overall oligonucleotide probe hybridization result per subtype.