CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, Mar. 2004, p. 430–432 Vol. 11, No. 2
1071-412X/04/$08.00+0 DOI: 10.1128/CDLI.11.2.430–432.2004
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Performance Characteristics of Updated INNO-LiPA Assays for Molecular Typing of Human Leukocyte Antigen A (HLA-A), HLA-B, and HLA-DQB1 Alleles

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Received 13 June 2003/Returned for modification 8 August 2003/Accepted 3 December 2003

We carried out a multicenter performance evaluation of three new DNA-based human leukocyte antigen (HLA) typing assays: INNO-LiPA HLA-A Update, INNO-LiPA HLA-B Update, and INNO-LiPA HLA-DQB1 Update. After optimization, the accuracy rates were all 100%, and the final observed resolutions were 99.4, 92.4, and 85.6%, respectively. These rapid and easy-to-perform assays yielded results fully concordant with other DNA-based tissue typing tests.

In order to optimize the selection of compatible organ and stem cell donors, DNA-based human leukocyte antigen (HLA) typing assays must not only be accurate but also take into account the continuing discovery of new HLA alleles (2). We recently updated three such HLA tests and then validated the new tests in two phases. First, we performed in-house assessments of the robustness of amplification and of the new probes by using well-characterized samples. In a second phase, reported below, we carried out external performance evaluations of the new assays.

The three upgraded tests, INNO-LiPA HLA-A Update, INNO-LiPA HLA-B Update, and INNO-LiPA HLA-DQB1 Update (Innogenetics, Ghent, Belgium), are line probe assays based on the reverse-hybridization principle, which can be described operationally as follows. Amplified, biotinylated DNA material is chemically denatured, and the single strands are hybridized with specific oligonucleotide probes immobilized as parallel lines on membrane-based strips. This process is followed by a stringent wash step to remove any mismatched amplified material. Thereafter, streptavidin conjugated with alkaline phosphatase is added and bound to any biotinylated hybrid previously formed. Incubation with a substrate solution containing a chromogen results in a purple-brown precipitate. The reaction is stopped by a wash step, and the reactivity pattern of the probes is recorded.

The performance assessments of INNO-LiPA HLA-A Update and INNO-LiPA HLA-B Update (to the allele group level, i.e., two digits) were carried out at four European tissue typing centers (Rennes and La Tronche, France; London, United Kingdom; Edegem, Belgium), while the evaluations of INNO-LiPA HLA-DQB1 Update (to the allelic level, i.e., four digits) were performed at three centers (Rennes and Paris, France; London, United Kingdom). Study objectives included the determination of probe reactivity, accuracy (percentage of observed concordance with the results of alternative DNA reference assays), and resolution (percentage of alleles or allele groups that are unambiguously typed in heterozygous combinations).

Each lab was asked to select approximately 50 (Edegem) or 100 (other centers) routine samples for which an HLA typing result was available and to test their own samples in a blind manner with INNO-LiPA HLA-A Update and INNO-LiPA HLA-B Update (at the four centers), or with INNO-LiPA HLA-DQB1 Update (at the three centers). Totals of 346 (HLA-A), 347 (HLA-B), and 311 (HLA-DQB1) routine DNA samples (extracted from EDTA- or acid citrate dextrose-anticoagulated blood), made nonlinkable to the donor subject, were analyzed. Prior to amplification, DNA was extracted from fresh or frozen samples and then diluted to concentrations between 20 and 250 ng/μl (for HLA-A and HLA-B) or between 20 and 1,500 ng/μl (for HLA-DQB1). One sample for HLA-A, 5 samples for HLA-B, and 12 samples for HLA-DQB1 were excluded from the analysis due to protocol violations. An *Auto*-LiPA instrument (Innogenetics) was used to perform the hybridization. The INNO-LiPA HLA Update result was compared with a result obtained from at least one or a combination of alternative DNA typing methods using PCR sequence-specific oligonucleotides, PCR sequence-specific primers, or sequencing technology (HLA-A and -B only). Results were analyzed by using the clinical trial version of the LiRAS (Innogenetics) interpretation software for LiPA HLA. The nomenclature was based on "Nomenclature for Factors of the HLA System, 2000" (1). An overview of the alleles encountered is given in Tables 1 to 3.

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TABLE 1. Frequency of HLA-A alleles with a unique typing result included in the external evaluation

Allele[a]	No. of occurrences (%)
A*01	93 (13.4)
A*02	168 (24.3)
A*03	81 (11.7)
A*0303N	1 (0.1)
A*11	40 (5.8)
A*23	27 (3.9)
A*24	72 (10.4)
A*25	7 (1.0)
A*26	21 (3.0)
A*29	27 (3.9)
A*30	33 (4.8)
A*31	20 (2.9)
A*32	39 (5.6)
A*33	13 (1.9)
A*34	1 (0.1)
A*36	1 (0.1)
A*66	5 (0.7)
A*68	31 (4.5)
A*6811N	2 (0.3)
A*69	4 (0.6)
A*74	5 (0.7)
A*80	1 (0.1)

[a] $n = 692$ alleles.

TABLE 2. Frequency of HLA-B alleles with a unique typing result included in the external evaluation

Allele[a]	No. of occurrences (%)
B*07	90 (13.1)
B*08	63 (9.2)
B*13	14 (2.0)
B*14	32 (4.7)
B*15	41 (6.0)
B*18	32 (4.7)
B*27	28 (4.1)
B*35	75 (10.9)
B*37	6 (0.9)
B*38	14 (2.0)
B*39	7 (1.0)
B*40	54 (7.9)
B*41	4 (0.6)
B*42	4 (0.6)
B*44	87 (12.7)
B*45	4 (0.6)
B*46	2 (0.3)
B*47	6 (0.9)
B*48	1 (0.1)
B*49	15 (2.2)
B*50	5 (0.7)
B*51	48 (7.0)
B*52	3 (0.4)
B*53	6 (0.9)
B*54	1 (0.1)
B*55	16 (2.3)
B*56	1 (0.1)
B*57	13 (1.9)
B*58	11 (1.6)
B*67	1 (0.1)
B*73	2 (0.3)
B*78	1 (0.1)

[a] $n = 687$ alleles.

All centers involved in the same evaluation were provided with a proficiency panel (five samples with known reactivities and interpretations). The primary objective of this panel was for the performance of a system suitability check to ensure that the external user could perform the assay proficiently and to check all of the settings of the instrumentation.

As the same panel was provided to all participating centers, the results obtained could be used to reflect interlab variability for this subset of probes. Each center obtained identical typing results. The panel was also tested with two different batches of products. The same typing results were obtained for each sample independent of the batch used.

With respect to probe reactivity and accuracy for INNO-LiPA HLA-A Update, the initial accuracy was found to be 99.1% (342 of 345 samples). After discrepancy testing, this result increased to 99.7% (344 of 345 samples). Although false reactivities were observed for some probes, the LiRAS software provided correct typing results, except in three instances. These included INNO-LiPA HLA-A Update determinations of the presence of A*03xA*68, A*31012xA*31012, and A*32xA*32, which were determined by reference methods to be A*03xA*6811N, A*3001xA*31012, and A*32xA*74, respectively. For the first two samples, discrepancies occurred due to a false-negative probe reaction (probes 1 and 4, respectively). Upon retesting, these probes no longer produced a false-negative reaction but rather a weak reaction. To ensure correct typing results, the LiRAS software was amended to include the possibility of these weak reactions.

As for the third sample, sequence analysis of cloned material confirmed the presence of the A*7403 allele. Based on the reactivity observed with a synthetic sample, a positive reactivity was assigned to probe 17 for this allele in the typing table. However, the result obtained in this study showed that probe 17 does not react at all with A*7403. The typing table was therefore adapted to prevent mistyping, with probe 17 being shown to react negatively with A*7403. Henceforth, two typing possibilities will be given for the probe pattern without probe 17, namely, A*3201xA*3201 or A*3201xA*7403. After this modification, the overall accuracy of the HLA-A Update assay increased to 100% (Table 4).

TABLE 3. Frequency of HLA-DQB1 alleles with a unique typing result included in the external evaluation

Allele[a]	No. of occurrences (%)
DQB1*0201	51 (9.1)
DQB1*0202	60 (10.7)
DQB1*0203	1 (0.2)
DQB1*0301	104 (18.5)
DQB1*0302	48 (8.6)
DQB1*0303	19 (3.4)
DQB1*0304	3 (0.5)
DQB1*0305	6 (1.1)
DQB1*0401	1 (0.2)
DQB1*0402	18 (3.2)
DQB1*0501	49 (8.7)
DQB1*0502	13 (2.3)
DQB1*0503	25 (4.5)
DQB1*0504	4 (0.7)
DQB1*0601	9 (1.6)
DQB1*0602	66 (11.8)
DQB1*0603	39 (7.0)
DQB1*0604	20 (3.6)
DQB1*0609	25 (4.5)

[a] $n = 561$ alleles.

TABLE 4. Overall performance characteristics of the INNO-LiPA HLA Update tests

Test	Accuracy (%)	Observed resolution (%)
INNO-LiPA HLA-A Update	100[a]	99.4 (at group level)[a]
INNO-LiPA HLA-B Update	100	92.4 (at group level)
INNO-LiPA HLA-DQB1 Update	100	85.6 (at allelic level)

[a] After discrepancy testing and adaptation of the typing table.

For HLA-A Update, the observed resolution at the allele group level, obtained after discrepancy analysis, was 99.4% (343 of 345 samples). Initially, one ambiguity was observed (A*02xA*66 or A*02xA*26), resulting in an initial observed resolution of 99.7% (344 of 345 samples). A second ambiguity arose as a result of the adaptation of the typing table (A*3201xA*3201 or A*3201xA*7403). This ambiguity, as well as the other ambiguity encountered, was not seen with the reference methods (A*32xA*74 and A*02xA*66). By contrast, 14 samples (five ambiguities) whose results were ambiguous by the reference methods could be reduced to clear-cut typing results (A*02xA*24, A*03xA*68, A*23xA*24, A*24xA*24, and A*24xA*32) with INNO-LiPA HLA-A Update. The INNO-LiPA HLA-A Update assay was also able to identify two null alleles in this study: A*0303N and A*6811N.

For INNO-LiPA HLA-B Update, the accuracy was 100% (342 of 342 samples). The false reactivities observed were included in the LiRAS software to provide correct typing results, and the observed resolution with the results of INNO-LiPA HLA-B Update at the allele group level was 92.4% (316 of 342 samples). For 6 of the 13 ambiguities encountered, the reference method also yielded an ambiguous answer. For another two discrepancies observed with the reference method results (B*07x40 or B*40x81, two samples; B*07x08 or B*07x42, four samples), the INNO-LiPA HLA-B Update assay provided single clear-cut typing results (B*07x40 and B*07x08, respectively).

With respect to INNO-LiPA HLA-DQB1 Update, the accuracy was 100%. One probe gave a weak false-positive reaction, and another gave a weak-to-false-negative reaction on one or several occasions. However, these false reactivities were identified as such with the LiRAS software, and a concordant typing at the allelic level was achieved when the interpretation software was used.

At the allelic level, the observed resolution was 85.6% (256 of 299 samples). A total of eight different ambiguities were encountered (43 samples). Among these ambiguities, some cannot be resolved with any method that is not capable of separating the alleles. The other ambiguities contain at least one infrequent allele. In addition, for the ambiguities observed, INNO-LiPA HLA-DQB1 Update showed a higher resolution than the earlier-generation LiPA HLA-DQB (Innogenetics; eight different ambiguities, 21 samples) or Dynal RELI (Dynal Biotech, Oslo, Norway; five different ambiguities, 11 samples). There were also two samples for which INNO-LiPA HLA-DQB1 Update and Dynal RELI revealed the same ambiguity. Finally, the resolution was lower than that with the reference Micro SSP (One Lambda, Canoga Park, Calif.) for five separate ambiguities (nine samples). In no less than 199 cases, the ambiguities observed with the reference methods could be reduced to a single clear-cut typing result with INNO-LiPA HLA-DQB1 Update.

Given the continual discovery of new HLA alleles, the updating of HLA assays and their software support is an ongoing process to ensure optimal organ and stem cell donor matching. For probe-based assays such as INNO-LiPA HLA-A and -B, this means ensuring the robustness of the amplifications and probes, improving resolutions at the allele group level, and enabling the detection of null and low-expression alleles. The amelioration of robustness includes the redesign of some probes as well as the multiplex amplification of smaller fragments in exons 1, 2, 3, and 4 for HLA-A and exons 2, 3, and 4 for HLA-B to increase the amplification efficiency. To enable the detection of null and low-expression alleles and to improve resolution, a rational selection of probes was made, thereby improving the theoretical resolution at the allele group level from 95.8 to 99.5% for INNO-LiPA HLA-A Update and from 97.2 to 98.8% for INNO-LiPA HLA-B Update. In addition, the INNO-LiPA HLA-B Update assay can discriminate splits of B14 (B64, B65), B15 (B62, B63, B75, B76, B77), B40 (B60, B61), and B70 (B71, B72).

As for DQB1 Update, the resolution at the allelic level was enhanced by the addition of 16 probes to a total of 37, thereby improving the theoretical resolution from 19.2 to 78.1%. The test was also upgraded to detect polymorphisms in exon 3 to differentiate DQB1*0201 from DQB1*0202/DQB1*0203 as well as DQB1*03011 from DQB1*0309. For this, a multiplex amplification was optimized for amplifying exons 2 and 3.

In conclusion, external multicenter performance evaluations of INNO-LiPA HLA-A Update, HLA-B Update, and HLA-DQB1 Update showed that all tests performed exceedingly well with respect to probe reactivity, accuracy, and observed resolution. For all three INNO-LiPA tests, the LiRAS software enables accurate, traceable, validated, and automatable interpretation of results.

We thank Fred Shapiro (Innogenetics) for coordinating the writing of the manuscript. We are also grateful to A. Rombout, N. Thienpont, N. Vande Casteele, W. Van Cauter, A. Verhelst, W. Van De Zande, and K. Lebeer (all from Innogenetics) for their contributions in assay development.

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