

# **Reference panels for Sequencing Based Typing: selection criteria for HLA-A and HLA-B**

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## **Summary.**

Typing reference panels are used to validate typing approaches. Basically, one would like to type all alleles and allele combinations. Since this is impossible, an efficient reference panel should be used. For Sequencing Based Typing (SBT), besides testing whether all alleles will be amplified, validation should include whether all heterozygous sequence patterns can be discriminated. Since sequence profiles mainly are defined by the local nucleotide sequence, SBT reference panels should be optimized to represent as many sequence motifs as possible, in both homozygous as well as heterozygous combinations, in a limited number of samples. Following these criteria, an HLA-A reference panel of 13 samples, and an HLA-B reference panel of 15 samples have been selected.

## **Introduction**

In Sequencing Based Typing (SBT), automated comparison of the sequences with an allele sequence library ensures a systematic and precise identification of HLA alleles. The computerized allele assignment showed that the identification of heterozygotes should be validated for each polymorphic position, since single nucleotide differences may result in a different allele assignment. To validate an SBT approach, a reference panel should be used which contains for each polymorphic position samples which are homozygous, and samples which are heterozygous at that position. Since the sequence at neighboring positions may influence the sequence profiles, all motifs present at a variable region should be present in a reference panel (1). Such reference panel does not necessarily represent all alleles and their combinations.

Based on the samples available through the 13th International Histocompatibility Workshop, reference panels for HLA-A and HLA-B have been selected to represent as many polymorphisms as possible. Quantification of the number of polymorphisms is obtained by comparing the HLA sequences of the samples with the allele sequence database, as downloaded from the IMGT/HLA database (2). This database, which is accessible through <http://www.ebi.ac.uk/imgt/hla/>, contains all sequence information of all HLA alleles recognized by the WHO Nomenclature committee for Factors of the HLA system. Selection criteria are restricted to polymorphisms in exon 2 and 3, since in most approaches typing is restricted to those exons, unless the results indicate the presence of alleles which only uniquely can be identified based on sequencing other exons.

For HLA-A a reference panel of 13 samples is selected, which covers more than 72% of all polymorphisms. For HLA-B a panel of 15 samples is selected, which covers more than 69%

of all polymorphisms. Most of polymorphisms not covered by these panels are unique for a single allele. These small reference panels are efficient resources for validation of HLA-A and HLA-B SBT approaches.

## **Material and Methods**

The HLA-A database is constructed from the exon 2 and exon 3 sequences downloaded from the IMGT/HLA database (2). The HLA-A database used contains 175 allele sequences with 98 polymorphic positions. The HLA-B database is constructed from the exon 2 and exon 3 sequences downloaded from the IMGT/HLA database (2), and contains 328 allele sequences with 116 polymorphic positions. Deletions and insertions are not considered.

From the cells available through the Cell and Gene bank of the 13 IHWC, subsets are selected. For each sample the heterozygous sequence of exon 2 and exon 3 are generated. The sequence at positions where samples differ are compiled. These sequences are compared with the allele sequences, and polymorphisms not represented in the panel are inspected. The subset which covers most of the polymorphisms is selected as reference panel.

## Results

### *HLA-A reference panel*

**Table 1:** The HLA-A reference panel

Ref. Cell Number	Workshop Number	Designation Name	HLA-A alleles
2	9273	LADA	A*02011,8001
3	9263	G085	A*0101,2901
4	9373	FH1	A*0205,6802
7	9045	TUBO	A*0216,03011
9	9076	T7527	A*0206,0207
11	9103	KT14	A*2402101,2602
14	9364	GRC212	A*0211,68012
16	9368	280599	A*2402101,2604
17	9367	LCK	A*0203,1102
23	9253	THAI742	A*24031,3303
37	9092	BM92	A*2501
41	9267	LE023	A*3201,6601
46	9021	RSH	A*3001,6802

Table 1 shows the HLA-A reference panel, which consists of 13 samples. All but one are heterozygous.

In Figure 1, the sequences of these samples are shown at those positions where the samples show differences in sequence. Heterozygous positions are encoded by the NC-IUB codes, which are listed in Table 2.

**Table 2:** NC-IUB nomenclature according to (3)

R = G & A	D = G & A & T
K = G & T	V = G & A & C
S = G & C	B = G & T & C
W = A & T	H = A & T & C
M = A & C	N = G & A & T & C
Y = T & C	

This panel covers 78 polymorphic positions. At many positions, all nucleotides are present both homozygous as well as all heterozygous combinations. For instance, at position 98, samples could have either A, C and T, as well as Y (=TC), W (=AT) and M (=AC). However, there are also positions where all nucleotides possible are present in individual alleles, but not in all combinations: at position 97, samples have either T or W, but no samples are homozygous A (Figure 1).

**Figure 1:** Compilation of the nucleotides of the sequences of the HLA-A reference panel. Position numbers are shown on top in vertical orientation. The consensus sequence is constructed based on the most common nucleotide at that position. Dots indicate identity to the consensus. Heterozygous positions are indicated by their NC-IUB code. The ‘Pool’ sequence is for each position a compilation of all nucleotides occurring at least once in a sample.

11111111	1112222222	2222222222	2333333333	3333333344	4444444445	5555555555	5555	
7990022224	6780013344	5556677899	9000111114	5666789901	1111245890	0122223345	5677	
8782613674	3600398903	6791501202	9127134791	5238651721	2348383972	6714678955	9001	
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CTAAGCCGGA	AGCAGGGGGG	GGGGCAGGCG	TGAGCCTGGA	AGGAGTGTCC	CAGGCCAGTA	GGCAGATGCG	AGGT	Consensus
Y.T.....	WR.....RK.	.R...W.C.M	YR.....M	RKR...K...	.RSK..MRY.	R..RSKYT..	RMSK	<b>LADA</b>
.WY.....C	.Y.RR....	CWS..TRS..	CA.....M	.TR.SY....	.G...YCR.M	.YR.YYKY.	MSSK	<b>G085</b>
Y..CR...M	.R...K.	S.RS.W.S.S	.....C	M...YK.S.	.CT...AC.	A...T....	.C..	<b>FH1</b>
Y.T.....M	.....K.	SR...W.S.S	.....C	RKR...K...	.RSK..MRY.	R...W.T.K	RM..	<b>TUBO</b>
T.WM.....	.....T.	.....C.C	.....C	G..R..T...	.CT...AC.	A...T.T..	.C..	<b>T7527</b>
.MM.....C	.....	SRRSSW.C..	MA.SYSYKSM	MK.W.Y....	.SWY.M..M	.R...WYR..	MSSK	<b>KT14</b>
Y.WW...M	.....K.	S.RS.W.SY.	.....C	RK...K...	.RSK...AC.	A...T.K..	.C..	<b>GRC212</b>
.MM.....C	.....	SRRSSW.C..	MA.SYSYKSM	MK.W.Y....	.SKY.M..M	.R...WYR..	MYSK	<b>280599</b>
Y.WM...RM	.....K.	SR...W.S.S	.....M	RKR..YK...	.RSK..MAY.	RR...MYR..	MS..	<b>LCK</b>
.WC.....C	.....	SRRSSW.CY.	WR.SYSYKSC	MT.W.....	.SK.YM..M	.R..TYW..	.C..	<b>THAI742</b>
.C.....C	.....	C.AC.T.C..	AAGCTGCTC.	.....C....	.T.C..C	.A.....C..	.C..	<b>BM92</b>
.WM.....C	.Y.....	CRRS.T.S..	WRRSYSYKSM	.K...C.Y..	...YYC..C	.R.R.W.K..	MS..	<b>LE023</b>
.MMRM.Y.M	.....R..K	CRRS.T....	.....C	.KR..Y...Y	S.MY..MRYM	R..RKK.K..	.C..	<b>RSH</b>
YWHHRMYRRM	WRYRRRRRKK	SDVSSWRSYV	HRRSYSYKSM	VKRDSYKYSY	SRVNYMRYM	RRYRBNDYK	VNSK	Pool

In Table 3 all 98 polymorphic positions of the HLA-A database are shown, with for each position how many alleles have a G, A, T or C, respectively, at that position. In yellow it is indicated which polymorphism is *not* present in the reference panel. At 71 out of 98 polymorphic positions (72%), all polymorphisms are represented in the reference panel. At 22 out of the remaining 27 positions, all but one allele have the same nucleotide. The allele with the

alternative nucleotide is not present in the panel. At the remaining 5 polymorphic positions, two or three alleles are not represented in the panel.

**Table 3:** Nucleotide distribution over all HLA-A alleles for each of the 98 polymorphic position. In yellow indicated are nucleotides not covered by the reference panel.

Pos	Lib	G	A	T	C	Alleles
78	Y	-	-	48	127	
81	V	I	2	-	172	A*0230, A*2408, A*2420
92	R	2	173	-	-	A*2305, A*2425
97	W	-	14	161	-	
98	H	-	58	63	54	
102	H	-	117	10	48	
106	R	173	2	-	-	
121	M	-	9	-	166	
123	Y	-	-	9	166	
126	R	166	9	-	-	
127	R	174	1	-	-	A*1102
142	K	174	-	I	-	A*0242
144	M	-	57	-	118	
160	R	174	I	-	-	A*0221
163	D	I	173	1	-	A*3006
176	R	174	1	-	-	A*8001
180	Y	-	-	12	163	
194	S	I	-	-	174	A*0231
200	R	4	171	-	-	
203	R	167	8	-	-	
219	R	171	4	-	-	
233	R	I	174	-	-	A*3305
238	R	161	14	-	-	
239	R	174	1	-	-	A*8001
240	K	126	-	49	-	
243	K	162	-	13	-	
256	S	85	-	-	90	
257	D	93	77	5	-	
259	V	128	42	-	5	
261	S	133	-	-	42	
265	S	33	-	-	142	
268	M	-	173	-	2	A*0229, A*2904
270	W	-	83	92	-	
271	R	168	7	-	-	
282	S	44	-	-	131	
290	Y	-	-	11	164	
292	V	124	1	-	50	
294	Y	-	-	I	174	A*02013
299	H	-	48	102	25	
301	R	102	73	-	-	
302	R	7	168	-	-	
307	S	135	-	-	40	
308	S	174	-	-	I	A*7403
311	Y	-	-	40	135	
313	S	40	-	-	135	
314	Y	-	-	135	40	
317	K	135	-	40	-	
319	S	135	-	-	40	
331	S	I	-	-	174	A*2306
341	M	-	37	-	138	
355	V	43	95	-	37	
362	K	70	-	105	-	
363	R	141	34	-	-	
368	N	3	137	34	1	
376	S	173	-	-	I	A*2901
385	Y	-	-	127	48	
391	K	128	-	47	-	

397	Y	-	-	167	8	
402	S	4	-	-	171	
404	R	174	I	-	-	A*2426
411	Y	-	-	9	166	
412	S	9	-	-	166	
413	R	47	128	-	-	
414	V	86	9	-	80	
416	R	I	174	-	-	A*2606
418	N	83	1	81	10	
419	W	-	174	I	-	A*6817
423	Y	-	-	25	150	
448	Y	-	-	21	154	
453	M	-	96	-	79	
463	M	-	I	-	174	A*3304
468	Y	-	-	174	I	A*3304
477	R	174	I	-	-	A*02014
480	R	174	I	-	-	A*11012
485	D	I	I	173	-	A*0218, A*2427
489	R	95	80	-	-	
493	Y	-	-	I	174	A*0232N
497	Y	-	-	113	62	
502	V	I	113	-	61	A*1105
506	R	111	64	-	-	
517	R	150	24	-	-	
519	S	174	-	-	I	A*2611N
521	Y	-	-	8	167	
524	R	37	138	-	-	
526	B	169	-	2	4	
527	N	6	35	119	15	
538	Y	-	-	126	49	
539	D	54	39	82	-	
545	Y	-	-	8	167	
555	K	167	-	8	-	
559	V	4	138	-	33	
560	N	32	4	1	138	
570	S	136	-	-	39	
571	K	38	-	137	-	
583	Y	-	-	172	3	A*3301, A*3304, A*3305
595	S	174	-	-	I	A*0304
616	R	I	173	-	-	A*6814
618	K	172	-	I	-	A*24032

*HLA-B reference panel*

**Table 4:** The HLA-B reference panel

Ref. Cell Number	Workshop Number	Designation Name	HLA-A alleles
1	9215	M7	B*3501,5301
2	9273	LADA	B*07021,5703
6	9035	JBush	B*3801
12	9374	FH2	B*4001,4402
14	9364	GRC212	B*3505,4004
17	9367	LCK	B*38021,4601
23	9253	THAI742	B*1512,4601
24	9369	ISH3	B*1526N
25	9380	FH6	B*0705,2702
26	9376	FH4	B*2703,27052
32	9381	FH7	B*1801,3908
41	9267	LE023	B*51011,7301
42	9382	FH8	B*27052,8201
47	9024	KT17	B*15011,3501
48	9016	RML	B*51011

Table 4 shows the HLA-B reference panel, which consists of 15 samples. Three samples are homozygous, and 12 are heterozygous. In Figure 2, the sequences of these samples are shown at those positions where the samples show differences in sequence. This panel covers 88 polymorphic positions. Similar to the HLA-A reference panel, at many positions, all nucleotides are present both homozygous as well as all heterozygous combinations.

**Figure 2:** Compilation of the nucleotides of the sequences of the HLA-B reference panel. Position numbers are shown on top in vertical orientation. The consensus sequence is constructed based on the most common nucleotide at that position. Dots indicate identity to the consensus. Heterozygous positions are indicated by their NC-IUB code. The ‘Pool’ sequence is for each position a compilation of all nucleotides occurring at least once in a sample.

1111111111	1122222222	2222222222	2222222223	3333333333	3333333333	4444444444	4555555555	55556666		
9000144466	6900001234	4556667777	7788888990	0011111455	5556666678	0111123678	9123344556	77780011		
7356712415	7345693246	7691690123	7801235291	2913479134	5671238997	9128905376	9278904790	01233508		
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TGCGCCGAAG	AGGGAAGGGG	TCGGATCTGC	ACAACAATAA	GGAGTGGCCC	CTCAGGATGG	CTGTACGAGG	AGACGGGGGA	GTGTGCCG	Consensus	
. . . A . . . . . C	. . . AC . CA . .	. . AC . . . . T	. . . . . . . . . .	R . WSYKS . T	A . . . . . CC	. . . . C . . . C	. . T . T . . . CT	. . . . . . . . . .	<b>M7</b>	
. K . R . . K . . C	. . RRWMSR . .	. SRSRW . WWS	G . YMSGRK . .	R . WSYKS . Y	M . . RKS . Y . .	. . R . . . . MS .	. . W . K . . . SW	. . . . SMSK	<b>LADA</b>	
. T . . . . T . . .	. . A . . . . . A	. . AC . . . . .	. . . . . . . . . .	A . T . CTC . . .	. . . . . . C . .	. . A . T . . . . C	. . T . T . A . AC	. . . . . . . . . .	<b>JBush</b>	
Y . . A . . AC . .	TA . A . . . A . .	. . . . . . . . C	. . . . . . . . . .	RSMSYKS . Y	M . . . . . C . .	Y . RK . . . MSS	WKTSWS . . SW	. . S . SMSK	<b>FH2</b>	
YK . R . . RM . S	WR . AM . SA . .	. . RS . . . . Y	. . . . . . . . . .	. . . C . . . . Y	M . . . . S . YCS	. . R . M . . MSS	. . T . T . . . SW	. . . . . . . . . .	<b>GRC212</b>	
. K . R . . K . . S	. . RRWMSR . R	. . RS . WS . R	MSM . SR . KW	RSMSYKS . . . .	. . . . . . C . .	. . R . Y . . . . S	. . WYK . R . MY	. . . . . . . . . .	<b>LCK</b>	
. . . A . . . . . C	. . . ATCCA . .	. . . . . WS . M	MSM . SR . KW	. . . C . . . . .	. . . . . . . . . .	. . . . C . . . . .	. . . T . . . . CT	SK . . . . . . . .	<b>THAI742</b>	
. . . A . . . . . C	. . . ATCCA . .	. . . . . . . . C	. . . . . . . . . .	. . . C . . . . .	. . . . . . . . A . .	. . . . C . . . . .	. . . T . . . . CT	. . . . . . . . . .	<b>ISH3</b>	
YT . . . . WM . S	W . A . . . . . . .	. . RS . . . . R	G . M . GG . G . .	R . WSYKS . . . .	. . . . RY . Y . .	YYMK . . . MC .	. . W . K . . . . .	. . . . SMSK	<b>FH6</b>	
CT . . . . AC . .	T . A . . . . . . .	Y . . . . . . . . .	G . . . GG . G . G	A . CC . TC . . .	. . . . AT . . . .	TCCG . . . . C .	. . T . T . . . . .	. . . . . . . . . .	<b>FH4</b>	
YT . . . . T . RS	. . RRM . S . RR	. . RS . . . . C	. . . . . . . . . .	. . . C . . . . .	. . . . . . . . C . .	. . R . Y . . . . C	. . T . K . R . AC	. . . Y . . . . .	<b>FH7</b>	
YK . RY . YRM . C	. . RRM . SR . .	. . AC . . . . K	R . . . SR . KWR	R . WSYKSM . T	TGG . C . . . R	Y . A . W . RMSS	. . W . T . . . SW	. . . C . . . . .	<b>LE023</b>	
YKYR . . WM . .	W . A . . . . . . .	. . RS . . . . R	G . M . GG . G . R	R . MC . KS . . .	. . . . RKW . SS	YYMKWM . . SS	. . TSWS . RSW	. . S . . . . . .	<b>FH8</b>	
. . . A . . . . . C	. . . AYMCA . .	. . RS . . . . Y	. . . . . . . . . .	. . . C . . . . Y	M . . . . . YSS	. . . . C . . . S	. . WYK . . . CT	. . . . . . . . . .	<b>KT17</b>	
. . . A . T . . . C	. . . AC . CA . .	. . AC . . . . T	. . . . . . . . . .	A . T . CTC . . T	TGG . C . . . . .	. . A . . . A . . C	. . . . T . . . CT	. . . C . . . . .	<b>RML</b>	
YKYRYDDMRS	WRRRHMSRRR	YSRSRWSWNS	VSHMSRRKWR	RSHSYKSMYY	HKSRNBWHVS	YYVKHMRMSS	WKWBDSRRVH	SKSYSMSK	<b>Pool</b>	

In Table 5 all 116 polymorphic positions of the HLA-B database are shown, with for each position how many alleles have a G, A, T or C, respectively, at that position. In yellow it is indicated which polymorphism is *not* present in the reference panel. At 80 out of 116 polymorphic positions (69%), all polymorphism is represented in the reference panel. At 25 out of the remaining 36 positions, all but one allele have the same nucleotide. The allele with the alternative nucleotide is not present in the panel. At an additional 7 positions, two alleles are not represented. At the remaining 4 positions 3,4,5 and 11 alleles are not represented.

**Table 5:** Nucleotide distribution over all HLA-B alleles for each of the 116 polymorphic position. In yellow indicated are nucleotides not covered by the reference panel.

Pos	Lib	G	A	T	C	Alleles
83	Y	-	-	1	318	B*1516
91	Y	-	-	321	1	B*1404
97	B	11	-	249	65	
103	K	222	-	103	-	
105	Y	-	-	2	323	
106	R	109	216	-	-	
117	Y	-	-	1	324	B*7301
119	K	324	-	1	-	B*3507
126	R	323	2	-	-	B*3912, B*4103
141	Y	-	-	30	295	
142	D	148	75	102	-	
144	M	-	249	-	76	
161	R	9	317	-	-	
165	S	148	-	-	178	
167	W	-	255	71	-	
175	M	-	325	-	1	B*5401
193	R	271	56	-	-	
204	R	200	127	-	-	
205	R	127	200	-	-	
206	N	3	178	58	88	B*0713, B*5401, B*5507
209	M	-	269	-	58	
213	S	180	-	-	147	
222	R	137	191	-	-	
226	R	2	326	-	-	B*0712, B*5401
228	R	2	326	-	-	B*0712, B*5401
234	R	319	9	-	-	
245	M	-	327	-	1	B*5505
246	R	300	28	-	-	
247	Y	-	-	327	1	B*2703
255	S	1	-	-	327	B*4413
256	S	9	-	-	319	
257	R	327	1	-	-	B*5804
259	R	150	178	-	-	

261	S	149	-	-	179	
263	Y	-	-	1	327	B*1528
266	R	11	317	-	-	
269	H	-	15	312	1	B*0805
270	S	3	-	-	325	
271	W	-	11	317	-	
272	N	56	46	98	128	
273	S	11	-	-	317	
277	V	65	260	-	3	
278	S	3	-	-	325	
280	H	-	277	11	40	
281	M	-	317	-	11	
282	S	57	-	-	271	
283	R	68	260	-	-	
285	R	11	317	-	-	
287	R	1	327	-	-	B*1549
289	R	2	326	-	-	B*0713, B*0715
292	K	98	-	230	-	
293	R	1	327	-	-	B*4602
299	W	-	320	8	-	
301	R	16	312	-	-	
302	R	230	98	-	-	
309	S	303	-	-	25	
311	H	-	228	56	44	
313	S	80	-	-	248	
314	Y	-	-	247	81	
317	K	227	-	101	-	
319	S	227	-	-	101	
322	K	1	-	327	-	B*5112
341	M	-	1	-	327	B*7301
353	Y	-	-	75	253	
354	Y	-	-	62	266	
355	H	-	76	62	190	
356	K	62	-	266	-	
357	S	62	-	-	266	
360	S	327	-	-	1	B*3916
361	D	4	319	5	-	
362	N	254	17	4	53	
363	B	250	-	17	61	
368	N	1	315	9	3	
369	H	-	1	148	179	
379	V	255	1	-	72	
387	S	272	-	-	56	
396	Y	-	-	1	327	B*5117
397	Y	-	-	1	327	B*3502
404	S	327	-	-	1	B*5118
408	K	327	-	1	-	B*5117
409	Y	-	-	66	262	
411	Y	-	-	308	20	
412	V	145	163	-	20	
414	M	-	1	-	327	B*5114
418	N	33	1	293	1	B*4027, B*2709
419	H	-	154	71	103	
420	M	-	26	-	302	
435	R	293	35	-	-	
453	B	1	-	1	326	B*1805, B*15013
463	M	-	250	-	78	
474	M	-	1	-	326	B*38022
477	S	246	-	-	82	
485	M	-	1	-	327	B*1533
486	S	147	-	-	181	
489	R	327	1	-	-	B*27053
499	W	-	315	13	-	
506	K	326	-	2	-	B*1301, B*1302
512	K	315	-	13	-	
527	H	-	134	193	1	B*5805
538	B	36	-	38	254	
539	D	73	36	219	-	
540	S	292	-	-	36	
544	R	296	32	-	-	

557	R	327	1	-	-	B*8201
559	V	85	75	-	168	
560	H	-	85	168	75	
570	B	325	-	1	2	B*4015
571	K	3	-	325	-	
572	S	304	-	-	24	
582	R	1	326	-	-	B*07022
583	Y	-	-	277	50	
603	S	275	-	-	50	
604	W	-	324	1	-	B*5118
605	M	-	35	-	290	
610	S	51	-	-	274	
618	K	287	-	35	-	

## Discussion

To validate an SBT approach, ideally all alleles in all combinations should be tested. Since this is impossible, reference panels are necessary to type. The sequences obtained typing this reference panel should represent all polymorphisms, both in homozygous as well as in heterozygous combinations. Since sequence profiles at a particular position mainly are influenced by the direct neighboring nucleotides, it is sufficient to include samples representing all polymorphisms *at this position* in the reference panel, independent of the polymorphisms at other positions in the samples. Therefore, one cell can represent several polymorphisms at different positions. Optimizing the sample selection, a limited number of cells are required to represent the majority of the polymorphisms. In this study we present minimum reference panels for HLA-A and HLA-B, which are of limited size (13 and 15 samples, respectively), but represent the majority of all polymorphisms known for HLA-A and HLA-B. Most of the polymorphisms not covered are represented by only one or two, mostly rare alleles, which are not present in the panels. Typing these reference panels is a minimum requisite to validate SBT approaches.

## Reference List

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