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CLASS I TYPING BY PCR-SSP IN OLOMOUC

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Summary

In this article, methodical experience and population data are given for application of PCR-SSP/ARMS method (Phototyping[1]) to HLA class I typing in Olomouc. Phototyping is reliable method suitable for typing of small to medium number of samples. Method is fast enough for use in on-call service, resolution is better than the level of good serology, and price of method is comparable with serology. Our experience and tips are described below. Population data of healthy unrelated individuals for HLA-A, -B, -Cw are given in the tables.

Introduction

Molecular genetics methods are becoming standards in HLA typing because of absence of problems accompanied with serological typing. Shortly, they are not affected by cells vitality and amount, their reagents are easily obtainable and material can be stored for years. Besides, they can define more alleles (with immunobiological significance) with greater precision than is ever achievable by serology. Class I molecular genetics typing started later than one for class II. The reasons were that[2]:

1) serological typing for class I seemed to be quite reliable (at least for HLA-A, -B) in comparison to class II typing.

2) sequence data were not available. Class I was sequenced later than class II because of technical problems (from where to start sequencing, how to handle two polymorphic exons, how to overcome secondary structure constraints within class I region).

3) class I region contain many interloci and intraloci similarities (the same sequence stretch occurs at several loci of one chromosome or in several alleles at one locus, respectively).

However, since 1992 technical obstacles have been overcome and DNA-based techniques have been applied to class I typing as well. The most popular ones are PCR-SSO[3] (PCR followed by hybridization with sequence-specific oligoprobes), SBT[4] (sequencing-based typing), and PCR-SSP (PCR with sequence-specific primers). Though many other methods have been applied to genotyping, they are not (yet) widely spread in HLA typing practice. Our project aimed on testing class I genotyping method which is suitable and affordable for our laboratory. The article summarizes our experience with introduction and adaptation of noncommercial class I genotyping method based on PCR-SSP principle (Phototyping) to Olomouc HLA laboratory.

Methods

Materials

The primers and Taq polymerase were obtained from the Czech suppliers (Generi Biotech and TopBio, respectively), nucleotides and agarose from Promega and other chemicals from Sigma. Plastics were purchased from Robbins.

Protocols

DNA was extracted by salting-out method from 10 ml of peripheral blood[5]. Concentrations of some primers were reduced after optimizing, other parameters of PCR remained as originally published for Phototyping. Amplicons were distinguished after electrophoresis in 2% agarose gel 0.5*TBE, at 5.6V/cm, for 20 min. Instant image was obtained by photographing with Polaroid camera. Master mixes with 14 days exspiration were prepared to reduce workload. Multichannel pipettes were used during primer aliquoting and gel loading, pipette dispensor was used to add template.

Method principle

In PCR-SSP or ARMS (amplification refractory mutation system), many PCR reactions are used to define HLA type of individual. Every reaction contains primer mix which is specific either for allele or for group of alleles. Only perfect match between both primers and template (with 3' end of primer being the most important) launches exponential amplification of the sequence of interest by PCR. Discriminatory ability of primer mixes allows to determine presence of specific allele by detection of specific amplicon (or amplicons pattern) after electrophoresis.

On the contrary, absent allele yields no amplicon detectable by electrophoresis. To avoid problems with false homozygote typing (lack of specific amplicon for second allele could mean PCR failure due to pipetting or other error), one more primer pair is added to every PCR reaction yielding constitutive human amplicon of different length than these of specific amplicons.

Subjects

Over 300 persons from Olomouc region were HLA-class I genotyped. 127 of them were healthy unrelated individuals, whose data were used to obtain population frequencies.

Results

Phototyping for class I typing in our hands is the method of choice because it is method with medium turn-over and medium workload (as compared with classical PCR-SSO and automated SBT), its resolution is adjustable from medium to high by number of primer mixes used, it lasts for 3 hours (starting from bleeding and ending with interpretation of result) and it uses basic laboratory equipment (cycler, electrophoresis, camera) without need for expensive investment.

Expertness needed

As in any other PCR set-up, stringent conditions to prevent contamination must be used. EFI defines necessary and optional conditions required for accreditation. Though these conditions may seem annoying to small laboratory, we found necessary to follow them to insure reliable result and prevent need for dramatic repairs. Moreover, we test every new batch of chemical (double distilled water included) for presence of PCR inhibitors.

Taq polymerase enzyme unit is defined as amount required to catalyse the incorporation of 10 nanomoles of nucleosides into an acid-insoluble product within 30 min under assay conditions. Assay conditions change among enzymes from different organisms and different suppliers. Even if two companies declare the same testing conditions, their polymerases fail to exhibit agreeing efficacy. Moreover, unit definition has not correspond to cycling nature of PCR and to our actual PCR conditions. That is why we standardise polymerase concentration by changing definition of polymerase unit: Enzyme has standard concentration of 5U/µl, if it amplifies optimally under Phototype conditions at dilution 1 part of Taq to 160 parts of total volume. Optimal amplification is described by thick band on the gel in allele-specific reaction and two thinner bands in control reaction. Second band in control reaction is used for band alignment in interpretation-difficult gels.

After above mentioned precautions, method is reliable to the extent that every tissue typist on-call (6 persons) can obtain correct result even at 3 a.m., what is confirmed externally by Prof. P. Terasaki and C. Cabacungan quality control (since 19th control run). We still keep microcytotoxicity test as standard method for class I typing because only combined results from both methods may point to serologically undetected and DNA method untested null allele. Additional aspect concerns our microlympho-cytotoxicity crossmatch method: good crossmatch results require experienced and running serology.

Turn-over

Number of samples which can be typed by one person is restricted by number of cyclers (or better number of wells), number of gel tanks and pipetting ability. Cyclers with 192, 384 wells or plates with partitioned tubes are already on market. For gel loading we use 12-channel pipette twice in one row. Our combs are constructed to allow loading with double density according to pipette spacing - second set of 12 wells is placed in between (partially superimposed) first set of 12 wells. For pre-pipetting of primers, 12-channel dispenser can be used advantageously. Full automated systems are available as well (Biomek Workstation), however not in our laboratory.

Resolution

Though authors of the original method increase its reliability by including more primer mixes (even for null alleles), we found satisfactory to use their original protocol with adjustment of several primer concentrations. Resolution under original condition is slightly better than that of good serology, what is fully acceptable for Czech organ transplantation program.

Time requirements

Under time pressure, we use 50 min modification of salting-out DNA extraction method, followed by 10 min PCR set-up and 80 min amplification protocol on MJR cycler. Electrophoresis, photographing and interpretation takes 30 min altogether. In essence, we can provide result after 3 hours from bleeding.

Price

The most expensive items of investment to method are in our conditions: primers, Taq polymerase and PCR microtubes. Price of primers decreases steadily and when we included all taxes, the Czech company Generi BioTech gave us the best price (54Kč ~ 1GBP per nucleotide for standard synthesis, 0.2mmol scale without purification). Large synthesis scale and large number of typings performed reduce price per typing. It is useful to share expenses with other laboratory.

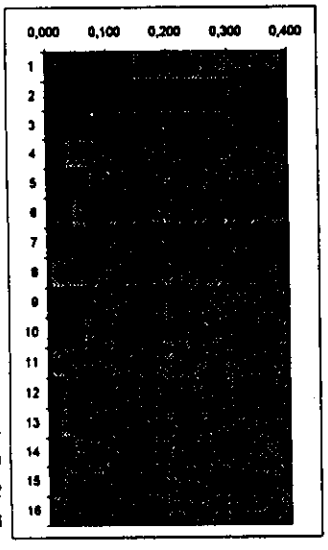
Company TopBio supplies Taq polymerase for less than 5Kč/U. Using our unit definition, we found that one batch of Taq had actual concentration 25U/µl (price being reduced to 1Kč/company-defined unit or 0.4Kč/PCR reaction).

It is economical to use 96 tube plates instead of separate tubes or tube strips. The cheapest plates are offered by Costar. However, thermal transfer through polycarbonate plate is slower than one through polypropylene tubes. Moreover, sealing of plates by thermowell sealers is not tight and sometimes leaks. These two drawbacks can be overcome by use of mineral oil outside (to improve thermal transfer) and inside (to prevent evaporation) of reaction tubes within plate. We prefer not to use oil and stick to Robbins plates because oil is messy and expels amplicon from our small gel wells during electrophoresis loading.

Frequency data

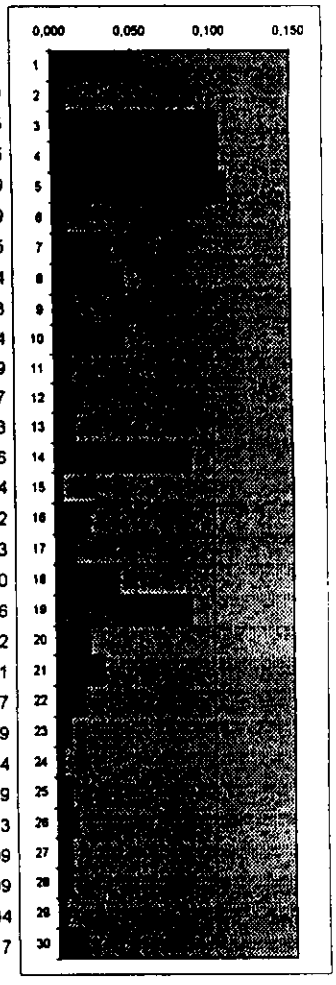
HLA-A*

no	A*	Count	Antigen	Gene
1	01	31	0,270	0,145
2	02	58	0,504	0,296
3	03	26	0,226	0,120
4	23	7	0,061	0,031
5	24	15	0,130	0,067
6	25	9	0,078	0,040
7	26	13	0,113	0,058
8	66	1	0,009	0,004
9	68	14	0,122	0,063
10	11	13	0,113	0,058
11	29	1	0,009	0,004
12	30	5	0,043	0,022
13	31	4	0,035	0,018
14	32	9	0,078	0,040
15	33	5	0,043	0,022
16	74	1	0,009	0,004



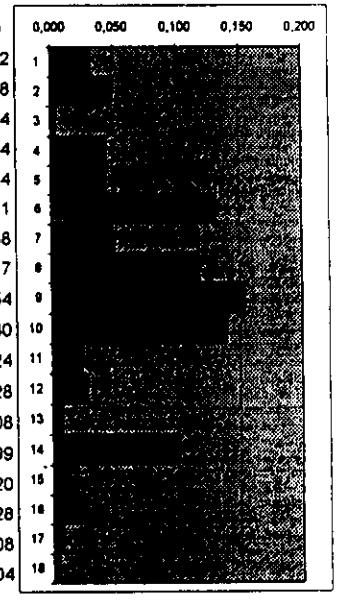
HLA-B*

no.	B*	Count	Antigen	Gene
1	51	13	0,112	0,058
2	52	2	0,017	0,009
3	07	23	0,198	0,105
4	08	23	0,198	0,105
5	44	24	0,207	0,109
6	45	2	0,017	0,009
7	13	8	0,069	0,035
8	62	10	0,086	0,044
9	63	3	0,026	0,013
10	38	10	0,086	0,044
11	39	2	0,017	0,009
12	57	4	0,034	0,017
13	58	3	0,026	0,013
14	18	19	0,164	0,086
15	49	1	0,009	0,004
16	50	5	0,043	0,022
17	56	3	0,026	0,013
18	27	9	0,078	0,040
19	35	19	0,164	0,086
20	37	5	0,043	0,022
21	60	7	0,060	0,031
22	61	4	0,034	0,017
23	41	2	0,017	0,009
24	47	1	0,009	0,004
25	48	2	0,017	0,009
26	53	3	0,026	0,013
27	71	2	0,017	0,009
28	55	2	0,017	0,009
29	64	1	0,009	0,004
30	65	4	0,034	0,017



HLA-Cw*

no.	Cw*	Count	Antigen	Gene
1	01	8	0,063	0,032
2	02	12	0,094	0,048
3	0302	1	0,008	0,004
4	0303	11	0,087	0,044
5	0304	11	0,087	0,044
6	04	31	0,244	0,131
7	05	12	0,094	0,048
8	06	28	0,220	0,117
9	0701	36	0,283	0,154
10	0702/03	33	0,260	0,140
11	0704	6	0,047	0,024
12	08	7	0,055	0,028
13	1202	2	0,016	0,008
14	1203	24	0,189	0,099
15	14	5	0,039	0,020
16	15	7	0,055	0,028
17	1601	2	0,016	0,008
18	1602	1	0,008	0,004



Conclusions

We introduced PCR-SSP method, known as Phototyping for HLA-A, -B, -Cw typing to Olomouc HLA laboratory at 1996. In our hands, method is fast (3h) and reliable (as checked by UCLA control organised by Prof. Terasaki), suitable for small to medium scale typing. 115 healthy unrelated persons from region of Olomouc was typed for HLA-A, 116 persons for HLA-B and 127 persons for HLA-Cw. Preliminary antigen and gene population frequencies are given in the tables.

Method is now used in our laboratory for confirmation of serologically abstruse samples, for pre-BMT typing and for cadaver donor typing.

Acknowledgements

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