WAKFlow

HLA typing kit user's manual

2004 Nov.

WAKUNAGA Pharmaceutical Co., Ltd.

Contents

1. Principle of the HLA typing

The WAKFlow HLA typing kit is based on the reverse sequence-specific oligonucleotide probes (SSO) method coupled with a microsphere beads array platform (xMAP technology of Luminex corp.) to identify HLA alleles encoded by the sample DNA.

First of all, the target DNA is amplified by polymerase chain reactions (PCR) with biotinylated primers specifically designed for each HLA locus. The PCR product is denatured and hybridized to complementary oligonucleotide probes immobilized on fluorescently coded microsphere beads. At same time, biotinylated PCR product is labeled with phycoerythrin-conjugated streptavidin to allow it to be detected with the Luminex 100 system. The entire process is performed in a single well of a 96-well PCR plate; thus, 96 samples can be treated at one time. The HLA typing is determined by analysis of the reaction (hybridization) pattern of the target sample.

2. Kit component

Product	**Size**
WAKFlow HLA typing kit	**95 tests / kit**
①. Amplification Reagent (different with HLA-A and B locus)	1,323 μL (2 tubes)
②. DNA Polymerase Solution	54 μL (1 tube)
③. Denaturation Solution	1,000 μL (1 tube)
④. Hybridization Solution	2,200 μL (1 tube)
⑤. Luminex Beads Solution(different with HLA-A and B locus)	330 μL (1 tube)
⑥. Streptavidin-Phycoerythrin Solution (SAPE)	220 μL (1 tube)
⑦. Wash Solution	50 mL (1 tube)
⑧. Plate Sealing Sheet	2 sheets

Storage conditions

- DNA polymerase solution should be stored at -20^{o}C for longer storage.
- SAPE solution must be stored at 2 - 8^{o}C under the dark condition.
- The other reagents can be stored at 2 - 8^{o}C.

3. Protocols

3.1 Attentions to avoid PCR contaminations.

Contamination of previously amplified DNA in PCR reaction may cause incorrect typing results.

To avoid PCR contaminations,

- Separate PCR preparation area from post-PCR area to handle amplified DNA.
- All materials, such as micropipette, thermal cycler, aspirator, to treat amplified DNA must be physically separated from materials used for preparing PCR reactions.
- When handling amplified DNA, wear a lab coat and disposable gloves only for post PCR area, and don't bring them out to another area.
- After PCR preparation, wipe a Platte with 0.5% sodium hypochloride solution or breech diluted with H_2O in ten-fold.

3.2 Devices and equipment to be supplied by user.

(1) PCR

- Thermal cycler
- Micropipette (1~20 μL, 10~200 μL, 100~1000 μL)
- Sterilized micropipette tips
- Sterilized 0.2mL PCR tubes or 96-well PCR tray

(2) Hybridization and labeling

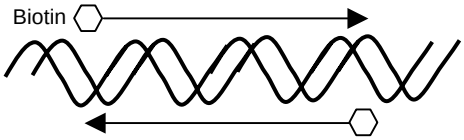
- Multi-channel micropipette
- Micropipette (1~20 μL, 10~200 μL, 100~1000 μL)
- Vortex mixer
- Centrifuge machine for a microtiter-plate

(3) Data acquisition and analysis

- Luminex 100 system
- Personal computer

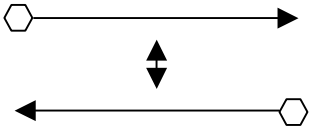
3.3 Procedure of the *WAKFlow* HLA typing kit.

PCR (1.5 hours)



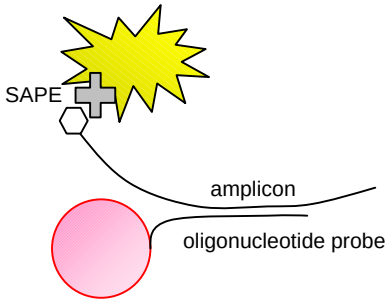
Amplification of target genes with biotinylated-locus specific primers.

Denaturation (5 minutes)



Denaturation of the amplified DNA into single strand.

Neutralization and Hybridization / fluorescent-labeling (30 minutes)



Hybridization of denatured-DNA to oligonucletide probe immobilized on microshpere beads and fluorescent labeling with streptavidin-phycoerythrin conjugate (SAPE) in a single step.

Washing (5 minutes)

Acquisition and data analysis with the Luminex 100 system

3.4 *WAKFlow* HLA typing kit protocol

(1) Preparation of sample DNA

Preparation of sample DNA should be done in a pre PCR area to avoid PCR contaminations. Sample DNA for PCR should be prepared at a concentration of 5 to 40 ng/μL.

(2) PCR (in pre-amplification area)

Note ･Wear gloves to avoid contamination of PCR product.
･Turn on a thermal cycler to warm up heated lid.
･Mix well "Amplification Reagent" before use.

2-1 Prepare appropriate number of the following reaction mixture.

Component of a 1x reaction
①. Amplification Reagent (specific for each HLA locus)･･･24.5μL
②. DNA Polymerase Solution ･･･････････････････････0.5μL

Note ･Preparation of PCR reaction should be done on ice to avoid non-specific gene amplification.

2-2 Aliquot 25 μL of the PCR reaction mixture of step 2-1 into a PCR reaction tube or a 96-well PCR tray, then add 2μL of sample DNA prepared in step 1.

Note ･Change pipette tip when adding sample DNA.

2-3 Cap or seal the PCR tray and set the PCR tubes in a thermal cycler, then run PCR program consisting of

93°C 3 min
↓
93°C 30 sec
60°C 30 sec 40 cycles
72°C 30 sec
↓
4°C Hold

Note ･This PCR reaction may take for 1.5 hours when utilizing the GeneAmp® PCR System 9700 Gold-96-well.

・For the GeneAmp^{R} PCR System 9700 Gold-96-well, set 「Reaction volume」 to "27"μL and 「Ramp Speed」 to "MAX" mode on 「Selection Method Options」 window before starting PCR reaction.
・For another type of thermal cycler, you may need to optimize a PCR cycle empirically.

(3) Hybridization and labeling

Note ・Wear gloves.
・Turn on a thermal cycler and run program of 55^{o}C HOLD.
・Turn on the Luminex 100 and XYPlatform, and perform warm up procedure.
・Make sure that the "Denaturation solution", "Hybridization solution", and "Wash solution" are at room temperature when used.

3-1) Aliquot 5μL of "Denaturation Solution" into a 96-well PCR tray.

Note ・"Denaturation solution" is alkaline reagent. Wear protection glasses and gloves to protect your eyes and skin.

3-2) Add 5 μL of the PCR amplified-DNA of step 2-3 into each well, then mix by pipetting or vortex, followed by keeping at room temperature for 5 minutes.

3-3) Prepare appropriate number of the following hybridization mixture in a sample tube, and mix well by vortex.

Component of a 1x hybridization mixture

④. Hybridization Solution・・・・・・・・・・・・・・・・・・・・・・・・・・・・・・・・20μL
⑤. Luminex Beads Solution (specific for each HLA locus)・・・・・・・3μL
⑥. Streptavidin-Phycoerythrin Solution (SAPE) ・・・・・・・・・・・・・・2μL

Note ・Mix "Luminex beads solution" firmly by vortex before use.

3-4) Add 25μL of the hybridization mixture prepared in the step 3-3 to the denatured-DNA of the step 3-2. Seal the 96-well PCR tray with a plate-sealing sheet, followed by mixing well with vortex mixer.

Note ・Apply of the hybridization mixture should be done quickly (within 3 minutes) because "SAPE Solution" is sensitive to light.
・Sealing of the PCR tray should be done tightly to avoid well-to-well sample contaminations.

・After mixing the PCR tray, snap down the plate to get solution attached to the plate seal sheet down to the bottom of the plate.

3-5) Place the 96-well PCR tray into a thermal cycler pre-warmed at 55°C, and put a PCR pad on top of the tray, then incubate for 30 minutes.

Note ・Make sure that the Luminex 100 system has been warmed up and ready for measurement of fluorescent signal.

3-6) Add 75μL of "Wash solution" to each well, then centrifuge the plate for 1 minute at 3,000 rpm (approx. 1000 x g).
Remove supernatant carefully by snapping or aspirating.

Note ・Be sure to leave small part of the solution, approx. 10 to 25μL, Not discard hybridized-beads from the PCR tray.

3-7) Add 75 μL of "Wash solution" to each well.

(4) Acquisition and data analysis

Note ・Acquisition should be carried out immediately. Store the samples under the dark condition, if they are not measured immediately.

4-1) Eject tray stage of the Luminex XY Platform, and place the PCR tray in step 3-7. Then retract the stage.

4-2) Select a template file corresponding to a product lot that you use, and enter the number of samples, then start acquisition.

4-3) Open an output.CSV file that is automatically created after acquisition with the HLA typing software, *WAKFlow* Manager. Positive or negative reaction is assigned based on the pre-set cut-off value for each typing probe. Fluorescent signal higher than the cut-off value is defined as positive reaction. Determination of HLA allele of the sample is performed automatically by matching a pattern of positive and negative reactions of each bead.

4. Warning or caution

1) This product is for research use only. Don't use for purpose of diagnosis and prognosis of disease.
2) Don't use reagents expired.
3) Don't drink or eat reagents. In case of contact to reagents, immediately wash eyes or skin with a large amount of water. Call a physician in case of burns, inhalations, ingestions and so on.
4) Pay attentions to PCR contamination. This may cause incorrect typing results. For the detail, see section 3.1 Attentions to avoid PCR contaminations.
5) It is possible to be changed the specifications to improve the kit without any notice.

<Effective period; 12 months>

< Packaging unit; 96 tests / kit>

WAKUNAGA Pharmaceutical Co., Ltd.
BioBusiness development

1624 Shimokotachi, Koda-cho, Akitakata-city
Hiroshima 739-1195 JAPAN

Phone; +81 (826)-45-4625
FAX; +81 (826)-45-4624
URL; http://www.wakunaga.co.jp