Revised in Jun .2022.
Issued in Jun 2021.

WAKFlow HLA Typing kit

Instruction manual

Research use only

5th Edition

Wakunaga Pharmaceutical Co., Ltd.

Contents

1. Principles of HLA typing

The ***WAKFlow*** HLA typing kit is based on the reverse sequence-specific oligonucleotide probes (SSO) method coupled with xMAP technology designed for use with the Luminex® system (http://www.luminexcorp.com/) to identify HLA alleles encoded by sample DNA.

The target DNA is first amplified by polymerase chain reaction (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 the same time, the biotinylated PCR product is labeled with phycoerythrin-conjugated streptavidin to allow it to be detected by the Luminex® 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 alleles are assigned by analysis of the reaction (hybridization) pattern of the target sample using the ***WAKFlow*** HLA typing software.

2. Kit components and storage conditions

Product	Size
***WAKFlow* HLA typing kit**	**96 tests / kit**

Amplification Package Store below –15℃

①. PCR pre-mix (specific for each HLA locus)················1,323 μL (2 tubes)

②. DNA Polymerase Soln. (specific for each HLA locus) ··············54 μL (1 tube)

Detection Package Store at 2～8℃

③. Denaturation Soln. ··1,000 μL (1 tube)

④. Hybridization Soln. ··2,200 μL (1 tube)

⑤. Beads mix (specific for each HLA locus)·······················330 μL (1 tube)

⑥. SAPE (Phycoerythrin-conjugated Streptavidin) ··················220 μL (1 tube)

⑦. Wash Soln. ···50 mL (1 tube)

・ Plate Sealing Sheet···2 sheets

Note

・PCR Pre-mix and DNA polymerase soln. should be stored below -15°C for longer storage.

・⑤ Beads mix and ⑥ SAPE must be stored at 2 - 8°C under dark conditions.

・Do not mix components form different lots or products.

3. Protocols

3.1 Attentions to avoid PCR contamination.

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

To avoid PCR contamination;

・Separate pre-PCR area from post-PCR area.

・Pre- and Post-PCR area must have their own separate set of lab coat, shoes, gloves, and equipment, including micropipettes, pipette tips, racks, thermal cycler, and so on. These items should not leave the area to which they are assigned.

・After PCR preparation, wipe the lab bench with 0.5% sodium hypochloride solution or bleach diluted ten-fold with H_2O.

3.2 Instrument and equipment requirements.

(1) PCR (in the pre-PCR area)

・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

Gold-96well GeneAmp® PCR System 9700

Micropipett

(2) Hybridization (in the post-PCR area)

・Multichannel micropipette

・Micropipette (1~20 μL, 10~200 μL, 100~1000 μL)

・Sterilized micropipette tips

・Repetitive micropipette

・Vortex mixer

・Sterilized 96-well low profile PCR tray

・Centrifuge with swing bucket rotor for 96-well microtiter-plate

Multichannel micropipette

Repetitive micropipette

Vortex mixer

Centrifuge

(3) Data acquisition (in the post-PCR area)

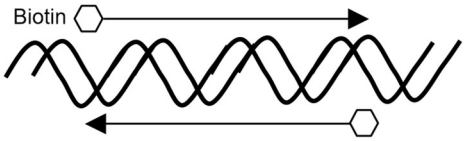
・Luminex system

・Personal Computer

Luminex System

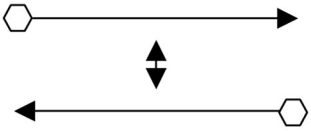
3.3 Procedure of the *WAKFlow* HLA typing kit.

PCR (1.5 hours)



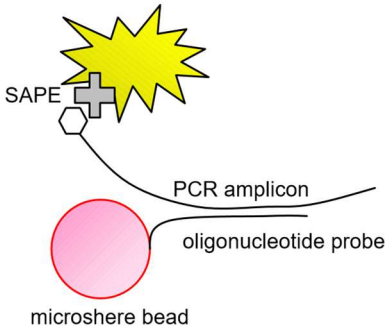
Amplification of target genes with biotinylated locus-specific primers.

Denaturation (5 minutes)



Denaturation of the amplified DNA into single strand.

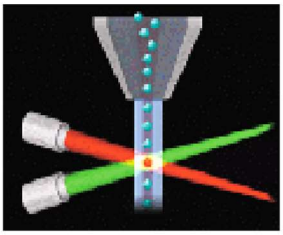
Neutralization, Hybridization, and fluorescent labeling (30 minutes)



Hybridization of denatured DNA to oligonucletide probes immobilized on microshpere beads and fluorescent labeling with SAPE in a single step.

Washing (5 minutes)

Data acquisition and anaiysis with the Luminex system



3.4 *WAKFlow* HLA typing kit protocol

(1) Preparation of sample DNA (in the pre-PCR area)

Sample DNA should be prepared in a pre PCR area to avoid PCR contamination. Recommended concentration of sample DNA is 20 ng/μL. Adjust concentration with nuclease-free water if necessary.

(2) PCR (in the pre-PCR area)

Note ・Wear lab coat and gloves dedicated for the pre-PCR area to avoid PCR contamination.
・Enter the PCR program below into your thermal cycler. Turn on the thermal cycler to warm up heated lid.
・Mix thoroughly "PCR Pre-mix" before use.

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

Components of a 1x PCR mixture
① PCR Pre-mix (specific for each HLA locus)・・・・・・・・・・・・・24.5μL
② DNA Polymerase Soln. (specific for each HLA locus)・・・・・・・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 for each sample DNA.

2-3 Cap or seal PCR tubes or PCR tray and place samples in a thermal cycler, then run the program below.

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

Note ・This PCR program may take about 1.5 hours when utilizing “GeneAmp® PCR System 9700 Gold-96-well”, “Veriti™ 96-well Thermal Cycler 0.2 mL” or “VeritiPro™ 96-well Thermal Cycler 0.2 mL”. For other types of thermal cyclers, it may be necessary to optimize the PCR cycle empirically.

・For “GeneAmp® PCR System 9700”, set “Reaction volume” to 27 µL and “Ramp Speed” to MAX mode on the “Selection Method Options” window before starting the PCR reaction.

・For “Veriti™ 96-well Thermal Cycler 0.2 mL”, use “9700 Mode” or “9700 Max Mode” (depending on the version) for setting the PCR program. For “VeritiPro™ 96-well Thermal Cycler 0.2 mL”, use “GeneAmp® PCR System 9700” for setting the PCR program.

・The reaction mixture should be stored at 4℃ when it does not proceed to hybridization process immediately after PCR. Storage at room temperature may cause low signals.

(3) Hybridization and data acquisition

Note ・Wear lab coat and gloves dedicated for the post-PCR area ・Turn on a thermal cycler and run the program of 55°C HOLD.

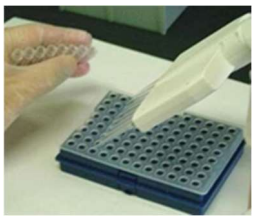
・Turn on the Luminex 100 and XYPlatform, and perform the warm-up procedure.

・Make sure that the "Denaturation soln.", "Hybridization soln.", and "Wash soln." are at room temperature when used.

・Denaturation soln. is an alkaline reagent. Wear protective glasses and gloves.

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

3-2) Add 5 µL of PCR product of step 2-3 into each well, then mix thoroughly by pipetting or vortex, and incubate at room temperature for 5 minutes.



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

Components of a 1x hybridization mixture

④ Hybridization Soln. ・・・20μL
⑤ Beads mix (specific for each HLA locus)・・・・・・・・・・・・・・・・・・・・・・・・3μL
⑥ SAPE ・・・2μL

Note ・Suspend the "Beads mix" thoroughly by vortex before use.

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

Note ・Apply of the hybridization mixture should be done quickly (within 3 minutes) because the “Beads mix” and "SAPE" are light sensitive.
・Sealing of the PCR tray should be done thoroughly to avoid well-to-well sample contamination.
・After mixing the PCR tray, collect the hybridization mixture to the bottom of the PCR tray by snapping.

3-5) Place the 96-well PCR tray onto the thermal cycler pre-warmed to 55°C, tighten the lid, and 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 soln." to each well, then centrifuge the plate for 1 minute at 3,000 rpm (approx. 1000x g).
Remove supernatant carefully by snapping or aspirating.

Note ・Be sure to leave a small amount of solution, approx, 10 to 25μL. Do not discard the hybridized beads from the PCR tray.

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

3-8) Eject the tray stage from the Luminex XY Platform, place the PCR tray in step 3-7, and then retract the stage.

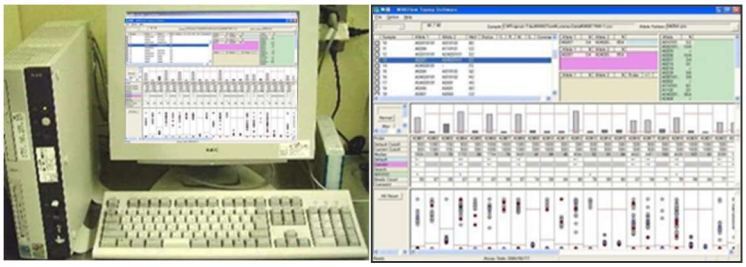
3-9) Choose a template file corresponding to the product lot, enter number of samples, and then start data acquisition.

(4) Data acquisition and analysis

Note ・Data acquisition should be carried out immediately. Store the samples under dark condition at 37℃, if they are not measured immediately.

4-1) Open an output.CSV file, which is automatically created after data acquisition, with HLA typing software, and perform genotyping. Positive or negative reaction is assigned based on the pre-set cut-off value for each typing probe. A fluorescent signal higher than the cut-off value is defined as a positive reaction. HLA alleles are assigned automatically by matching the pattern of positive and negative reactions of each bead.

HLA alleles used for the ***WAKFlow*** HLA typing kit are cited from the report of the Anthony Nolan Research Institute HLA Informatics Group（http://www.anthonynolan.org.uk/HIG/index.html）, and updated in January every year.



4. Warning or caution

1) This product is for research use only. Do not use for the diagnosis and prognosis of disease.
2) Do not use expired reagents.
3) Denaturation Soln. and Hybridization Soln. are corrosive and may cause burns. In case of contact with reagents, immediately wash the eyes or skin with a large amount of water. Call a physician in case of burns, inhalation, ingestion and so on.
4) PCR contamination may cause incorrect typing results. To avoid PCR contamination;
   - Separate pre-PCR area from post-PCR area.
   - Pre- and Post-PCR area must have their own separate set of lab coat, gloves, and equipment, including micropipettes, pipette tips, racks, thermal cycler, and so on. These items should not leave the area to which they are assigned.
   - After the work, wipe the lab bench with 0.5% sodium hypochloride solution or bleach diluted ten-fold with H_2O.
   - Avoid autoclaving used 96-well plates or tubes on which PCR products may be present. Complete degradation of PCR products is not achieved by autoclaving and aerosols containing PCR products from an autoclave may cause PCR contamination.
5) The specifications may be changed to improve the kit without notice.

≪Effective period≫ 18 months （indicated on product box）

≪Packaging unit≫ 96 tests / kit

≪Contact≫

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