≪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

≪Manufactured by≫

Wakunaga Pharmaceutical Co., Ltd.

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****Revised in Jun. 2022**

***Revised in Apr. 2021**

PRODUCT INSERT

WAKFlow HLA Typing Kit
HLA-A

Research use only

Please read this product insert before use and keep for your convenience.

≪Principles≫

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 of 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*** Typing Software.

≪Kit components and storage conditions≫ (96 tests / kit)

Amplification Package Store below −15℃

① PCR Pre-mix (HLA-A) ····· 1323 µL (2 tubes)
② DNA Polymerase Soln. (Class I) ····· 54 µL (1 tube)

Detection Package Store at 2 – 8℃

③ Denaturation Soln. ····· 1000 µL (1 tube)
④ Hybridization Soln. ····· 2200 µL (1 tube)
⑤ Beads mix (HLA-A) ····· 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℃ for longer storage.
⑤ Beads mix and ⑥ SAPE must be stored at 2 – 8℃ under dark conditions.
Do not mix components from different lots or products.

≪Instrument and equipment requirements≫

Information about instrument and equipment requirements is described in the ***WAKFlow*** HLA Typing kit Instruction manual, which can be downloaded for free from our web site, http://www.wakunagahla.jp/, as pdf format.

≪Directions for use≫

1. Preparation of sample DNA

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 a lab coat and gloves dedicated for a pre-PCR area to avoid PCR contamination.

2-1. Prepare the appropriate amount of the following PCR mixture. This step should be done on ice to avoid non-specific amplifications.

Components of a 1x PCR mixture	
① PCR Pre-mix (HLA-A)	24.5 μL
② DNA Polymerase Soln. (Class I)	0.5 μL

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

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

PCR program

93℃ →	93℃ →	60℃ →	72℃ →	4℃
3 min	30 sec	30 sec	30 sec	Hold
	↑		↓	
	40 cycles			

■ 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 a lab coat and gloves dedicated for a post-PCR area.

・Turn on the thermal cycler and run the program of 55℃ HOLD.

・Turn on the Luminex system, set temperature of XY Platform to 37℃, and perform the warm-up procedure.

・Make sure that "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 plate. 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 – 10** minutes.

3-2. 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.	20 μL
⑤ Beads mix (HLA-A)	3 μL
⑥ SAPE	2 μL

■ Thoroughly suspend the Beads mix by vortex before use.

3-3. Add 25 μL of the hybridization mixture prepared in step 3-2 to the denatured DNA from step 3-1. Seal the 96-well PCR plate tightly with a plate-sealing sheet, and mix thoroughly by vortex. Collect the hybridization mixture to the bottom of the PCR plate by snapping.

3-4. Place the 96-well PCR plate onto the thermal cycler pre-warmed to 55℃, tighten the lid of the thermal cycler, and then incubate for 30 minutes.

3-5. Add 75 μL of "Wash Soln." to each well, then centrifuge the plate for 1 minute at 3,000 rpm (approx. 1,000x g). Remove the supernatant by snapping (More than 5 μL of supernatant remaining after snapping may lead to high background signal. Additional washing step may be effective to lower the background signal).

3-6. Add 75 μL of "Wash Soln." to each well.

3-7. Make sure that XY Platform is set at 37℃. Place the PCR plate, choose a template file or a protocol file corresponding to the product lot, and then start data acquisition with the Luminex system.

Note: It will be a cause of non-specific hybridization if XY Platform and the sample plate are left at room temperature.

* 4. Data analysis

Open the csv file, which is automatically created after data acquisition in the "output" folder, with ***WAKFlow*** 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://hla.alleles.org/alleles/index.html), and updated in January every year.

≪Performance≫

1. **Specificity**
   The same results can be obtained when testing according to this operating procedure using defined number of HLA-known standard samples.
2. **Sensitivity**
   Hybridization signals specific for each probe can be obtained when testing with defined number of HLA-known standard genomic DNAs and standard plasmid DNAs.
3. **Within-run reproducibility**
   The same results can be obtained when repeating the test three times at once.