Morgan™ HLA SSP B27 Typing Kit

Instructions for Use

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1. INTENDED USE

This kit provides the HLA-B27 typing results by a DNA-based method.

2. SUMMARY AND EXPLANATION

The HLA molecules of the human major histocompatibility (MHC) antigens are key elements in restricting the specificity of T-cell mediated immune responses.\(^1\) HLA-B27 is one of the types of the HLA-B locus. The association between HLA-B27 and ankylosing spondylitis has been widely demonstrated.\(^2,3\) Ankylosing spondylitis is a form of chronic inflammation of the spine and the sacroiliac joints. The majority of patients with ankylosing spondylitis are B27 positive. HLA-B27 typing provides an aid to diagnosis of ankylosing spondylitis.

This Morgan\textsuperscript{TM} HLA SSP B27 Typing Kit provides a DNA-based method for determining HLA alleles involving PCR process using sequence specific primers (SSP).\(^4\)

\begin{center}
\framebox[1\textwidth]{{\textbf{WARNING:} The results obtained from the Morgan\textsuperscript{TM} HLA SSP B27 Typing Kit should not be used as the sole basis for making a clinical decision.}}
\end{center}
3. PRINCIPLE(S)

The Morgan™ HLA SSP B27 Typing Kit is for determining HLA alleles using PCR techniques with sequence specific primers (SSP). Allele sequence-specific primer pairs are designed to selectively amplify target sequences which are specific to a single allele or group of alleles. This PCR-SSP method is based on the principle that only primers with completely matched sequences to the target sequences result in amplified products under controlled PCR conditions. The presence of amplified DNA fragment is a positive indication of the existence of allele specific sequence in the genomic DNA. On the other hand, mismatched primers do not generate amplicons.

In addition to sequence specific primers, an internal control primer pair, which amplifies a conserved region of the house keeping gene, cystic fibrosis gene, is included in every PCR reaction mix, and the PCR product of the internal control primer pair serves as an indication of the integrity of PCR reaction. When the DNA fragment amplified by sequence specific primer is present, the product of the internal control primer pair is sometimes weak or absent due to the differences in amplifying efficiency between the specific primer pair and the internal control primer pair.

The PCR reaction products are examined by agarose gel electrophoresis which separates the DNA fragments by size. The amplified fragments are visualized by ethidium bromide staining followed by ultraviolet light exposure, and appear as bands.

The Morgan™ HLA SSP B27 Typing Kit supplies single primer mix which is designed to provide positive identification of HLA-B27 alleles.
4. REAGENTS

4.1 Contents of the Morgan™ HLA SSP B27 Typing Kit
The Morgan™ HLA SSP B27 Typing Kit provides sufficient reagents for 96 tests.

4.1.1 Typing Trays
Each tray is designed for 96 tests, consisting of PCR tubes that contain dried primers.

4.1.2 Master Buffer 1020μl
The buffer is optimized for PCR reactions, and contains nucleotides, salts, glycerol and cresol red (final concentration: 0.15 mM dNTP, 1.5 mM MgCl₂, 50 mM KCl, 15.75 mM Tris-HCl, 0.5 % glycerol). Each pre- aliquoted tube is for 96 tests.

4.1.3 PCR Plate Cover Membrane
The cover membrane is used to prevent evaporation loss during the PCR process.

4.2 Warning or Caution
For In Vitro Diagnostic Use

4.2.1 Do not use reagents past the expiration date printed on the label.

4.2.2 The test must be performed by well-trained and authorized laboratory staff.

4.2.3 Reagents should be handled in accordance with good laboratory practice using appropriate precautions.

4.2.4 Pipettes used for post-PCR manipulations should not be used for pre-PCR manipulations.

4.2.5 Biohazard Warning: the ethidium bromide used for staining of DNA is a potential carcinogen. Always wear gloves when handling stained gels.

4.2.6 Biohazard Warning: all blood products should be treated as potentially infectious.

4.2.7 Caution: wear UV-blocking eye protection and do not view UV light source directly when viewing or photographing gels.

See Material Safety Data Sheets for detailed information.

4.3 Storage
The Typing Trays are sealed in pouches and should be stored at 2-8°C. The Master Buffer should be store at -20°C. Any unused Typing Tray should be returned to their original pouch and sealed using the zipper. Once the pouch is opened, the Typing Tray should be used within 4 weeks.

4.4 Instability Indications
Do not use Typing Trays with cracks in the tubes.
5. INSTRUMENT REQUIREMENTS

5.1 Programming the Thermal Cycler
The Morgan™ HLA SSP B27 Typing Kit is standardized for the following program using Applied Biosystems GeneAmp® 9600 and 9700. Thermal cyclers other than the recommended model and/or brand have to be user-validated. The program runs in approximately 1 hr 25 min.

Morgan HLA Program

<table>
<thead>
<tr>
<th>Segment</th>
<th>Cycle Number</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>96°C</td>
<td>2.5 min</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>96°C</td>
<td>15 sec</td>
</tr>
<tr>
<td></td>
<td></td>
<td>65°C</td>
<td>60 sec</td>
</tr>
<tr>
<td>3</td>
<td>22</td>
<td>95°C</td>
<td>15 sec</td>
</tr>
<tr>
<td></td>
<td></td>
<td>62°C</td>
<td>50 sec</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72°C</td>
<td>30 sec</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>4°C</td>
<td>until removed</td>
</tr>
</tbody>
</table>

Please program the thermal cycler according to manufacture’s instruction.

5.2 Gel Electrophoresis
Refer to Section 7.6 Gel Electrophoresis.

6. SPECIMEN COLLECTION AND PREPARATION

6.1 Blood Sample Collection
The peripheral blood can be collected using blood collection tubes with sodium citrate and EDTA as anticoagulants. Contamination of the DNA by heparin can result in interference with the PCR reaction. For this reason, heparinized blood should not be used as a starting material for DNA isolation. Please refer to manufacturer’s instruction for blood sample storage and stability.

6.2 DNA Isolation
Genomic DNA may be obtained from all nucleated cells. The source of cell includes, but is not limited to, whole blood, buffy coat and cultured cells. DNA isolation can be performed by any validated protocol that produces DNA with adequate quality and quantity of DNA followed by repeated user validation.

6.3 DNA Quantity
The DNA sample should be re-suspended in sterile distilled water or appropriate buffer solution at a concentration of 10-40 ng/µl. DNA should
not be re-suspended in solutions containing chelating agents, such as EDTA, above 0.5 mM in concentration.

6.4 DNA Quality
The DNA sample should have an A260/A280 ratio between 1.65 and 1.8. DNA samples may be used immediately after isolation or stored at −20°C or below for extended periods of time (over 1 year) with no adverse effects on the HLA typing results.

7. DIRECTIONS FOR USE

7.1 Materials Provided
Refer to Section 4. REAGENTS

7.2 Materials Required, But Not Provided

7.2.1 PCR
7.2.1.1 Taq DNA polymerase (5 U/µl) (e.g. Fermentas, Cat. #EP0401 or EP0402)
7.2.1.2 Distilled water or DNA diluent
7.2.1.3 Adjustable pipettes
7.2.1.4 Disposable pipette tips
7.2.1.5 Vortex mixer
7.2.1.6 Microcentrifuge
7.2.1.7 Table top centrifuge for 96 well PCR plates
7.2.1.8 Thermal Cycler with heated cover (e.g. Applied Biosystems GeneAmp 9600 and 9700)
7.2.1.9 Pressure pad

7.2.2 Gel Electrophoresis
7.2.2.1 Molecular biology grade agarose
7.2.2.2 0.5x TAE or TBE buffer
7.2.2.3 Ethidium bromide solution (10 mg/ml)
7.2.2.4 Hot plate or microwave oven for heating agarose solutions
7.2.2.5 Gel tray and electrophoresis chamber Power supply
7.2.2.6 UV transilluminator
7.2.2.7 Photographic or image documentation system.

7.3 Sample Preparation
Refer to Section 6. SPECIMEN COLLECTION AND PREPARATION.

7.4 Reagent/Equipment Preparation
Refer to Section 5. INSTRUMENT REQUIREMENTS.
7.5 Performing PCR

7.5.1 Remove the tube of pre- aliquoted Master Buffer from freezer and thaw at room temperature (20-25°C). Each tube is for 96 tests.

7.5.2 Once the Master Buffer is thawed, vortex to mix well. Pulse-spin the buffer tube in a microcentrifuge to bring all liquid down to the bottom.

7.5.3 Remove Typing Trays from fridge. Take out the trays from the pouch. Return any unused tray to pouch and seal with the pouch zipper for future use.

7.5.4 Remove Taq DNA polymerase (5 U/μl) from freezer and keep on ice.

7.5.5 It is recommended to perform 16 tests at one time. If less than 16 tests are to be performed, it is still recommended to prepare the master mix for 16 tests. Prepare the master mix (Master Buffer/Taq polymerase) for 16 tests as followed. Combine 1 μl of Taq polymerase and 180 μl of Master Buffer within a clean eppendorf tube. Cap tube and vortex to mix well. Pulse-spin in a microcentrifuge to bring all liquid down to the bottom.

7.5.6 Dispense 10 μl of the Master Buffer/Taq polymerase mixture into each reaction tube of the Typing Tray. Care should be taken to avoid cross-contamination between wells by applying the master mix to the walls of the tube.

7.5.7 Add 2 μl of DNA sample (10-40 ng/μl) into the reaction tube of the Typing Tray. Each tube is for 1 test. Make sure the DNA sample mix with the master mix in the tube.

7.5.8 Cover the Typing Tray with PCR Plate Cover Membrane. Make sure all tubes are properly covered by the membrane to prevent evaporative loss during the PCR process. Make sure the dispensed solution comes in contact with the dried primers on bottom of each tube by tapping or centrifugation.

7.5.9 Place a pressure pad on top of the PCR Plate Cover Membrane before closing the thermal cycler lid.

7.5.10 Close the heated thermal cycler lid and start the program shown in Section 5.1 of this manual.

7.5.11 After the PCR process is completed, you may proceed to gel electrophoresis immediately or store the tray at –20 ºC and continue to gel electrophoresis at a later time.
7.6 Gel Electrophoresis

7.6.1 Gel Preparation
7.6.1.1 Prepare sufficient 2% agarose gel to resolve PCR reactions according to the original gel electrophoresis system manufacture’s instructions.
7.6.1.2 Make appropriate adjustments to ensure that the gel contains a final ethidium bromide concentration of 0.5 μg/ml.
7.6.1.3 Make sure that each well of the gel creates a minimum volume of 15 μl.
7.6.1.4 Make sure that the running buffer used for electrophoresis is consistent with the buffer used for gel preparation.

7.6.2 Performing Gel Electrophoresis
7.6.2.1 Transfer the entire volume of each PCR reaction mix (12 μl) into the wells in the proper sequence.
7.6.2.2 The electrophoresis takes place at 10V/cm until the cresol red has migrated about 0.7-1.0 cm into the gel.
7.6.2.3 After the electrophoresis is completed, place the agarose gel on an UV transilluminator. Photograph for documentation and HLA type interpretation.

8. Results

8.1 Data Analysis
8.1.1 The DNA fragments amplified the B27-specific primer pairs are designed to have a size of 140 bp. The internal control fragment is 600 bp in size. Therefore, the internal control band is the slower migrating band compared to the specific band. The presence of the specific band is scored as positive for B27. The absence of the specific band is scored as negative.
8.1.2 The internal control band may be weak or absent when the specific band is present. As the presence of the specific band is also an indication to the success of the PCR reaction, this is not a limitation to the test.
8.1.3 The internal control band should always be visible in negative lanes. Absence of both the control band and specific band indicates failed reaction and can void the test result.

8.2 Gel Interpretation
8.3 Limitation of Procedure

8.3.1 Performance of the test can only be guaranteed if the enclosed instructions are strictly followed.

8.3.2 All instruments, including the thermal cycler and pipetting devices, must be calibrated according to the manufacturer’s instructions.

8.3.3 The Morgan™ HLA SSP B27 Typing Kit is standardized for the Taq polymerase from Fermentas. The use of other Taq polymerase may result in non-specific false positive PCR reactions.

8.3.4 The quality and quantity of the DNA samples have to be within the range as specified. The DNA samples should be kept according to instruction.

8.3.5 The results obtained from the Morgan™ HLA SSP B27 Typing Kit should not be used as the sole basis for making a clinical decision.

8.4 Quality Control

Each manufactured lot is checked against a panel of standard DNA samples. The alleles carried by these DNA samples react with most of the primer mixes in the kit. The report is available upon request.
9. PERFORMANCE CHARACTERISTICS

9.1 Specific Performance
Performance was evaluated by testing 88 random whole blood specimens and comparing with one flow cytometry B27 typing system. The results showed 97.7% concordance (86/88) for the HLA-B27 alleles, as summarized in the following table.

<table>
<thead>
<tr>
<th>Allele</th>
<th>Number of Concordant Tests</th>
<th>Number of Tests</th>
<th>Concordance</th>
<th>95% Confidence Limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-B27</td>
<td>88</td>
<td>86</td>
<td>97.7%a</td>
<td>94.6% 100%</td>
</tr>
</tbody>
</table>

a) Further analysis of the two non-concordant test results for the HLA-B27 alleles using one FDA cleared HLA SSP typing product confirmed the two samples carry B*27 alleles.

9.2 Reproducibility
Ten standard DNA samples from International Histocompatibility Working Group (IHWG) were typed using the Morgan™ HLA SSP B27 Typing Kit for 5 times for the within-lot and inter-lot reproducibility studies. The data show 100% reproducibility for HLA-B27 alleles.
## 10. TROUBLESHOOTING

<table>
<thead>
<tr>
<th>Problem</th>
<th>Potential Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weak bands or absence of bands</td>
<td>Poor DNA quality</td>
<td>Repeat DNA purification and make sure the A260/A280 ratio is between 1.65-1.80.</td>
</tr>
<tr>
<td></td>
<td>Insufficient amount of DNA</td>
<td>Make sure the DNA concentration is within the suggested range of 10~40ng/μl.</td>
</tr>
<tr>
<td></td>
<td>Insufficient Taq polymerase activity</td>
<td>Make sure there is sufficient amount of Taq polymerase added and the Taq polymerase has not been degraded.</td>
</tr>
<tr>
<td></td>
<td>Improperly calibrated thermal cycler</td>
<td>Calibrate the thermal cycler.</td>
</tr>
<tr>
<td></td>
<td>Unevenly mixed PCR master mix</td>
<td>Make sure the master mix is thoroughly mixed before use.</td>
</tr>
<tr>
<td></td>
<td>Insufficient ethidium bromide staining</td>
<td>Re-stain the agarose gel in fresh 0.5XTBE buffer with 0.5 μg/ml ethidium bromide.</td>
</tr>
<tr>
<td>Presence of false positive band(s)</td>
<td>Overly high DNA concentration</td>
<td>Make sure the DNA concentration is within the suggested range of 10~40ng/μl.</td>
</tr>
<tr>
<td></td>
<td>Excessive Taq polymerase activity</td>
<td>Make sure the amount of Taq polymerase added is as instructed on page 8.</td>
</tr>
<tr>
<td></td>
<td>Improperly calibrated thermal cycler</td>
<td>Calibrate the thermal cycler.</td>
</tr>
</tbody>
</table>
11. BIBLIOGRAPHY


12. TRADEMARKS USED IN THIS DOCUMENT

Morgan™
TBG Biotechnology Corp.
Applied Biosystems
GeneAmp®

13. PATENTS USED IN THIS DOCUMENT

This product is optimized for use in the Polymerase Chain Reaction (“PCR”) Process which is covered by patents owned by Roche Molecular Systems, Inc. and F. Hoffmann-La Roche Ltd (“Roche”). No license under these patents to use the PCR Process is conveyed expressly or by implication to the purchaser by the purchase of this product. Further information on purchasing licenses to practice PCR may be obtained by contacting, in the United States, the Director of Licensing at Roche Molecular Systems, Inc. 1145 Atlantic Avenue, Alameda, California 94501, and outside the United States, the PCR Licensing Manager, F. Hoffmann-La Roche Ltd, Grenzacherstr. 124, CH-4070 Basel, Switzerland.

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