13th IHWG HLA Class I Reverse Line Strip Typing Protocol:

TABLE OF CONTENTS

	Page
I. DNA Amplification	
A. PCR Materials & Preparation	2
B. PCR Amplification Procedure	2-4
C. Setting the Thermal Cycler	4
D. Gel Electrophoresis	4-5
II. Hybridization	
A. Hybridization Materials	5
B. Solution Preparation	6
C. Hybridization / Reverse Line Strip Detection Assay	7-9
III. Allele Determination	
A. Strip Interpretation Software & Accessories	9-10
B. Using the Strip Interpretation Software	10-13
C. Using the Hit Tables	13-15
D. Positive Control Probes	15
E. Undetermined Alleles	15-17
F. Troubleshooting Messages in the Interpretation Program	17-18
IV. Appendices	
A. HLA Class I Probe Region Tables	18-19
B. Allele Associated Faint Probe Signal Tables	20-21
C. Abbreviated Reverse Line Strip Detection Assay	22

January 30, 2001

13th IHWG HLA Class I Reverse Line Strip Typing Protocol:

I. DNA AMPLIFICATION

A. PCR Materials & Preparation:

Provided:

1. HLA Locus Specific Master Mixes (These mixes consist of a Tris-HCl Solution containing glycerol, KCl, dATP, dCTP, dGTP, dUTP, biotinylated locus specific primers, Taq polymerase, and sodium azide.)
2. Positive Control / Reference Cell DNA GO85 (See more below.)
3. 6.0mM MgCl2 Solution

You will need to supply:

4. Sterile water
5. Pre-extracted DNA Samples / The DNA should be at a concentration of approximately 10ng per ul. In addition, these pre-extracted samples should be free of Magnesium ion (Mg^{++}).

B. PCR Amplification Procedure:

1. Wear clean gloves and work in a sanitary environment, as free from post-PCR materials as possible, to avoid contamination at this step. Clean all working surfaces thoroughly before use with a 10% bleach solution.

2. Solutions may precipitate during shipping and/or freezing. Therefore, all DNA samples and HLA Premixes should be completely defrosted, vortexed, and centrifuged prior to PCR setup. This process will ensure that all components of the HLA Master Mixes and DNA from all of the samples is collected and transferred to the PCR tube during pipetting.

3. Positive Control DNA / Reference Cell DNA GO85

   a. A tube of Reverse Line Strip Reference Cell DNA (GO85) has been included with your Quality Control cell panel. Reference Cells are used as positive controls to assess the specificity of probes and primers over the course of the data generation phase of the workshop. Your laboratory will need to amplify and type this Reference Cell DNA along with the workshop samples that are typed using the Reverse Line Strip tests.

   b. Each time you amplify a set of workshop samples for a particular class I locus, perform one positive control amplification for that locus using the GO85 Reference Cell DNA in addition to any other control amplifications you may

perform. Type the DNA amplified in this positive control sample along with the amplified workshop sample DNA.

c. You have received 3 micrograms (ug) of GO85 Reference Cell DNA, at a concentration of 10 nanograms per microliter (ng/ul). Because each Reverse Line Strip PCR amplification requires 150 ng (15 ul) of template DNA, there should be sufficient GO85 DNA for 20 PCR amplifications. Assuming that amplifications will be performed in sets of 96 samples, there should be sufficient GO85 DNA to last for the typing of 560 samples.

4. Assemble the Thermal Cycler tray with enough tubes for all samples plus the positive control GO85 and a negative control. Each reaction is optimized for 150 ng of starting template DNA.

5. Pipette the following into each reaction tube:

   a. Use this mixture for the HLA-A & HLA-C locus amplifications.

      1) 15 ul Pre-extracted DNA sample (10ng/ul)

      2) 30 ul HLA Master Mix (specific to the HLA-A locus or the HLA-C locus)

      3) Add the MgCl2 last. All reaction tubes receive 15 ul of 6.0mM MgCl2.

      4) The total volume for each tube will be 60 ul.

      5) The negative control tube will have 15 ul sterile water, 30 ul Master Mix, and 15 ul 6.0mM MgCl2.

      6) The positive control tube will contain 15 ul GO85 Control DNA at a concentration of 10 ng/ul, 30 ul HLA Locus Specific Master Mix, and 15 ul 6.0mM MgCl2.

   b. Because the HLA-B locus has two Reverse Line Strips used for typing, one for each Exon, there is a need to amplify more DNA to accommodate all of the strip reactions. Use the following combination for the HLA-B locus amplification. Each reaction is optimized for 150 ng of starting template DNA.

      1) 25 ul Pre-extracted DNA sample (10ng/ul)

      2) 50 ul HLA-B Master Mix

      3) Add the MgCl2 last. All reaction tubes receive 25 ul 6.0mM MgCl2.

      4) The total volume for each tube will be 100 ul.

5) The negative control tube will have 25 ul sterile water, 50 ul Master Mix, and 25 ul 6.0mM MgCl2.

6) The positive control tube will contain 25 ul GO85 Control DNA, 50 ul HLA-B Master Mix, and 25 ul 6.0mM MgCl2.

C. SETTING THE THERMAL CYCLER:

Set the Thermal Cycler for the following heat cycles.

1. Set the cycle program for 35 cycles of:
   a. 15 seconds at 95 ^{O} Celsius.
   b. 45 seconds at 60 ^{O} Celsius.
   c. 15 seconds at 72 ^{O} Celsius.

2. Set the hold program for 5 minutes at 72 ^{O} Celsius.

3. Set another hold program for 15 ^{O} Celsius indefinitely.

4. Link the 3 programs together into a Method program before proceeding so that the 35 cycles are run first, then the hold program at 72 ^{O} Celsius, and finally the 15 ^{O} Celsius hold program.

5. In the cycle programs, the ramp times should be left at the default setting (0:00), which is the maximum rate, and the allowed setpoint error is at the default setting (2 ^{O} Celsius).

The entire sequence will run for approximately 1.6 hours. The PCR product can be removed shortly after reaching the 15 ^{O} Celsius hold program or may be left overnight.

Consult the User's Manual on your particular Thermal Cycler model for additional information on programming and operation of the Thermal Cycler.

D. GEL ELECTROPHORESIS

Following amplification, the PCR product can be stored at 2-8 ^{O} Celsius until it is needed for the hybridization procedure. To verify sample amplification at this stage, 5 ul of each amplified PCR sample, along with 2 ul running dye, can be run on an electrophoretic gel. Good resolution is obtained using 15 ul Ethidium Bromide at a concentration of 10mg/ml in the gel and 75 ul Ethidium Bromide added to the running buffer. The approximate sizes of the product bands for each locus are as follows:

LOCI:	FRAGMENT	SIZES:
	Exon 2	Exon 3
HLA-A	480bp	314bp
HLA-B	376bp	355bp
HLA-C	440bp	618bp

An appropriately sized molecular marker for these fragments is the Phage X174 digested with restriction enzyme Hae III.

For more detailed information on the process of gel electrophoresis, please refer to the SSOP Protocol on the IHWG website.

II. HYBRIDIZATION

A. Hybridization Materials:

Provided:

1. DNA Denaturing Solution (This solution contains EDTA, sodium hydroxide, and thymol blue. Caution: This solution is an irritant.)
2. SSPE Concentrate is a 20X sodium phosphate solution with NaCl, EDTA and ProClin 150 (a preservative).
3. SDS Concentrate is a 20% sodium dodecyl sulfate solution with ProClin 150.
4. Streptavidin-HRP Conjugate – This is a Streptavidin-horseradish peroxidase conjugate in an ACES solution with NaCl and ProClin 150 preservative.
5. Substrate A is a citrate solution containing hydrogen peroxide and ProClin 150.
6. Substrate B contains 3,3',5,5'-tetramethylbenzidine (TMB) in dimethlyformamide (DMF). Caution: This compound is toxic if swallowed.
7. Citrate Concentrate is a 20X sodium citrate solution.
8. Locus Specific HLA Reverse Line Strips
9. Polystyrene typing tray (This is a 24 well tray with a transparent lid.)
10. An abbreviated strip detection assay protocol is provided in the Appendix of this document for quick reference. The more comprehensive version follows here.

You will need to supply:

11. Distilled water
12. Waterproof marking pen – An erasable *black ink* pen works well.
13. Rotating hot water bath

B. Solution Preparation:

Prepare the following solutions in advance with the concentrates provided:

1. **4X Hybridization Buffer**
   a. Mix the distilled water, SSPE concentrate, and SDS concentrate together in the following order. To aid dissolution, the concentrates may be heated to 50°C.

      1) 55 ml SSPE Concentrate

      2) 213 ml distilled or deionized water

      3) 6.9 ml SDS Concentrate

   b. Mix well. The prepared Hybridization buffer can be stored at room temperature for 3 months.

2. **Ambient Wash & Stringent Wash Buffers**
   a. Mix the SSPE and SDS concentrates together with the distilled water in the following order.

      1) 65 ml SSPE concentrate

      2) 1228.5 ml distilled or deionized water

      3) 6.5 ml SDS concentrate

   b. Mix well.

   c. Divide the Wash Buffer into 2 separate dispensers. Use 275 ml for Stringent Wash Buffer and 1025 ml for Ambient Wash Buffer. Wash buffers can be stored at room temperature for 3 months.

3. **Working Citrate Buffer**
   a. Mix 30 ml of Citrate concentrate with 570 ml distilled or deionized water.

   b. Mix well. The Citrate solution can be stored at room temperature for up to 3 months.

   The final volumes of these solutions are sufficient for 50 hybridizations.

C. Hybridization / Detection Assay Procedure:

1. Warm the water bath to 50 ^{O} Celsius. Adjust the water level to ½-1cm above the shaking platform. Check, at each step, to see that water does not splash into the tray wells at a shaking speed of 60rpm.

2. Add the Denaturing Solution to each amplified PCR sample in a one to one ratio. For HLA-A & C, add 55 ul Denaturing Solution to each well. HLA-B samples will need 95 ul per well. (Each calculation assumes 5 ul of PCR sample was used for gel electrophoresis.) Mix by pipetting up and down. Allow 10 minutes at room temperature for complete denaturation. Denatured PCR samples may be stored for up to 1 week at 2-8 ^{O} Celsius.

3. Label the Reverse Line Strips with a waterproof pen at the blank end. Place them lined side up into the tray wells. Use gloves and clean forceps. **Each strip should be placed in an individual well.** For HLA-B, there are two strips per locus (one for each Exon). Each HLA-B strip must be placed in an individual well.

4. Warm the 4X Hybridization Buffer and Stringent Wash to 50 ^{O} Celsius. (All other reagents will be used at room temperature.)

5. Add 5ml of 50 ^{O} Celsius 4X Hybridization Buffer to each tray well.

6. Add 70ul of each denatured PCR sample to each designated well. Make sure to add the denatured PCR sample to the unlined end of each strip. Gently shake each sample as it is added to the corresponding well. For HLA-B, 70 ul of denatured sample will be added to both the well containing the Exon 2 HLA-B strip and the well containing the Exon 3 HLA-B strip.

7. Put the lid on the tray and place the tray into the 50 ^{O} Celsius water bath for 30 minutes at a shaking speed of 60rpm. Use a weight to hold the tray in place. (The tray may be incubated in the hot water bath for longer than 30 minutes and the results of the hybridization will not be affected. However, the strips should not be left longer than 2 hours to ensure proper results. Longer incubation times create concerns about the water level in the bath being too low - due to evaporation. In addition, excessive condensation on the tray lid can cause cross-contamination between samples.)

8. Remove the tray from the water bath and wipe the inside of the lid. Remove the liquid contents of each well, via suction with a vacuum aspirator, being careful not to touch the strips. It is helpful to tilt the tray and aspirate at the corner near the blank end of the strip (the end of the strip which has the sample number and information written on it.)

9. Add 5ml Ambient wash to each well. Then, aspirate each well again.

10. Add 5ml of the 50 ^{O} Stringent wash to each well.

11. Place the tray in the hot water bath for 15 minutes at a shaking speed of 60 rpm. **The time and temperature of this step is critical to the performance of the assay. (If the strips are left longer than 15 minutes at this step, the strips should be discarded and the assay performed again from the beginning with new strips).**

12. Prepare Working Conjugate solution in a separate beaker:
    a. Add (5.3 ml X Number of Reverse Line Strips) = Number ml Ambient wash buffer
    b. Add (16 ul X Number of Reverse Line Strips) = Number ul Streptavidin-HRP Conjugate

    Do not prepare this solution more than 15 minutes prior to use.

13. Remove the tray from the hot water bath. Rinse the bottom of the lid and wipe the lid dry. Aspirate the well contents. Pipette 5ml of Working Conjugate solution into each well.

14. Place the tray onto the room temperature/dry orbital shaker for 15 minutes at 60rpm.

15. Aspirate the wells. Pipette 5ml Ambient wash into each well. Place the tray on the room temperature shaker at 60rpm for 5 minutes.

16. Repeat step 15 once.

17. Aspirate contents of wells. Pipette 5ml of Citrate buffer into each well.

18. Place on room temperature shaker for 5 minutes at 60rpm.

19. Prepare Working Substrate solution in a separate beaker:
    a. Add (4.4 ml X Number of Reverse Line Strips) = Number ml Substrate A
    b. Add (1.1 ml X Number of Reverse Line Strips) = Number ml Substrate B

    Do not prepare the Working Substrate more than 3 hours prior to use and protect the solution from exposure to direct light. The reagents should be at room temperature prior to mixing.

20. Aspirate the tray wells. Pipette 5 ml of Working Substrate into each well & place onto the room temperature shaker for 10 minutes at 60 rpm. During this step, the blue signal lines should resolve on each Reverse Line Strip.

21. Aspirate the wells. Add 5ml of distilled water to each well. Shake for 5 minutes at room temperature and 60rpm. Then, aspirate the contents.

22. Repeat step 21 two additional times.

23. Add 5ml Citrate buffer to each well. The strips are ready to be interpreted and can be stored in the Citrate buffer, at 2-8 ^{O} Celsius, for up to 3 days.

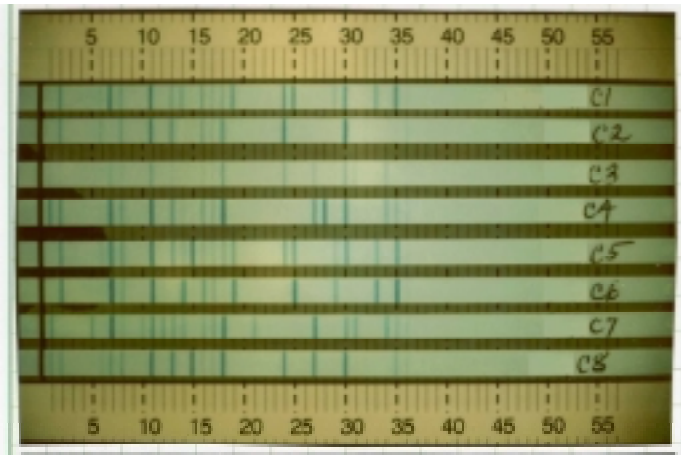
III. ALLELE DETERMINATION

A. Strip Interpretation Software & Accessories

Provided:

1. CD ROM disk / Enclosed with the Reverse Line Strip materials is a CD ROM disk, which contains the software to type each Reverse Line Strip.

2. Strip Interpretation Pad / This pad is used to align multiple strips in order to photograph the Reverse Line Strips for later interpretation, as shown below. Up to twelve strips can be aligned on the pad, by aligning the black reference line on the strips with the black reference line on the overlay. When the reference lines on the pad and strips are correctly aligned, the numbered positions of the positive probes will be indicated.



3. Strip Interpretation Overlay / The Strip Interpretation Overlay is designed to be held over a single strip for interpretation purposes. Align the black reference line on the strip with the black reference line on the overlay, and the numbered positions of the positive probes on your strip will be indicated.

4. HLA Locus-Specific Hit Tables / Hit tables for each locus are included on the CD ROM disk (in Excel file format) or can be accessed via the IHWG website. The hit tables allow for manual strip interpretation and can be used in conjunction with the software to aid in resolving difficult to interpret probe patterns.

You will need to supply:

5. System Requirements: The HLA Software Interpretation Program can be run on Windows 95, 98, 2000 or NT.

6. Minimum Hardware Requirements: Pentium 100MHz with 32Mb of memory.

If your computer system does not meet the minimum system requirements for using the HLA Software, hit tables for each locus are included on the CD ROM disk or can be accessed via the IHWG website for download and/or printing. These hit tables will allow manual allele determination for each locus.

Users with Macintosh systems can obtain the program core on a 3.5 inch floppy disk by sending an email request to 'steve.mack@roche.com'. This program core will function in conjunction with the Macintosh version of the Java Runtime Environment, which can be downloaded for free from www.javasoft.com.

B. Using the Strip Interpretation Software:

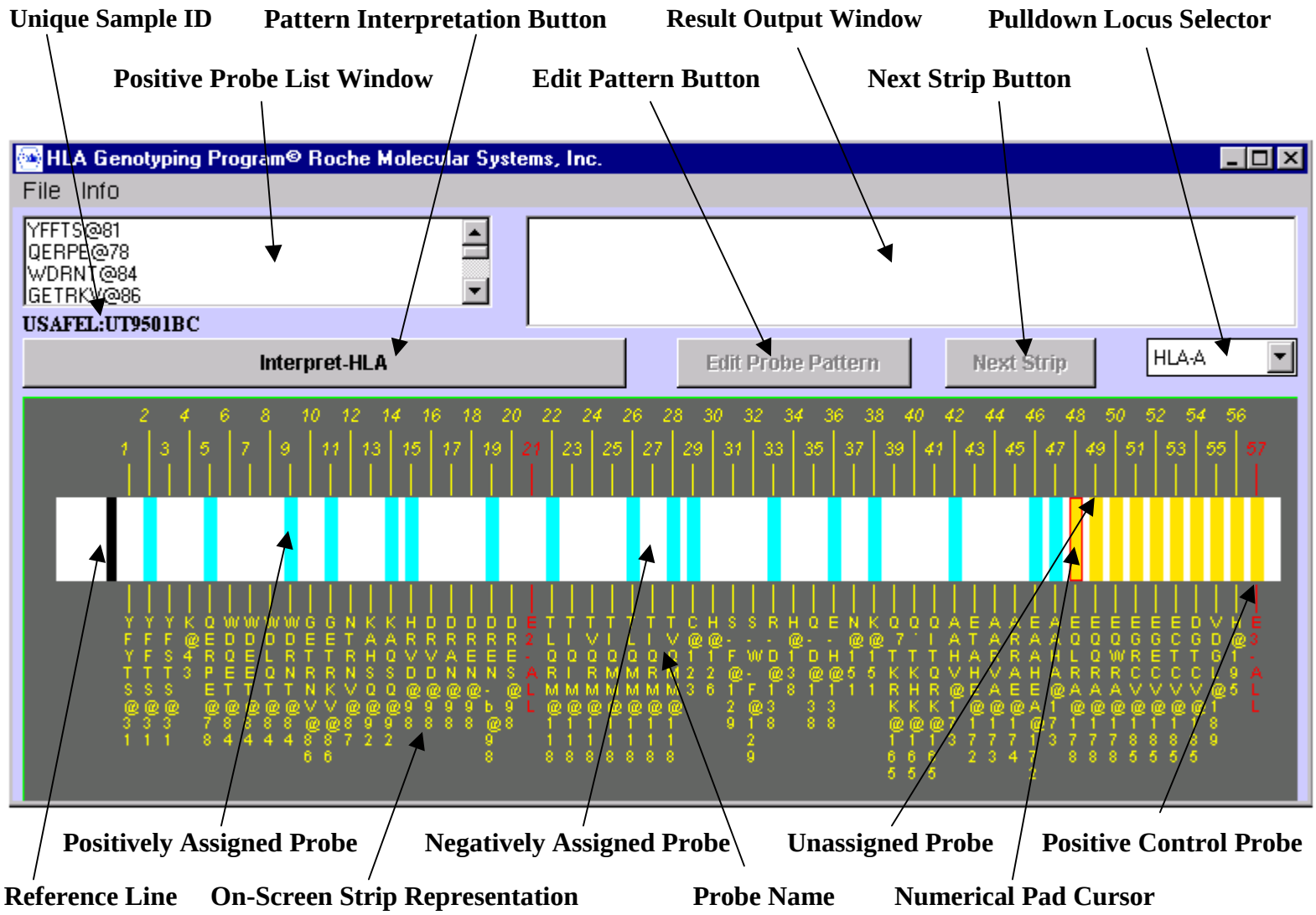
1. To start the HLA typing software, use the mouse to click on the 'HLA.exe' file. An onscreen representation of an HLA-A numbered strip will appear along with a dialogue box titled, "Enter the sample ID". (See page 11 below for a diagrammed pictorial of the opened HLA Interpretation Program to aid in using the software.)

2. Enter the six-character laboratory code and the sample identifier for the strip that you will be interpreting. Click on 'OK' or press return.

3. The default locus is HLA-A. If you will not be typing the HLA-A locus, use the pulldown menu on the right hand side of the window to choose the alternate locus that is being typed for that session, i.e. HLA-B or C. Point and click on the locus to access its hit pattern. A representation of a numbered strip will appear in the main box. The black reference line on this strip will be on the left.

13th IHWG HLA Class I Reverse Line Strip Typing Protocol:

4. Identify the numbered positions of the positive probes (blue lines) on your strip by using either the interpretation overlay, or the interpretation pad.



5. The HLA type is assigned by entering the pattern positive probes on the typing strip into the computer representation of the strip, as seen above. There are two ways in which to enter this information:

a. Using the mouse, click on the appropriately numbered position of each positive probe, which should correspond to the positive probes on the hybridized strip. Each time, a yellow line should change to a blue line, indicating that that probe has been marked as positive. As each probe is selected, its name will appear in the upper left-hand window. Positive probes appear as blue lines on the strip representation, while negative probes will remain yellow.

b. Positive and negative probes can also be entered by using the computer's numeric keypad. Proceeding along the strip from left to right, beginning at the black reference line, enter '1' for a positive probe or '2' for a negative probe. Keys '4' and '6' are marked with left and right arrows and will allow left and right movement between the lines on the probe strip as well. Positive probes appear as blue lines on the strip representation as the 1 key is pressed, while negative probes will vanish as the 2 key is pressed.

6. After entering all of the positive probes for a particular strip, click on the 'Interpret-HLA' button or press enter. The Exon 2 and Exon 3 control lines must be entered as positive for interpretation to occur. (See more information in section D on control lines.) The software will present all possible allelic combinations which exactly match the probe pattern in the upper right-hand window (output window). Potential allele combinations which do not exactly match the probe pattern will appear in a separate window titled 'List of potentially matching allele pairs'. These potential allele combinations are provided to assist in making correct allele calls but should <u>not</u> be construed as accurate data. If necessary (see section E below), the probe pattern information can be edited or re-entered by pressing the 'Edit Probe Pattern' button.

7. Individual strip data may be printed at this time using the 'File' pulldown menu and selecting 'Print strip info'. This will print the probe pattern –an actual color representation of the strip data entered - and associated data shown in the adjacent windows. Note: this may require a color printer. (Allelic data for <u>all</u> the strip information entered will be retained by the software and can be printed at one time at the end of the session. This subject is addressed below.)

8. The software is now ready for the next strip's probe pattern information to be entered. Click on 'Next Strip'. You will be prompted to enter the sample identifier for the next strip to be interpreted. The lab code will be remembered between strips. Click on 'OK' or enter to continue, and the representation of the strip will be cleared of all positive and negative probe assignments.

9. Enter the positive and negative probes from the next sample strip as previously covered in steps 4, 5, and 6.

10. To print the data which has been entered for all the strips interpreted during the session, click on the 'File' menu and select 'Print all entries'.

11. To save all the data which has been entered during a session, click on the 'File' menu and select 'Save as (all the entries)…'. A new window titled 'Save as a text file' will appear. Specify the directory in which you want to save the file, and then enter the name of the file in the 'File name:' box. NOTE: Enter ONLY the filename. Do not add a file-type suffix. For example, if you enter the file name 'antarctica', the file will be named 'antarctica.txt' on the disk. Do not specify the .txt suffix.

12. There is an 'info' pulldown menu on the typing program which is next to the 'file' pulldown menu. It keeps track of how many strips have been typed since the software has been opened and the data last saved. This function allows you to clear the buffer of typing information, setting the number of strips to zero. Do not clear the buffer until after the data has been saved.

    Data from several interpretation sessions can be appended to a given file by supplying the filename (without a suffix) in the 'File name:' box. In general, it is recommended that you append all data for the same population at the same locus to the same file, and that you save data for different populations and different loci to different files.

13. Data files can later be viewed with a standard text-editor, printed, or submitted via email to the central database at database@ihwg.org. To save additional data or append a file's data, the file must be closed first before saving. (i.e. You may not have the data file open for viewing while trying to save information to the file.)

14. If there is an attempt to change the HLA loci during a typing session, a dialogue box titled 'Decision' will appear. This box asks if the user has saved the current loci data that has been generated during the session. If you answer 'No', you will be allowed to save the data as described in step 11. If you answer 'Yes', any unsaved data will be lost as you proceed to the new locus.

NOTE: The HLA-B probe in position number 28 will not affect strip interpretation when toggled. This probe has been eliminated from consideration by the strip interpretation software, as it is used to distinguish ambiguities between alleles which are not resolved as part of the IHWG allele set. This will not affect allelic results for the workshop typing and is mentioned only to keep the software user from being concerned that they are getting inaccurate results.

C. Using the Hit Tables

An excel file containing a probe reactivity table (hit table) has been included for each HLA locus. Each of these tables relates the unique pattern of positive probes for each allele (or group of related alleles).

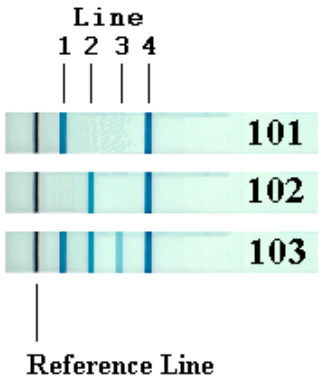
Probes which are positive for a given allele are indicated as colored boxes on a given line, and probes which are negative for that allele are indicated as empty or white boxes on that line.

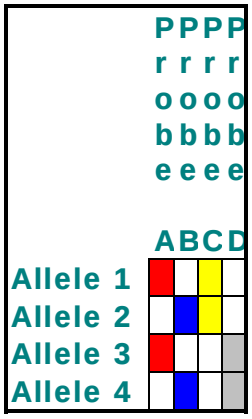
The order of probes on the Reverse Line Strip corresponds exactly to the order of probes on the hit table; each column represents the reactivity for a given probe. Each positive blue-line probe signal on a Reverse Line Strip will correlate to the appropriate colored probe box on the hit table.

The identity of a given allele may be 'read' off of a strip by matching the positive probe lines on the strip with the colored boxes on the hit table. Since most individuals are heterozygous at a given locus, the task identifying the identity of the alleles becomes one of finding a **pair** of allele-specific probe reactivity patterns (row of colored boxes) which matches the pattern of positive probes on the Reverse Line strip (see example below).

It should be noted that because some pairs of allele-specific probe reactivity patterns can be generated from different sets of alleles, some patterns of probe reactivity on the reverse lineblot will be consistent with more than one genotype. Consequently, not all allele pairs can be resolved using this system.

Here the probe reactivities for a set of three strips (labeled 101-103) can be compared to the hit table for four alleles (Allele 1-4), which are distinguished by four probes (A-D). Because the order of the probe lines on the strip corresponds to the order of the probes on the hit table, it can be inferred that probes A and D are positive on strip 101, probes B and D on strip 102, and all four probes A, B, C, and D on strip 103.





Looking at the pattern of positive probes (colored boxes) on the hit table, we can see that probes A & D are positive for Allele 3. Therefore the individual typed using

strip 101 must be homozygous for Allele 3. Similarly, probes B & D are positive for Allele 4, so the individual typed using strip 102 must be homozygous for Allele 4.

Because we know that each of these alleles is only positive for two probes, we can tell that the person typed using strip 103 is heterozygous. However, it is not possible to distinguish between the probe patterns for two sets of heterozygous alleles. The person may be heterozygous for Alleles 1 and 4 (for which probes A & C and B & D are positive) or they may be heterozygous for Alleles 2 and 3 (for which probes B & C and A & D are positive).

D. Positive Control Probes

The position of the positive control probes on the Reverse Line Strips for each HLA Class I locus is indicated in the table below.

REVERSE LINE STRIP:	CONTROL	POSITION:
	EXON 2	EXON 3
HLA-A	Probe 21	Probe 57
HLA-B	Probe 42	Probe 40
HLA-C	Probe 17	Probe 36

There is one positive control line per Exon for each of the Reverse Line Strips. The signal intensity for these control lines is designed to be lighter than all other positive lines on the strip. Therefore, an absent control line could mean that other positive probe signals are not being detected. If the control lines are not visible on a particular strip, it is recommended that typing be repeated for that sample. The presence of the control lines provides assurance that the appropriate probe lines are clearly visible.

E. Undetermined Alleles

1. When alleles cannot be determined from the entered probe pattern, one of several messages may appear in the upper-right hand window. If the entered probe pattern bears no resemblance to any allele pairs, the message 'No exact match. Possible new allele.' will be displayed in the output window. In this case, there may be a novel allele pattern, which confounds interpretation, or there may have been several transcription errors made when the data was entered into the strip representation. Take a moment to review the pattern of positive probes on the strip, and compare it to the representation on the screen. It may be necessary to edit the pattern in order to determine the alleles.

2. If the entered probe pattern resembles a particular allele pair but differs at one or more probe positions, the message 'No exact match.' will appear in the output window. The 'List of Potential Alleles' window will also indicate each potential pair of alleles for each Exon or Exons in which positive probe lines may have inadvertently been entered or missed. For example, '<Possibly extra lines entered in both Exons, or new allele>' means that the pattern might represent a new allele, or that extra probes could have been entered for each Exon. As in the example above, the strip representation should be compared to the probe pattern on the strip, and edited if necessary.

3. Although considerable effort has been made to have all blue positive probe lines develop at equal intensities, there is variation between the intensity of blue positive signals on each strip; some positive blue signals may be stronger than others. There are several contributing factors to this variation in intensity and some understanding of the confounding factors may be helpful in analyzing strip results.

   a. Cross hybridization occurs when one probe hybridizes weakly with non-complementary DNA. This weak hybridization results in a faint false positive probe line. The control line for each Exon and locus are designed to aid in determining which signals are actually positive and which can be attributed to cross hybridization. A signal as dark as or darker than the control (equal to or stronger than the control) is most likely positive. A fainter signal is most likely cross hybridization and should be considered negative for strip interpretation purposes.

   b. Secondary structure interactions are the result of base pairing in the amplified DNA and may affect hybridization with the probes and reduce the strength of valid positive signals. Secondary structure can contribute to false negatives during interpretation. These signals are not as easily distinguished as cross-hybridization signals but must be entered as positive probes for accurate strip interpretation to occur. Here are some guidelines to help distinguish secondary structure interference.
      1) Use the locus specific hit tables in conjunction with the HLA Interpretation Software to help resolve the false negatives for a particular Reverse Line Strip allele pattern. First, using the software, determine all plausible allele patterns. Second, use the hit table to look at each of these allele patterns more closely. Further examination of the Reverse Line Strip may reveal a very faint positive at an appropriate probe for a particular allele.
      2) Some faint signals, due to secondary structure interactions, are associated with particular alleles. Thus, a given probe will give a strong signal for one allele but a weaker signal for another allele, both of which are complementary to the probe. Usually, these problematic alleles are rare and will not be encountered. Some of these are documented in Appendix B for reference.

   c. Contamination between samples may result in extra signals that are false positives. To avoid potential contamination during hybridization, the water level

in the bath must be adjusted so that it does not enter the top of the tray while the tray is shaking at 60 rpm. The strip interpretation software may be able to detect contamination when more than two allele combinations are represented in any one probe region of the Reverse Line Strip. Use the HLA hit tables in conjunction with the software to determine where contamination has occurred. No more than two probes should be positive in any given region. The hit table will allow the user to distinguish extra signals in a probe region, i.e. more than two positive probes per probe region (one from each parent) is evidence of contamination between samples during hybridization. Probe regions are delineated for each locus in Appendix A.

4. Samples which appear to be new alleles, presenting novel probe patterns should be referred to the IHWG sequencing core for further investigation.

F. Troubleshooting Messages in the Interpretation Program:

Messages which appear in the Result Output Window:	Action:
'No exact match. Possible new allele.'	(1) Make sure the pattern on the strip matches the pattern on the screen. (2) Make certain that the locus specified in the pulldown corresponds to the locus specific strip being interpreted. (3) Make sure that the reference line on the strip is properly aligned with the reference line on the interpretation overlay or pad.
'[N] too many positive probes in region [X]. Contamination likely.'	Check the on-screen strip representation to make sure that all of the lines which have been entered as positive are positive on the Reverse Line Strip. Refer to the section on sample contamination. (See section E on Undetermined Alleles, no. 3, part C.) New Reverse Line Strips may have to be run for these samples.
'Matches found for Exon[X], but no matches found for Exon[Y]'	Check to see that the strip entries for Exon [Y] are all entered correctly and match the blue positive signals on the Reverse Line Strip.

Messages which appear in the List of Potential Allele Pairs Window:	Action:
'Possibly one or more lines missed in Exon 2, or new allele'	Check the strip to make sure that all of the lines which are positive at Exon 2 are entered as positive in the strip representation.
'Possibly one or more lines missed in Exon 3, or new allele'	Check the strip to make sure that all of the lines which are positive at Exon 3 are entered as positive in the strip representation.
'Possibly extra lines entered in Exon 2, or new allele'	Check the on-screen strip representation to make sure that all of the lines which have been entered as positive at Exon 2 are positive on the Reverse Line Strip.
'Possibly extra lines entered in Exon 3, or new allele'	Check the on-screen strip representation to make sure that all of the lines which have been entered as positive at Exon 3 are positive on the Reverse Line Strip.
Possibly XXXX + XXxx(N) cases	As the window titled, 'List of Potentially Matching Allele Pairs' opens, potential alleles and allele family pairs will appear at the top of the window to assist you in confining potential errors that are made while entering the pattern from the strip. Consult the hit tables for these allele combinations and compare the positive probe patterns to the pattern saved on the strip.

IV. Appendices

A. HLA Class I Probe Region Tables

The message '[N] too many positive probes in region [X]. Contamination likely.' will appear if 3 or more probes come up in the following regions:

13th IHWG HLA Class I
Reverse Line Strip Typing Protocol:

1. HLA-A

HLA – A Probes:	Region:
Probes1-3	A
Probes 6-9	B
Probes 10-12	C
Probes 15-20	D
Probes 22-28	E
Probes 33-36	F
Probes 39-41	G
Probes 42-47	H
Probes 48-50	I
Probes 51-54	J

2. HLA-B

EXON 2

HLA – B Probes:	Region:
Probes 1-6	A
Probes 7-10	B
Probes 11-14	C
Probes 17-21	D
Probes 22-24	E
Probes 25-32	F
Probes 33-37	G
Probes 38-41	H

EXON 3

HLA – B Probes:	Region:
Probes 1-10	I
Probes 13-21	J
Probes 22-24	K
Probes 25-29	L
Probes 30-32	M
Probes 37-39	N

3. HLA-C

HLA – C Probes:	Region:
Probes 1-5	A
Probes 8-10	B
Probes 18-23	C
Probes 24-28	D
Probes 30-33	E

B. Allele Associated Faint Probe Signal Tables

For each table, the Probe Motifs are shown beneath the on screen strip representation. The probe names are listed on the hit tables.

1. HLA-A

LINE POS.	PROBE MOTIF	PROBE NAME	** COMMENTS
3	YFSTS@31	DB352BSA	Faint signal on A*3002
6	WDQET@84	HLAA226BSA	Very slight cross-hyb with WDRNT,WDGET, WDLQT;slightly faint signal on A*03's
10	GETRNV@86	HLAA712BSA	Slight cross-hyb with GETRKV (probe #11)
11	GETRKV@86	HLAA603BSA	Slight cross-hyb with GETRNV (probe #10)
14	KAQSQ@92	HLAA573BSA	Faint signal on A*03 (accounted for by software)
19	DREN-b@98	HLAA705BSA	Faint signal overall
25	TIQMM@118	HLAA596BSA	Faint on A*3201
27	TIQRM@118	HLAA664BSA	May be faint on A*25/26/68...
28	TVQMM@118	HLAA672BSA	Slightly faint signal
33	R-D@138	HLAA685BSA	Faint on A*29 and A*68
35	Q-D@138	HLAA629BSA	Faint on A*3201
41	QITQRK@165	HLAA632BSA	Faint on A*3002, A*3201 / cross-hyb with A*2402 - should be negative
44	AARVA@173	HLAA244BSA	Faint on A*3201
46	EAAHEA@172	HLAA633BSA	slight cross-hyb with AAHV
48	EQLRA@178	HLAA61BSA	Faint signal on A*3002 and A*3201
			** Weak signals on certain alleles can be masked in heterozygous combinations;
			a strong signal will be observed if the other allele is positive for the same motif.

2. HLA-B

LINE POS.	PROBE MOTIF	PROBE NAME	COMMENTS
11	DTQFVa	DB818B	Weak with B*1511 & B*5401
14	DTLFV	DB1121B	Weak with B*44xx, B*0702x & B*4901, B*4701, and B*4101 & B*7301
25	IF-TNT	DB1158B	Weak w/B*08xx & B*18xx, B*0702x & B*3503, B*0802 & B*2708, B*5101, B*0801, B*3901, B*35XX
77	RRYLEN	RAP374B	Weak with (B*3701), (B*5401), and (B*39xx & B*55xx)

Probe 25
Although the HLA-B faint allele table addresses probe 25, there have been several miscalls due to weak signal intensity on probe 25. Please consider probe 25 carefully during strip interpretation.

3. HLA-C

LINE POS.	PROBE MOTIF	PROBE NAME	COMMENTS
6	ASPRGE(a)	DB1019B	Weak w/C*03031, C*03041, C*0302, C*14XX
7	ASPRGE(b)	DB961B	Weak with C*0501, C*0701/06 and C*0803
8	PRAPW(a)	DB1016B	Very faint cross-hyb with C*0602
10	PREPW	DB1018B	Weak with C*0401, C*0304
11	AQTDR	DB1098B	Weak with C*04XX, C*03XX
12	AQADR	DB964B	Weak with C*0803
13	RNLRG	DB1089B	Weak with C*08XX, C*12XX
14	RKLRG(a)	DB1090B	Probe is faint on this batch (with all alleles)***
16	SEAGE / QSEA	DB1329B	Weak with C*0602, C*0701
18	QRMYG(a)	DB1091B	Weak with C*03031, C*0302/6, C*0304
19	QRMYGB	JCC009	Weak with C*03XX
22	QRMSG	DB990B	Weak with C*0501, C*0702/3
31	EQRRAY	DB1083B	Weak with C*0501, C*0802
33	EQWRAY	RAP455	Weak with C*02024. C*06XX

Probe 14

**** The Allele Associated Faint Probe Signal Table for HLA-C indicates that probe 14 is faint on this batch of Reverse Line Strips. Regardless of the availability of this information, probe 14 has been miscalled several times during the Quality Control phase of the Workshop. Please be aware of this caveat and pay careful attention to probe14 during strip interpretation.*

Probe 15

*Probe 15 has consistently shown up as a faint signal. It appears that, unless probe 15 is quite dark, this probe should be called negative. As shown on the C-locus hit table, this probe should only be positive for C*03031/032/11 and C*0312 alleles. The weak nature of probe 14 on this lot of strips, may make it difficult to accurately call probe 15. Please consider this probe carefully while interpreting strips.*

Probe 19

Probe 19 was miscalled by lab participants and has been identified above as a possible false negative call. However, probe 19 has also been called falsely positive. Please consider this probe carefully during future strip interpretations and consult the hit table for HLA-C, when necessary, to help make a determination on probe 19.

C. Abbreviated Reverse Line Strip Detection Assay:

1. Warm the water bath to 50°C and adjust the water level to ½-1cm above the shaking platform.

2. Add Denaturing Solution to each amplified PCR sample in a one to one ratio.

3. Label the Reverse Line Strips. Place each strip in an individual well, lined side up.

4. Warm the 4X Hybridization Buffer and Stringent Wash to 50°C.

5. Add 5ml of 50°C 4X Hybridization Buffer to each tray well.

6. Add 70ul of denatured PCR sample to each designated well. Gently shake the tray as each sample is added.

7. Cover the tray with the lid and place the tray into the 50°C water bath for 30 minutes at a shaking speed of 60rpm.

8. Remove the tray from the water bath and wipe the inside of the lid. Aspirate the liquid contents of each well.

9. Add 5ml Ambient wash to each well. Then, aspirate.

10. Add 5ml of 50°C Stringent wash to each well.

11. Cover the tray and place it in the 50°C water bath for 15 minutes at a shaking speed of 60 rpm. *The time and temperature of this step is critical.*

12. Prepare Working Conjugate:
    Mix (5.3 ml X No. of strips) = ml of Ambient Wash with (16 ul X No. of strips) = ul of Streptavidin-HRP Conjugate.

13. Remove the tray from the hot water bath and wipe the lid dry. Aspirate the wells.

14. Pipette 5ml of Working Conjugate into each well.

15. Place the tray onto the room temperature shaker for 15 minutes at 60rpm.

16. Prepare the Working Substrate:
    Mix (4.4 ml X No. of strips) = ml of Substrate A with (1.1 ml X No. of strips) = ml of Substrate B.

17. Aspirate the wells. Pipette 5ml Ambient wash into each and place onto the room temperature shaker at 60rpm for 5 minutes.

18. Repeat step 17 once.

19. Aspirate the wells. Then, add 5ml Citrate buffer to each.

20. Place on the room temperature shaker for 5 minutes at 60rpm.

21. Aspirate the wells. Pipette 5 ml of Working Substrate into each.

22. Place onto the room temperature shaker for 10 minutes at 60 rpm.

23. Aspirate the wells. Add 5ml of distilled water to each well. Agitate for 5 minutes at room temperature and 60rpm.

24. Repeat step 23 two additional times.

25. Add 5ml Citrate buffer to each well.

26. Interpret the strips.