

InnoTyper[®] 21 Human DNA Analysis Kit – User Guide v3.7

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Amplification Kit Contents for 100 x 25 µL reactions

DNA Control (1 clear top tube)	100 µL @ 0.12ng/µL
InnoTyper [®] 21 Primer Mix (1 red top tube)	385 µL
InnoTyper [®] 21 Master Mix (1 brown top tube)	550 µL
IGT DNA Polymerase (1 green top tube)	55 µL
InnoTyper [®] 21 Allelic Ladder (1 blue top tube)	30 µL

Amplification Kit Contents for 50 x 25 µL reactions

DNA Control (1 clear top tube)	50 µL @ 0.12ng/µL
InnoTyper [®] 21 Primer Mix (1 red top tube)	193 µL
InnoTyper [®] 21 Master Mix (1 brown top tube)	275 µL
IGT DNA Polymerase (1 green top tube)	28 µL
InnoTyper [®] 21 Allelic Ladder (1 blue top tube)	15 µL

Storage Conditions

Upon receipt, store the kit at -20°C. After initial use, store the kit at +4°C.

IMPORTANT! The fluorescent dyes attached to the primers are light-sensitive. Protect the primer set, amplified DNA, allelic ladder, and size standard from light when not in use.

Please note that the InnoTyper[®] 21 Kit is designed to be highly sensitive. All reasonable precautions including clean laboratory techniques should be employed.

I. PCR Reaction Setup

I-1. Calculate the volume of each component needed to prepare the reactions using the table below:

PCR Components for Multiplex	Volume per Reaction 25 µL Total Reaction Mix
InnoTyper [®] Primer Mix	3.5 µL
InnoTyper [®] Master Mix	5.0 µL
IGT DNA polymerase	0.5 µL
DNA Template/Pos. Control/TE ⁻⁴ buffer [†]	Up to 16.0 µL

Note: Include additional reactions (1.1x) in your calculations to provide excess volume to account for the loss that occurs during reagent transfers.

[†]**Note:** It is recommended to use TE⁻⁴ buffer (10mM Tris-HCl [pH 8.0], 0.1mM EDTA). The pH and EDTA concentration of the TE buffer is critical because PCR amplification efficiency and quality can be greatly altered by changes in pH (due to added Tris-HCl), available magnesium concentration (due to chelation by EDTA) or other PCR inhibitors, which may be present at low concentrations. TE⁻⁴ may be ordered from Teknova (Cat # T0223).

I-2. Prepare reagents as follows. Thaw the InnoTyper[®] Master Mix, Primer Mix, and DNA polymerase and equilibrate them to room temperature. Vortex the InnoTyper[®] Master Mix, Primer Mix, and DNA polymerase for 3 to 5 seconds. Briefly centrifuge (~2 seconds) the tubes before opening if the content is not at the bottom of the tubes.

NOTE: Avoid centrifuging the Primer Mix extensively.

- I-3. Pipette the required volumes of Master Mix and Primer Mix multiplex components into a sterile 1.5 mL polypropylene tube. Pulse vortex the mix for 5-10 seconds, then centrifuge briefly (~2 seconds). Add the required volume of the DNA polymerase.
- I-4. Pulse vortex the reaction mix for 5-10 seconds, then centrifuge briefly (~2 seconds).
- I-5. Dispense 9.0 μL of the reaction mix into each reaction well of PCR reaction tubes or 96-well reaction plate.
- I-6. Prepare samples as shown in the following table and add to the reaction mix.
 - Use a final reaction volume of 25 μL .
 - Use TE⁻⁴ buffer[†] for DNA sample dilution, if needed.

Sample	Components and Volume
Negative control	Add 16.0 μL of TE ⁻⁴ buffer (10 mM Tris, 0.1 mM EDTA, pH8.0)
Positive control	Add 4.0 μL of the provided DNA Control plus 12.0 μL of TE ⁻⁴ buffer (Note: ensure the DNA Positive Control has been equilibrated to room temperature, vortexed, and centrifuged briefly prior to adding into the reaction)
Test sample	Add up to 16.0 μL of the DNA sample to the reaction mix.* If <16.0 μL of sample is used, the user should bring the total sample volume up to 16.0 μL using TE ⁻⁴ buffer

***Note:** During development, InnoGenomics has observed an optimal DNA template target range between 0.2 – 0.5 ng on an Applied Biosystems® 3130 Genetic Analyzer. These quantity values are based on the InnoQuant® Quantification & Degradation Assessment Kit. The optimal DNA template target range may vary depending on the user’s instrumentation and protocols, and should be determined based on the user’s internal validation studies.

- I-7. Mix the final reaction mix by pipetting up and down several times.
- I-8. Cover the 96-well reaction plate with clear or optical adhesive film, or cap the tubes.
- I-9. Pulse vortex the reaction mix for 5-10 seconds, then centrifuge the tubes or plates at 3000 rpm for 20 seconds to remove bubbles.

II. Perform PCR Amplification

Note: The InnoTyper[®] 21 kit has been developed and validated using the GeneAmp[®] PCR System 9700 with gold-plated silver 96-Well Block (Life Technologies), using the 9600 Emulation Mode. Use of other thermal cyclers and settings may affect performance of the InnoTyper[®] 21 kit. If you intend to use other thermal cyclers and/or settings, please contact InnoGenomics for further instructions.

Program the thermal cycling conditions:

- Using the GeneAmp[®] PCR System 9700 with either 96-well silver or gold-plated silver block, select the **9600 Emulation Mode**.

Initial Incubation	31 Cycles			Final Extension	Final Hold
	Denature	Anneal	Extend		
HOLD	CYCLE			HOLD	HOLD
95 °C 15 min	95 °C 30 sec	58 °C 30 sec	72 °C 1 min	60 °C 60 min	4°C ∞

II-1. Load the plate/tubes into the thermal cycler and close the heated cover.

Note: If using adhesive clear film instead of caps to seal the plate wells, be sure to place a MicroAmp[®] compression pad (Life Technologies P/N 4312639, or comparable product) on top of the plate to prevent evaporation during thermal cycling.

II-2. Start the run.

IMPORTANT! If storing amplified product long term, store all amplified product at –20 °C and away from light.

III. Genetic Analysis

Required Reagents: Hi-Di[™] Formamide, InnoGenomics ILS-155 internal lane standard (P/N: 21109ILS), InnoGenomics IGT 5 Dye Matrix Standard (P/N: 21109M), POP-4[™] Polymer, Genetic Analyzer Buffer (10X) with EDTA, 36cm capillary arrays.

Allelic ladder requirements: To accurately genotype samples, an allelic ladder sample must be run along with the unknown samples. When genotyping, it is critical to use an allelic ladder run under the same conditions as the samples because size values obtained for the same sample can differ between instrument platforms due to different polymer matrices and electrophoretic conditions.

Recommended number of allelic ladders to run depending on the Applied Biosystems[®] Genetic Analyzer being used:

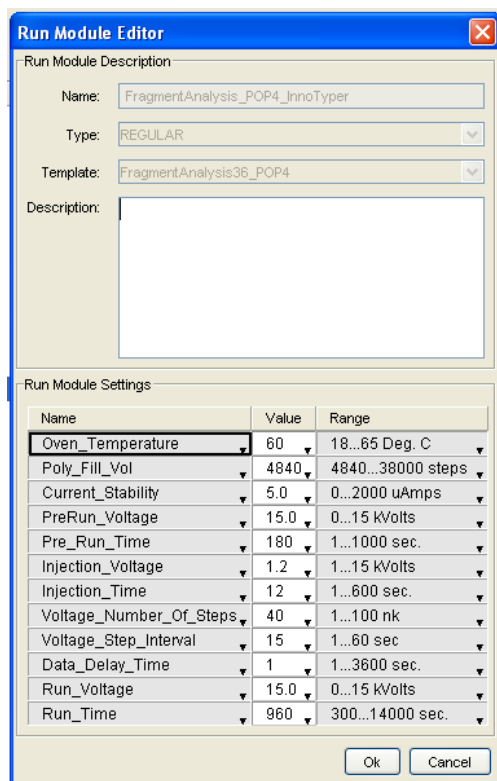
Instrument	Number of allelic ladders to run	One Injection equals	Number of samples per allelic ladder(s)
3500	1 per 3 injections	8 samples	23 samples + 1 allelic ladder
3500xl	1 per injection	24 samples	23 samples + 1 allelic ladder
3130 or 3100- <i>Avant</i>	1 per 4 injections	4 samples	15 samples + 1 allelic ladder
3130xl or 3100	1 per injection	16 samples	15 samples + 1 allelic ladder
310	1 per 10 injections	1 sample	9 samples + 1 allelic ladder

III-A. Instrument Preparation Using the 3130/3130xl:

IMPORTANT! Prior to running samples, ensure that a current InnoGenomics IGT 5 Dye Matrix Standard (P/N: 21109M) matrix file exists. For optimal sensitivity, it is recommended to run the matrix standards every time the polymer bottle is changed.

III-A-1. Open the Data Collection software.

III-A-2. Under *Module Manager*, create a new module “**FragmentAnalysis36_POP4_InnoTyper**” using “FragmentAnalysis36_POP4_1” as a template. During development and validation, InnoGenomics used the default “FragmentAnalysis36_POP4” Run Module Settings for the 4-capillary 3130 instrument, except for one minor change: the Run Time was shortened from 1500 to 960. (The small amplicon size range of the InnoTyper® 21 kit allows for a shorter run time, but this is an optional modification intended to accelerate analysis time.) Below is a screen shot displaying the Run Module Settings used at InnoGenomics with the 4-capillary 3130 instrument. For use with other instrument platforms, it is recommended that the user start with the standard run module settings for fragment analysis using a 36cm capillary and POP 4 polymer on that instrument (i.e. HIDFragmentAnalysis36_POP4_1 or HID36_POP4) and adjust if needed.



Note: The screen shot above displays the Run Module Settings used at InnoGenomics with a 4-capillary 3130 instrument. Injection and run conditions may be modified based on the instrument (i.e. number of capillaries) being used and the laboratory’s internal validation. Please note that the default injection conditions for the 3130xl would have a longer injection time of 18 seconds.

III-A-3. Under *Protocol Manager*, create a new protocol, **Fragment_36cm_InnoTyper**. In the protocol editor box make the following selections:

- “Regular” in the Type drop-down list
- “FragmentAnalysis36_POP4_InnoTyper” for Run Module
- “G5” in the Dye Set drop-down list

III-A-4. In the *Plate Manager*, create a new plate record according to your normal laboratory procedures.

III-A-5. In the *Plate Editor* dialog box, place sample names in the appropriate cells. In the Instrument Protocol column, select **Fragment_36cm_InnoTyper**. Ensure that this information is present for each row that contains a sample name. Select “OK.”

III-A-6. Go to the Spectral Viewer. Choose G5 from the Dye Set drop-down list. From the List of Calibrations for Dye Set G5, select the **InnoGenomics IGT 5 Dye Matrix Standard**, and click “set”.

III-B. Instrument Preparation Using the 3500/3500xl:

III-B-1. To create size standard for **IGT ILS-155** internal lane standard: Refer to the “Size standard library” section in *3500/3500xL Genetic Analyzer User Guide*.

1.1. Go to Library and then click “Size Standards” in “Analyze” window.

1.2. Click “Create” icon.

1.3. Give a name in “Size Standard”, such as “**IGT_ILS-155**”.

1.4. Choose "Orange" from the "Dye Color" dropdown box.

1.5. Enter new Size Standard definitions. Enter sizes in the list on the left. Separate sizes with a comma, space or return. IGT ILS-155 internal lane standard is composed of 11 fragments of 55, 60, 70, 85, 95, 105, 115, 125, 135, 145, and 155bp.

1.6. Click "Add Size(s)", click "Save", and click "Close".

III-B-2. To create an instrument protocol to run the InnoTyper[®] 21 kit: Refer to the “Instrument protocol library” section in *3500/3500xL Genetic Analyzer User Guide*.

2.1. Go to Library and click "Instrument Protocols" in the "Analyze" window.

2.2. Click "Create" icon.

2.3. Give a name in the “Protocol Name”, such as “**InnoTyper21**”.

2.4. Choose "HID" from the “Application Type”, "36" from the “Capillary Length”, and choose "POP4" from the “Polymer” dropdown window. The run module selection list is filtered based on the application you select.

2.5. Choose "IGT-G5" from the “Dye Set” drop-down window.

2.6. Set the following parameters as shown:

- Oven Temperature (°C): **60**
- Run Voltage (kVolts): **15.0**
- PreRun Voltage (kVolts): **15**
- Injection Voltage (kVolts): **1.2**
- Run Time (sec.): **1210**
- PreRun Time (sec.): **180**
- Injection Time (sec.): **15** (*see note below)
- Data Delay (sec.): **1**

***Note:** The instrument protocol for InnoTyper[®] 21 uses the run module “HID36_POP4xl” as a template for a 3500xL Genetic Analyzer. The above setting is the factory provided default setting except “Injection Time”. During development and validation with the 24-capillary 3500xL instrument, InnoGenomics determined that 15 sec injection time gives the best performance for the InnoTyper[®] 21 kit on the ABI 3500xL Genetic Analyzer. The Run Time may be shortened since the small amplicon size range of the InnoTyper[®] 21 kit

allows for a shorter run time, but this is an optional modification intended to accelerate analysis time. For use with other instrument platforms, it is recommended that the user start with the standard run module settings. Injection and run conditions may be modified based on the instrument being used and the laboratory's internal validation.

2.7. Click "Save" and then "Close".

III-B-3. To create a QC protocol to run the InnoTyper[®] 21 kit: [Refer to the "QC protocol library" section in 3500/3500xL Genetic Analyzer User Guide.](#)

(**Note:** A QC protocol is the required primary analysis protocol for HID applications. When you create an HID assay, it is required to add a QC protocol to the assay.)

- 3.1. Go to Library and click "QC Protocols" in the "Analyze" window.
- 3.2. Click "Create" icon. The "Create New QC Protocol" dialog box opens.
- 3.3. Provide a new QC protocol name in "Protocol Name", such as "**InnoTyper21_QC**".
- 3.4. Choose "**IGT_ILS-155**" for the size standard from the dropdown box.
- 3.5. In the "Analysis Settings" tab of the Create New QC Protocol dialog box, uncheck the check box for "Purple" and keep the rest checked. Select "Partial" for the "Sizing Range", then specify 50 to 160 to limit the fragment sizes evaluated for the size standard. If you specify sizes outside this range, the Sizing Quality may fail. For the rest of the values, use the default values to start with. You must optimize and validate peak amplitude threshold values during internal InnoTyper[®] 21 kit validation.
- 3.6. In the "QC Settings" tab, keep the default values.
- 3.7. Click "Save to Library".

III-B-4. To create an assay to run the InnoTyper[®] 21 kit: [Refer to the "Assay library" section in 3500/3500xL Genetic Analyzer User Guide.](#)

- 4.1. Go to Library and click "Assays" in the "Manage" window.
- 4.2. Click "Create" icon. The "Create New Assay" dialog opens.
- 4.3. Enter an Assay Name such as "**InnoTyper21-assay**".
- 4.4. Choose "HID" from the "Application Type" dropdown box. Select a color code for the assay from the "Color" dropdown box.
- 4.5. Select "No" for the "Do you wish to assign multiple instrument protocols to this assay?" question.
- 4.6. Select "**InnoTyper21**" from the "Instrument Protocol" dropdown box. Select "**InnoTyper21_QC**" from the "QC Protocol" dropdown box.
- 4.7. Click "Save".

III-B-5. To create a plate to run the InnoTyper[®] 21 kit: [Refer to the "Plates library" in 3500/3500xL Genetic Analyzer User Guide.](#)

- 5.1. Go to Library and click "Plates" in the "Manage" window.
- 5.2. Click "Create" icon. The software switches to the Main workflow and displays the Define Plate Properties screen.
- 5.3. Give a Plate Name. Names must be unique.
- 5.4. Select your plate type (96 well, 96 Fast tube, or 384 well).
- 5.5. Choose "HID" from the "Plate Type" dropdown box, "36" from the "Capillary Length" dropdown box, and "POP4" from the "Polymer" dropdown box. (Note: Secondary Analysis is optional.)
- 5.6. Click "Save Plate" icon and select a save option (Save, Save As..., or Save As

Template...).

III-B-6. To set up and run the InnoTyper[®] 21 kit: (Note: There are several ways to set up and run a plate. The following instructions show one way to run a plate.): Refer to the “Set up and run” section in 3500/3500xL Genetic Analyzer User Guide.

- 6.1. In the Dashboard, check consumable status. Ensure that consumables are not expired and that adequate buffer levels are at the fill lines.
- 6.2. Set the oven temperature to 60 °C, then click Start Pre-heat. (Note: Preheating the oven and detection cell helps mitigate subtle first-run migration rate effects. Applied Biosystems recommends that you pre-heat the oven for at least 30 minutes before you start a run if the instrument is cold.)
- 6.3. **IF** you have saved the plate using “Save as Template...” in Step 5, click “Create Plate From Template” icon to display the “Open Plate Template from Library” dialog box in the Dashboard. Select the template, then click “Open”.
IF you have saved the plate using “Save as...” in Step 5, click “Edit Existing Plate” icon. Select the plate, then click “Open”.
- 6.4. Click “Assign Plate Contents”.
- 6.5. You must assign the following information to the wells in a plate before you can run the plate:
 - Sample names and sample types (Sample, Positive Control, Negative Control, Allelic Ladder, or HiDi) (required) – Identifies the well positions of each sample for data collection and processing.
 - Assay (required). Select “**InnoTyper21-assay**” – Specifies the parameters that control data collection and primary analysis. All named wells on a plate must have an assigned assay.
 - Filename convention (optional) – Specifies file naming.
 - Results group (optional) – Specifies sample data file storage.
- 6.6. Load the plate in the instrument.
- 6.7. In the Assign Plates for Run screen, click “Link Plate for Run”.
- 6.8. In the Load Plates for Run screen, enter a “Run Name”.
- 6.9. Click either of the following:
 - “Create Injection List” – Displays the Preview Run screen where you can modify the injection list before starting the run.
 - “Start Run” – Displays the Monitor Run screen.

III-C. Sample Preparation:

III-C-1. Calculate the volume of Hi-Di™ Formamide and ILS-155 Internal Lane Standard needed to prepare the reactions using the table below:

For 3130/3130xl Genetic Analyzers:

Reagent	Volume per Reaction
Hi-Di™ Formamide	10.8 µL
ILS-155 Internal Lane Standard	0.2 µL*

For 3500/3500xL Genetic Analyzers:

Reagent	Volume per Reaction
Hi-Di™ Formamide	10.6 µL
ILS-155 Internal Lane Standard	0.4 µL*

Note: In order to provide excess volume for pipetting loss, include additional reactions (1.1x) in your calculations.

***Note:** The volumes of size standard indicated in the tables above are suggested amounts. Determine the appropriate amount of size standard based on your results and experiments.

III-C-2. Pipette the required volumes of components into an appropriately sized polypropylene tube. Vortex the tube, then centrifuge briefly.

III-C-3. Dispense 11 µL of the formamide/size standard mixture and 1 µL of PCR product or allelic ladder into 0.2 ml sample tubes or a 96-well sample plate. Seal the reaction plate or tubes with appropriate septa, then briefly centrifuge to ensure that the contents of each well are collected at the bottom.

Note: For blank wells, add 12 µL of Hi-Di Formamide.

III-C-4. Heat the reaction plate in a thermal cycler for 3 minutes at 95 °C and then immediately place it on ice for 3 minutes.

III-C-5. Assemble the tubes or 96-well sample plate in the autosampler tray.

III-C-6. Place the autosampler tray in the instrument, and close the instrument doors. Start the electrophoresis run.

IV. Data Analysis Using GeneMapper®, GeneMapper® ID or GeneMapper® ID-X

Note: Prior to data analysis, ensure that InnoTyper® 21 panels and bins are imported, as well as the IGT ILS-155 Internal Lane Standard.

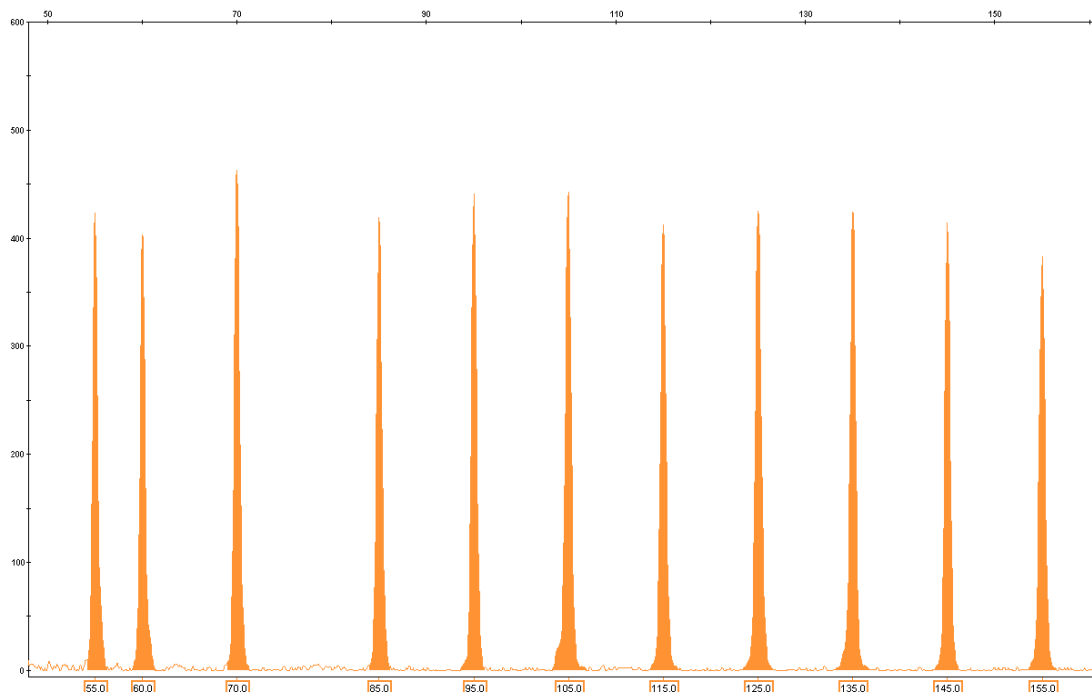
- IV-1. Open the GeneMapper®, GeneMapper® ID or GeneMapper® ID-X Analysis software.
- IV-2. Select *File* then *New Project* and under *Project Type*, select **Microsatellite**.
- IV-3. Under *File*, select *Add samples to Project* and select all samples to be analyzed. Select the sample type for each sample.
- IV-4. Under *Analysis Method*, select **Microsatellite Default**. Go to Peak Detector tab and select the **Advanced** peak detection algorithm.

Note: During development and validation, InnoGenomics used a 30 points baseline window under the Analysis Method Peak Detector tab (the default is 51 points). Users may wish to change the baseline window setting to 30 points if a high baseline is noted and/or for consistency with developmental validation studies.

- IV-5. Under *Panels*, select **InnoTyper_21 Marker Multiplex**.
- IV-6. Under *Size Standard*, select **IGT ILS-155**. (IGT ILS-155 internal lane standard is composed of 11 fragments of 55, 60, 70, 85, 95, 105, 115, 125, 135, 145, and 155bp. If you have not created a size standard, create one for “**ILS-155**” using “**Orange**” for Size Standard Dye.)
- IV-7. Select **Analyze**. Verify that the controls produced expected results (allelic ladder, positive control, and negative control).

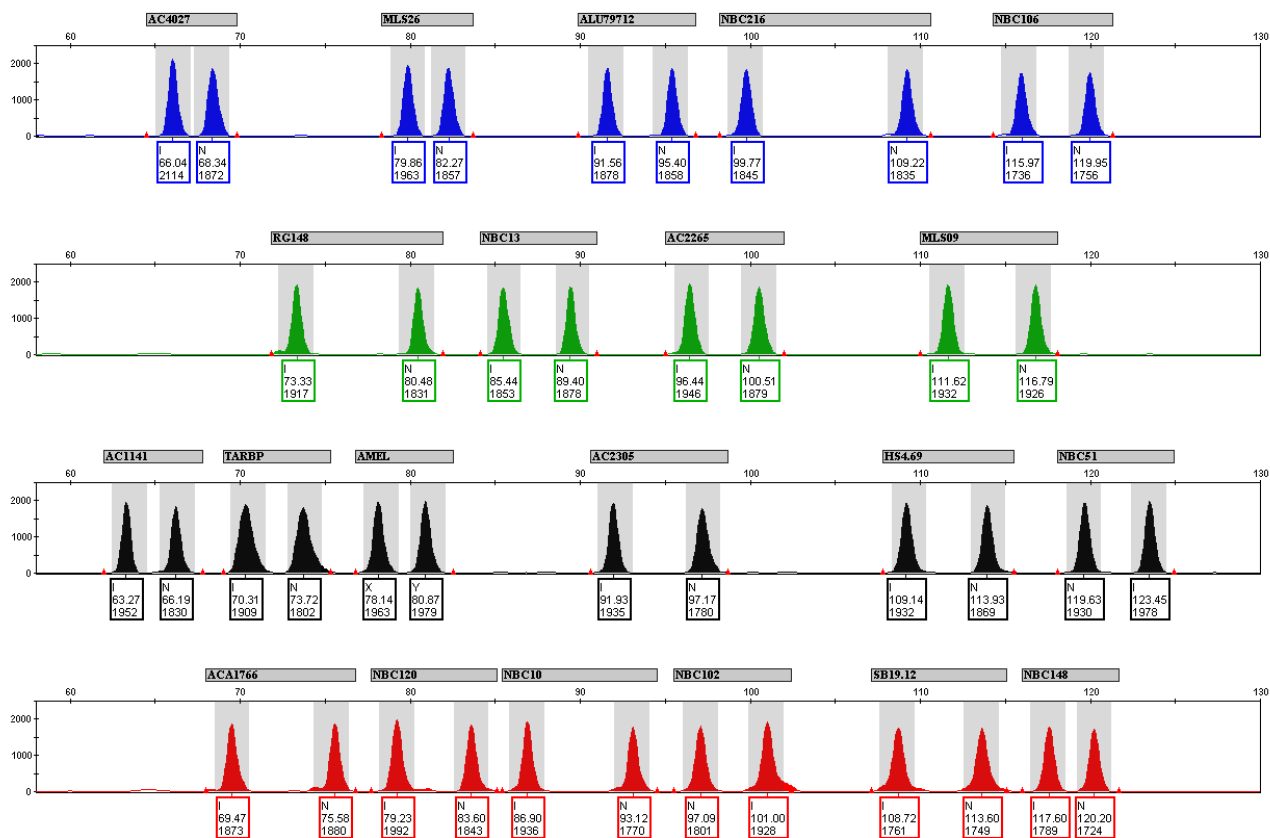
ILS-155 Internal Lane Standard Information

ILS-155 contains single stranded DNA with the TGI-Orange fluorophore attached. The bands are 55, 60, 70, 85, 95, 105, 115, 125, 135, 145, and 155bp.



Allelic Ladder Information

The allelic ladder for the InnoTyper[®] 21 Kit is shown below.



Note regarding loci configuration: the Insertion (“I”) amplicon has a smaller size than the No Insertion (“N”) amplicon in all but two markers, NBC51 and NBC102. For these two markers, the No Insertion amplicon is smaller than the Insertion amplicon. This is due to the unique “mini-primer” design used for this kit, with a single forward primer and two different reverse primers for the Insertion and No Insertion amplicons.

Marker Information

The following table shows the loci amplified and the corresponding fluorescent marker dyes. The observed allele sizes (in base pairs) and the genotype of the DNA Positive Control are also listed in the table.

	Marker	Dye	Chromosome	Band	Amplicon Size* (I = Insertion)	Amplicon Size* (N = No Insertion)	Positive Control Profile
1	AC4027	FAM	7	7q21.11	66.1	68.4	I,I
2	MLS26	FAM	3	3p22.1	79.8	82.3	I,N
3	ALU79712	FAM	20	20p12.2	91.6	95.4	N,N
4	NBC216	FAM	7	7p14.1	99.8	109.2	I,I
5	NBC106	FAM	21	21q22.2	115.9	120.0	I,N
6	RG148	JOE	2	2q23.3	73.3	80.5	I,N
7	NBC13	JOE	16	16p12.1	85.4	89.4	N,N
8	AC2265	JOE	13	13q33.1	96.4	100.5	I,N
9	MLS09	JOE	1	1q25.3	111.6	116.7	I,N
10	AC1141	TMR	3	3q11.2	63.4	66.2	I,I
11	TARBP	TMR	1	1q42.2	70.3	73.7	I,N
12	AMEL	TMR	X, Y	Xp22.1-22.3 Yp11.2	X=78.1	Y=80.9	X,Y
13	AC2305	TMR	13	13q13.3	92.0	97.2	I,N
14	HS4.69	TMR	5	5q34	109.2	113.9	I,N
15	NBC51	TMR	3	3q28	123.5	119.7	N,N
16	ACA1766	ROX	8	8q12.1	69.5	75.5	I,N
17	NBC120	ROX	22	22q11.21	79.2	83.6	I,N
18	NBC10	ROX	4	4q31.21	86.9	93.1	N,N
19	NBC102	ROX	17	17q23.3	101.0	97.1	N,N
20	SB19.12	ROX	19	19q13.43	108.7	113.6	I,N
21	NBC148	ROX	14	14q31.1	117.5	120.2	I,I

* Observed sizes on 3130 Genetic Analyzer

NOTE: During the development of products for forensic DNA analysis, InnoGenomics Technologies performs developmental validation studies. However, it is the responsibility of the customer laboratory to perform its own analysis and internal validation studies, and develop its own standard operating procedures and interpretation guidelines, to ensure that the products and services it obtains from InnoGenomics Technologies satisfy or will satisfy the applicable guidelines used by the forensic community and are fit for the customer laboratory's human identification applications.

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