THE CONTI-VECCHIOTTI REPORT: INTRODUCTION

The following is a complete translation of the report submitted by Profs. Carla Vecchiotti and Stefano Conti of the University of Rome — La Sapienza to the *Corte di Assise di Appello* (Court of Appeals) of Perugia, Italy, regarding DNA evidence in the case against Amanda Knox and Raffaele Sollecito. Vecchiotti and Conti were appointed by the presiding judge in Knox and Sollecito's appeal trial, Claudio Pratillo Hellmann, to conduct an independent review of this evidence, which (like most of the evidence in the case) had long been seriously questioned by Knox and Sollecito's defense teams and by outside observers.

The conclusions reached by Conti and Vecchiotti constitute a damning indictment of the investigation conducted by Italy's Scientific Police, and in particular of the methods employed by the prosecution's main forensic scientist, Dr. Patrizia Stefanoni. They lend official support to the already-widespread perception that Knox and Sollecito have been the victims of a scandalous miscarriage of justice.

Two translators worked on this project: **komponisto** and **katy_did**. Anyone with questions, comments, or corrections is invited to contact komponisto via private message on the Injustice in Perugia forum, JREF, or Less Wrong.

For more information on the wrongful prosecution of Amanda Knox and Raffaele Sollecito, and the terrible crime which led to it, see the website Injustice in Perugia.

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ASSIGNMENT

[1] On January 22, 2011, the undersigned Prof. Stefano Conti and Prof. Carla Vecchiotti, Specialists in Forensic Medicine employed in the Forensic Medicine Section of the Department of Anatomical, Histological, Forensic, and Locomotive Sciences of the University of Rome – La Sapienza, were appointed by Judge Claudio Pratillo Hellmann of the *Corte di Assise di Appello* of Perugia, Criminal Section, to conduct laboratory investigations relative to criminal case no. 10/2010 R.G. in order to provide answers to the following inquiries:

"*Having examined the record and conducted such technical investigations as shall be necessary, the Expert Panel shall ascertain:*

- *"whether it is possible, by means of a new technical analysis, to identify the DNA present on items 165b (bra clasp) and 36 (knife), and to determine the reliability of any such identification"*
- *"if it is not possible to carry out a new technical analysis, shall evaluate, on the basis of the record, the degree of reliability of the genetic analysis performed by the Scientific Police on the aforementioned items, including with respect to possible contamination."*

As it was necessary to conduct investigations outside of the presence of the Office [i.e. Court], 90 days were allotted, with a subsequent extension, for the submission of the expert report.

TESTS

[2] Retesting of the items [*Le operazioni peritali*] began on February 09, 2011, at 11:15 am, at the Forensic Genetics Laboratory of the Section of Forensic Medicine of Sapienza University of Rome, 336 Viale Regina Elena.

On that date, the following consultants for the parties were present:

-Dr. Patrizia Stefanoni, consultant for the Prosecutor's Office [*Procura Generale*];

-Prof. Giuseppe Novelli, consultant for the Prosecutor's Office;

-Dr. Emiliano Giardina, consultant for the Prosecutor's Office;

-Prof. Francesca Torricelli, consultant for the civil plaintiff [i.e. the victim's family];

-Prof. Adriano Tagliabracci, consultant for Raffaele Sollecito;

-Dr. Valerio Onofri, consultant for Raffaele Sollecito;

-Prof. Carlo Torre, consultant for Amanda M. Knox;

-Dr. Sarah Gino, consultant for Amanda M. Knox;

-Dr. Walter Patumi, consultant for Amanda M. Knox.

In the memorandum drafted at this session, it was noted "that the items labeled Item 36 and Item 165/b were transferred according to regulation on 2/8/2011, as ordered by His Honor Presiding Judge Hellmann, and as attested in the transfer memorandum dated 2/8/2011. It is recorded that at the moment of transfer, both items were regularly sealed in a single envelope (*in involucro singolo*), and in particular Item 36, no. 00015662 [was] locked in a carton [*scatola di cartone*] by means of a red-colored safety seal with the number 0000179, and Item 165/b no. 00012877 was contained in a tube with a red-colored top, as also revealed by the visual documentation [*documentazione iconografica*] by both the appointed experts and the Assigned Officer [*Agente Scelto*] of the State Police (Ugo Arcuri)".

In the memorandum, the consultants for the parties expressed their requests in writing. These are reported below:

1. Dr. Sarah Gino: “Quantification with Real Time”;

2. Prof. Tagliabracci and Dr. Onofri: “If material is available, request that the experts repeat the DNA test on any extract from Item 165B in the possession of the Scientific Police”; **[3]**

3. Dr. Stefanoni, Prof. Novelli, Dr. Giardina, Prof. Torricelli: “utilize the most sensitive techniques in a parallel and in a manner comparable to those used in the previous experiment”;

4. Dr. Gino, Prof. Torre: “Request that upon opening the items, any traces (streaks etc.) present on the knife be compared with photographic images from the initial investigation (inspection, laboratory tests, etc.)”;

5. Prof Tagliabracci: “concur”;

6. Dr. Onofri: “concur”

7. Prof. Novelli, Dr. Giardina: “No statement on the matter”;

8. Prof. Torricelli: “No statement in reference to knife item”;

9. Dr. Stefanoni: “No statement regarding the viewing of the items”.

The appointed experts, taking note of the matters raised by the parties, communicate to the latter that they will be reconvened according to law for testing. 12:30 pm, Rome, 2/9/2011″.

Testing continued on 03/22/2011, at 9:45 am, at the Forensic Genetics Laboratory of the Department of Anatomical, Histological, Forensic, and Locomotive Sciences — Section of Forensic Medicine of the Sapienza University of Rome.

On this date the following consultants for the parties were present:

-Dr. Patrizia Stefanoni, consultant for the Prosecutor’s Office;

-Prof. Giuseppe Novelli, consultant for the Prosecutor’s Office;

-Prof. Francesca Torricelli, consultant for the civil plaintiff;

-Prof. Adriano Tagliabracci, consultant for Raffaele Sollecito;

-Dr. Valerio Onofri, consultant for Raffaele Sollecito;

-Prof. Carlo Torre, consultant for Amanda M. Knox;

-Dr. Sarah Gino, consultant for Amanda M. Knox;

-Dr. Walter Patumi, consultant for Amanda M. Knox.

[4] At this session, the experts explained the ***plan*** [*piano di lavoro*] which, in accordance with forensic methodology, was to perform the laboratory tests on Item 36 and Item 165B in the following manner:

1) generic test for blood, employing colorimetric tests;

2) specific test for blood;

3) search for cells by means of specific coloration and observation under the microscope;

4) extraction of DNA from each individual item;

5) quantification of DNA via Real Time PCR, as requested and agreed upon by all consultants.

In the memorandum drafted at this session, the following was recorded: "It is noted by all present that both items, labeled Item 36 and Item 165/B, are regularly sealed in a single envelope and in particular Item 36 no. 00015662 [is] locked in a carton by means of a red-colored safety seal with the number 0000179 and Item 165/b no.00012877 contained in a tube with red-colored top, as revealed by the visual documentation by the appointed experts. It is noted that Prof. Giuseppe Novelli removed himself from the testing session at 10:15 am. At 2:45 pm, Prof. Carlo Torre, Dr. Patumi, Prof. Tagliabracci, and Dr. Onofri also removed themselves. Testing was interrupted at 6:05 pm and it was agreed to continue on the next day 3/23/2011 at 9:45 am.

On 3/22/2011, the box containing Item 36 (knife) was opened first, followed by the envelope [*busta*] containing the tube inside of which the clasps (Item 165B) were placed.

DESCRIPTION OF ITEM 36 (KNIFE)

[5] We observed Item 36 (knife) to be locked inside of a transparent plastic bag of the State Police, sealed with a red-colored string [*laccio*] of rigid plastic. An initial observation of the object inside of the sealed bag revealed several narrow streaks on the right side that proceeded from the edge [*filo*] of the blade to around the halfway point of the blade itself. We thus proceeded to open the bag with the aid of sterile scissors.

The knife is of single-lip type [*tipo monotagliente*] with the brand indication "Marietti Stainless – Inox – Italy" affixed on the left side; the blade is casted [*inserita in lavorazione pressofusa*] into a plasticized handle of black color. The marketed characteristics of Marca Marietti knives, according to the manufacturer's specifications [*scheda della Casa costruttrice*], are "AISI 420 Steel Blades, printed and polished". The handle, of quadrilateral shape, has dimensions of 3 x 6.6 cm, with the blade (which is "tight" [*a tenuta*]) inserted in the middle; the length of the blade is 17.5 cm along the back and 18 cm. along the cutting side [*il tagliente*], with a width [*altezza*] of 3 cm, and border thickness of around 1.5mm.

On the right side we confirm the visible presence of thin [*sottili*] streaks, some of which [are] of curvilinear type, which extend from the tip to about halfway down the blade and [are] on the entire surface of the width [= width of the surface?]; at the level of the edge where the blade and the handle make contact, an area of lighter color is present on almost the entire width of the blade, for a thickness of about 0.5 cm (corresponding to the chamfer [*becco di flauto*, lit. "beak of flute"]). Also present on the right side, exactly at the level of the edge where the blade meets the handle, is dark-colored material that extends to the entire width **[6]** for a thickness of about 1 mm, and at the level of the chamfer about 2 mm. Still on the right side, at the level of the back at about 1.5 cm from the tip, are small diagonally-placed streaks which extend toward the center, for a length of about 6 cm. The upper edge, back of blade, does not show alterations.

On the left side, there are thin streaks starting from the tip which extend to about halfway down the blade. At the level of the edge where the blade and handle make contact, also on the left side, is an area of darker color extending throughout the width

of the blade with a maximal dimension of about 0.6 cm and for almost 0.6 cm at the lower level corresponding to the chamfer. Also detectable at the level of the contact point between the blade and the handle is material of darker color extending to the entire width of the blade for a thickness of about 1 mm. The upper edge, back of knife, does not show alterations; along the cutting side are numerous discontinuities, less than [number missing] mm. which extend for a length of about 11 cm.

LABORATORY ANALYSIS OF ITEM 36

After the inspection and photographic survey of the item, we proceeded to the laboratory analysis, as shall now be described.

We emphasize that the counters, shelves, and all equipment present in the laboratory where the items were viewed had been previously treated with 10% sodium hypochlorite solution, washed with water and finally with 70% ethanol, as unanimously recommended and practiced by molecular biology laboratories (Hayatsu H. et al., 1971; Prince A.M., Andrus L., 1992; Schmidt T. et al., 1995; Whiteman M. et al., 1997; Ohnishi S. et al., 2002; Whiteman M. et al., 2002; Kemp B. M,. Glenn Smith D., 2005; Frégeau C.J. 2008; Caragine T. et al., 2008; Toothman M.H. et al., 2008).

It is recorded that while the tests were being carried out, all those present wore masks, disposable gowns, and disposable gloves.

All the manual operations (from opening of the items, to extemporaneous investigations, to the taking of samples) were performed by one of the experts (Prof. Carla Vecchiotti), with the assistance of a technical collaborator, as authorized by the *Corte d'Assise*, and it should be noted that during each individual phase, the disposable gloves were changed.

On the basis of photographic comparison with the images included in the RTIGF [=Technical Report on the Forensic Genetic Tests] and specific indications by Dr. Stefanoni, the previously-examined areas on the item were identified for the taking of new samples.

The areas of interest were indicated with the same letters assigned by Dr. Stefanoni.

Thus, the areas on the ***handle of the knife*** were indicated with the ***letters A-D-F***, while the areas of interest on the ***blade*** were indicated with the letters ***B-C-E-G***.

With the agreement of the parties, two further samples were taken from the ***point of contact between the blade and the handle***, on opposite sides of the knife, and these samples were indicated with the letters H-I.

[7] On each individual area of interest, we proceeded to perform the **generic test for blood**.

The test was conducted by means of reactive strips [*strisce reattive*] soaked with dimethyl-dihydro-peroxysan [*dimetil-diidro-perossiesano*]
and a colorimetric indicator (tetramethylbenzidine) which, in the presence of hemoglobin, assume a green-blue coloration ("***Combur Test® E***" *Roche Diagnostics GmbH, Mannheim, Germany; product code 11896857191, site no. 20212701*).

The test requires the reactive area of the strip to be first dampened with distilled water and then put into contact with the surface to be tested, causing a slight rubbing. Within 60 seconds, in the case of a positive result, the reactive area of the strip will assume a green-blue coloration, while any color variations occurring after an interval of two minutes from the test are not significant.

The following results were obtained:

Generic test for blood

Trace **A** = **negative reaction**

Trace **B** = **negative reaction**

Trace **C** = **negative reaction**

Trace **D** = **negative reaction**

Trace **E** = **negative reaction**

Trace **F** = **negative reaction**

Trace **G** = **negative reaction**

Trace **H** = **negative reaction**

Trace **I** = **negative reaction**

The results obtained were documented photographically.

On these same areas, then, samples were taken using sterile swabs (in individual sealed packaging, opened at the very moment of sampling), damp with sterile bidistilled water.

Nine samples were taken, of which seven corresponded to the areas already examined by Dr. Stefanoni (letters A-B-C-D-E-F-G) and two **[8]** corresponded to the point of contact — not analyzed by Dr. Stefanoni — between the blade and the handle of the knife (letters H-I).

Each individual swab was immediately inserted into the appropriate tube, on which the letter corresponding to the extraction site had been placed.

We note once again that the above operations were performed while wearing masks, disposable gowns, and disposable gloves, which were regularly changed between one sampling and the next.

When the extraction operations were complete, the knife was wrapped in a sheet of tissue paper, inserted in a paper bag, and placed inside the original box, in which the plastic bag that the item had originally been sealed in was separately placed. The box was closed by means of adhesive tape of brown color, on which the signatures of the consultants for the parties and the experts were attached.

We then proceeded to the inspection and subsequent opening of Item 165B.

DESCRIPTION OF ITEM 165B (BRA CLASPS)

[9] The item to be examined was observed by us to be locked inside a transparent tube with a red top, contained in turn in small sealed bag [*bustina sigillata*], of transparent plastic, of the State Police.

We emphasize, moreover, that — as shown photographically — multiple components of dark-red color were present, scattered throughout the inside of the tube, both at the bottom and near the closing top.

There were two clasps, both lacking grommets [*senza l'occhiello di tenuta della controparte*], with extended parts of red-brownish color, others of reddish color; whiteish elements were also partially in evidence.

One of the clasps was recognizable in its original form, with the characteristic square upon the which corresponding round-edged hook is sewn; the other no longer had any kind of shape, as it was so completely deformed as to have been inserted into the first [clasp], with which it was partially fused due to the presence of rust; separation of the two elements would involve the fragmentation of certain rusted components.

LABORATORY ANALYSIS OF ITEM 165B

After the photographic survey, we proceeded to open the tube, extract the clasps, and perform the **generic test for blood** on two distinct points of the items, indicated respectively by the ***letters L*** and ***M***.

The test was conducted by means of "***Combur Test® E***" (*Roche Diagnostics GmbH, Mannheim, Germany; product code 11896857191, site no. 20212701*), in the manner previously described.

The following results were obtained:

Generic test for blood:

Trace **L** = **negative reaction**

Trace **M** = **negative reaction**

The results obtained were documented photographically.

[10] Then, on these same areas, samples were taken via sterile swabs (in individual sealed packaging and opened at the very moment of sampling) dampened with sterile bidistilled water.

Each individual swab was immediately inserted into the approriate tube, on which the letter corresponding to the extraction site was placed.

The above operations were performed while wearing masks, disposable gowns, and disposable gloves, which were regularly changed between each sampling and the next.

When the extraction operations were finished, the clasps were folded in a sheet of tissue paper, and together with the transparent tupe and the plastic bag [*busta*] in which they had originally been sealed, were inserted in a white paper envelope [*busta*] on which the following label [*intestazione*] was placed: "Department of Forensic Medicine – Sapienza University of Rome". The envelope was closed, on one side, by means of brown-colored adhesive tape on which the signatures of the experts and the consultants for the parties were placed.

At the end of the sampling of the items, a negative control (labeled "**C.N.**") was prepared by means of a sterile swab (in individual sealed packing and opened at the very moment of sampling) dampened with the same sterile bidistilled water used for the individual extractions on the aforementioned items.

EXTRACTION OF DNA FROM ITEM 36 (KNIFE) AND ITEM 165B (BRA CLASPS)

[11] The extraction of DNA from the items under examination occurred on the same day of 2/22/2011, in another laboratory assigned to the phase of DNA extraction, physically separated from the areas where forensic samples are prepared and amplified.

The counter, as well as all equipment and laboratory instruments, were first treated with 10% sodium hypochlorite solution, washed with water and finally with 70% ethanol, as unanimously recommended and practiced by molecular biology laboratories.

Prior to autoclave treatment, four sealed packages, each containing two metal tweezers and two clippers [forbicine], were first prepared; as were, in addition, two Becker containers, the first containing a 10% sodium hypochlorite solution for the disinfection of the instruments in use, and the second sterile bidistilled water for the rinsing of the instruments prior to the next use.

All the operations were carried out while wearing masks, disposable gowns, and disposable gloves which were regularly changed between one sample and the next.

On each individual swab (12 swabs in total including the extraction-negative [il negativo di estrazione], labeled with the letters from A to M in the case of the specimens taken from the items, and with the marking “C.N.” for the negative control) we proceeded to take a sample of a piece of cloth [tessuto], of dimensions about 2×2 mm, for the subsequent cytological analysis.

The pieces of swab taken for the cytological analysis were separately inserted into 1.5 mL tubes (each of which labeled on its top with the letter corresponding to the respective sample).

The tubes were placed inside a carton that was closed by means of brown-colored adhesive tape on which the signatures of the experts and the consultants for the parties were placed. The carton was refrigerated at +4°C until the time of the next laboratory tests.

The remaining swabs were removed by means of sterile tweezers and clippers, individually inserted into 1.5 mL tubes (on the top of which was placed the letter corresponding to the sample in question) and then subjected to the DNA extraction

procedure by means of the commercial kit labeled DNA IQ™ System (Promega Corporation, Madison, WI, USA), specifically designed for **[12]** forensic genetics laboratories, which enables DNA to be isolated via paramagnetic particles and can be used for extracting DNA from a large variety of samples, including dried traces and biological liquids.

For the extraction of DNA from the samples, the following protocol, indicated in the user's manual of the kit, was followed:

1) removal [prelievo] of the swab and insertion into a 1.5 mL tube;

2) addition to each individual tube of 250 µL of Lysis Buffer (preliminarily prepared via the addition of 1 µL of 1M DTT for each 100 µL of Lysis Buffer) and incubation at 70°C for 30 min.;

3) transfer of the Lysis Buffer and of the sample to a new 1.5 mL tube equipped with a centrifuge basket [cestello per centrifuga] and subsequent centrifugation at maximum velocity (13000 rpm) for 2 min.;

4) removal [rimozione] of the basket; addition of 7 µL of magnetic resin; vortex and incubation at room temperature for 5 min;

5) vortex and positioning of the tube on the magnetic support; discharge [rimozione] of the solution;

6) lavage with 100 µL of Lysis Buffer (working solution [sol. di lavoro]);

7) lavage with 100 µL of Wash Buffer 1X (previously prepared via the addition of 15 mL of absolute ethanol [etanolo assoluto] and 15 mL of isopropanol to the Wash Buffer 2X included in the kit), repeated for a total of 3 lavages.

8) accurate removal [rimozione] of the solution; drying with open top, on the magnetic support, at room temperature, for 5-10 min.;

9) addition of 30 µL of Elution Buffer and incubation at 65°C for 5 min.;

10) vortex and repositioning of the tube on the magnetic support;

11) transfer of the solution into a new tube;

12) storage of the extract at +4°C.

We note that the samples under examination were rigorously subjected to the DNA extraction procedures individually according to a process that was precisely organized in terms of location and timing [secondo una precisa organizzazione temporo-spaziale della lavorazione], using disposable sterile filter-equipped tips. The operations were conducted while wearing masks, disposable gowns, and disposable

gloves which were regularly changed upon each passage of the process from one sample to the next.

[13] At the end of the extraction procedure, the 12 1.5 mL tubes (each of which labeled on the top with the letter corresponding to the sample in question) were placed inside an appropriately identified carton, sealed with brown-colored adhesive tape on which were placed the signatures of the experts and the consultants for the parties, and refrigerated at +4°C pending the quantification of the extracted DNA.

At 6:05 pm the testing operations were interrupted; they resumed the next day 3/23/2011 at 10:00 am according to the memorandum which we quote below:

"The next day, 03/23/2011, at 10:00 am, Dr. Stefanoni, Dr. Gino, Dr. Onofri, as well as the appointed experts Prof. Vecchiotti and Prof. Conti were present; after checking the integrity of the adhesive tape of the container of the extracts performed on 3/22/2011, all reported to the histology section of the appointed experts' home Department. In the presence of the parties, preparations were made for a quantification reaction via Realtime 7500 PCR; at the end of the quantification, the results were viewed; they were then printed, and copies were given to the consultants present. Dr. Stefanoni notes that the Real Time PCR reaction was prepared on the counter without using a fume hood to guarantee the absence of contamination. Dr. Gino has nothing to state. Dr. Onofri has nothing to state. This memorandum ends at 2:15 pm."

QUANTIFICATION OF DNA BY "REAL-TIME PCR" TECHNIQUE

[14] For the amplification of DNA samples, the technique of quantitative PCR or "Real-Time PCR" was employed, using the *Applied Biosystems 7500 Real-Time PCR* system. Unlike a conventional PCR, where the amplified product, or amplicon, is detected after the reaction is finished by observing the relevant band of amplified DNA on an agarose gel, with the "Real-Time PCR" technique it is possible to measure the amplified DNA while the reaction is in progress. This allows one to determine the initial amount of DNA present in a sample much more accurately and with very high sensitivity. The quantization is performed by using a fluorescent probe [*sonda*] which is employed to obtain a measurement [*valutazione*] of the amount of PCR product present in each cycle of the reaction. In practice, the intensity of fluorescent signal measured during the exponential phase of the PCR reaction allows one to determine the amount of genetic material present at the beginning of the reaction. For the analysis of DNA samples, we used the "*Quantifiler® Duo DNA Quantification Kit*", (product number 4387746, site number 1101024) product, also acquired from *Applied Biosystems (Foster City, Ca, USA)*. This kit allows one to simultaneously identify the total amount of human and male human DNA in a sample. The results obtained by using this kit allow one to determine whether the sample contains a sufficient quantity to proceed with the STR (Short Tandem Repeat) analysis, and can be used to determine the relative amount of male and female DNA present in the sample under examination. Finally, the kit used in this analysis contains a specific internal control (IPC) that allows one to measure the presence of any inhibitors capable of compromising the PCR itself. The IPC is based on the use of a synthetic DNA sequence not present in nature. The "*Quantifier Duo DNA Quantification Kit*" is designed and optimized for usage with the *Applied Biosystems 7500 Real-Time PCR* system equipped with the SDS software, available at the Histology and Medical Embryology section of Sapienza University of Rome. The genes analyzed are the following: Ribonucleasis P RNA Component III (RPPH1), and the SRY gene, used respectively to amplify human and human male DNA.

PREPARATION OF SAMPLES FOR REAL-TIME PCR

[15] Before proceeding with the analysis of the samples, all the working surfaces were treated with disinfectant and antifungal solutions, left to act for about 30 minutes before cleaning. Furthermore, to limit contamination as much as possible, an appropriate support (*MicroAmp® Splash Free 96-Well Base, Applied Biosystems*) was used to prevent contact between the plate containing the samples and the working surfaces.

The "*Quantifiler Duo DNA Quantification Kit*" used for the amplications of the previously extracted DNA samples includes the reagents necessary for amplification, identification, and quantification of one specimen of human DNA [*uno specifico DNA umano*] and one specimen of human male DNA, as previously reported. All the reagents present in the kit are optimized for use with the Applied Biosystems 7500 Real-Time PCR System apparatus equipped with SDS software.

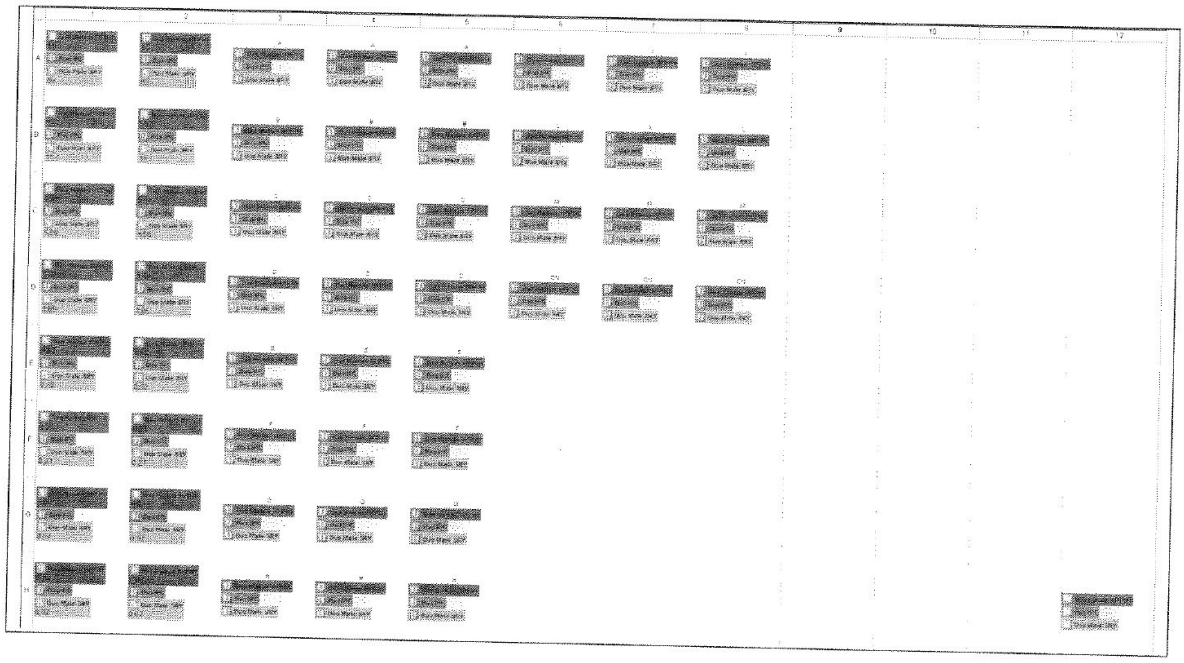
For each DNA sample and for each point of the standard curve, a reaction mixture was prepared according to the following scheme:

Components (provided with the kit)	**Quantity in Microliters (µl)**
Quantifiler Duo Primer Mix	10.5
Quantifiler Duo PCR Reaction Mix	12.5
Total	23.00

The necessary amounts of the components were prepared in appropriate sterile polypropylene tubes, and 23 µL of reaction were dispensed in each well [*pozzetto*] of a 96-well plate for Real-Time (*MicroAmp Optical 96-well reaction plate, Part number N801-0560, Applied Biosystems*). Subsequently, 2 µL of standard DNA, 2 µL of DNA to be analyzed, and 2 µL of control (NTC) were added to each well to obtain the final reaction volume of 25 µL/sample.

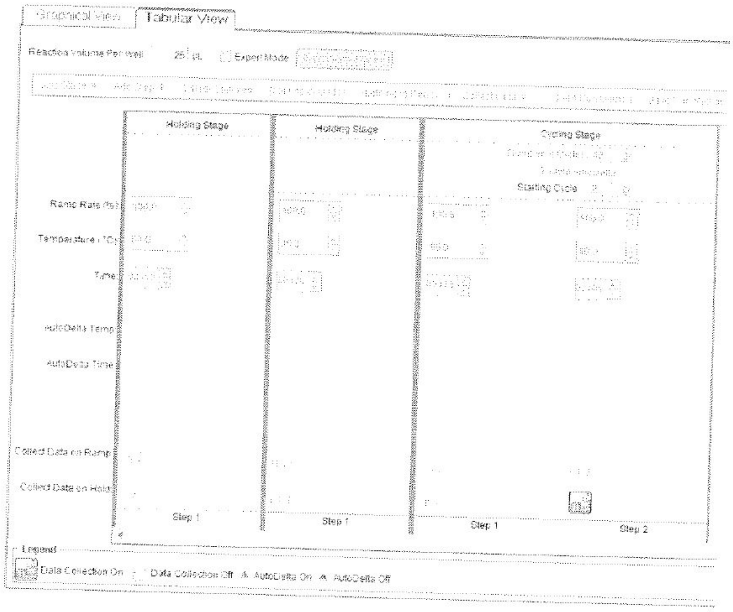
Before being processed, the plate was centrifugated at 300 rpm for about 30 seconds in order to remove any air bubbles in the wells.

[16] The samples, loaded onto the 96-well plate, were analyzed according to the following scheme:



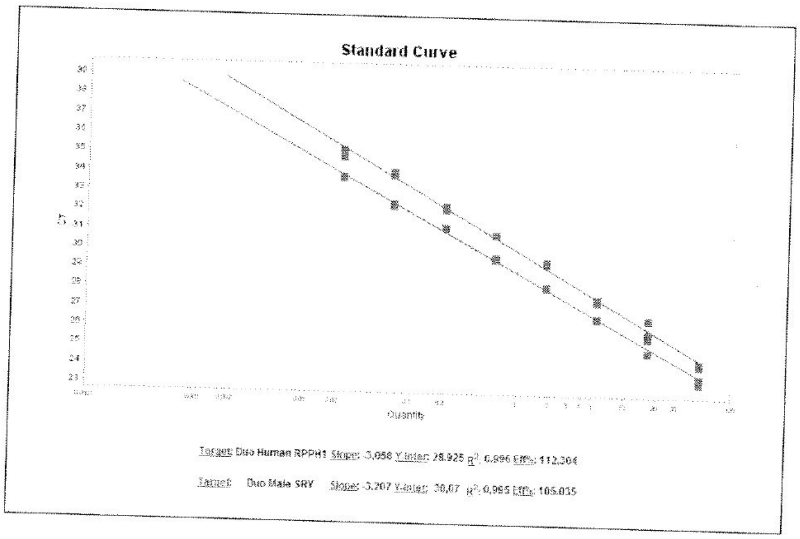
where: S stands for Standard and U for Unknown. All the DNA samples were analyzed in triplicate using 2 μL of volume for each reaction (total 25 μL) and are indicated with the letters A, B, C, D, E, F, G, H, I, L, and M. NC refers to a negative control sample while NTC identifies the Non Template Control, that is the PCR sample lacking amplifiable DNA. The standard curve was analyzed in duplicate.

[17] The parameters relevant to the PCR are the following:



Standard Curve

The standard curve was constructed via 8 noted concentrations of DNA (provided with the kit). The concentrations used run from 50 ng/µL (point 1) to 0.023 ng/µL (point 8). A duplicate for each standard point was used.



The analysis at the end of the run, performed via the SDS software, provided the results reported below.

ANALYSIS OF INDIVIDUAL DNA EXTRACTS (SAMPLES A-M)

[18] With regard to samples A-M, the real-time PCR analysis reveals a very low initial amount of DNA, with the maximum measurable DNA concentration at 0.005 ng/µL (5 pg/µL) in sample "I". The following table reports the measurements relative to the individual samples examined in triplicate. It should be noted that the analysis conducted on the internal control (IPC) does not reveal anything suggestive of an inhibition of the PCR reaction. In fact, the value of Ct relative to the IPC turns out to always lie in the interval between 28-31, in line with expectation. In other words, from the analysis performed one can rule out the presence of inhibitors of the PCR reaction capable of compromising the DNA amplification in the individual samples analyzed. **The concentration of DNA in the majority of the samples examined turned out to be undeterminable (Undetermined).**

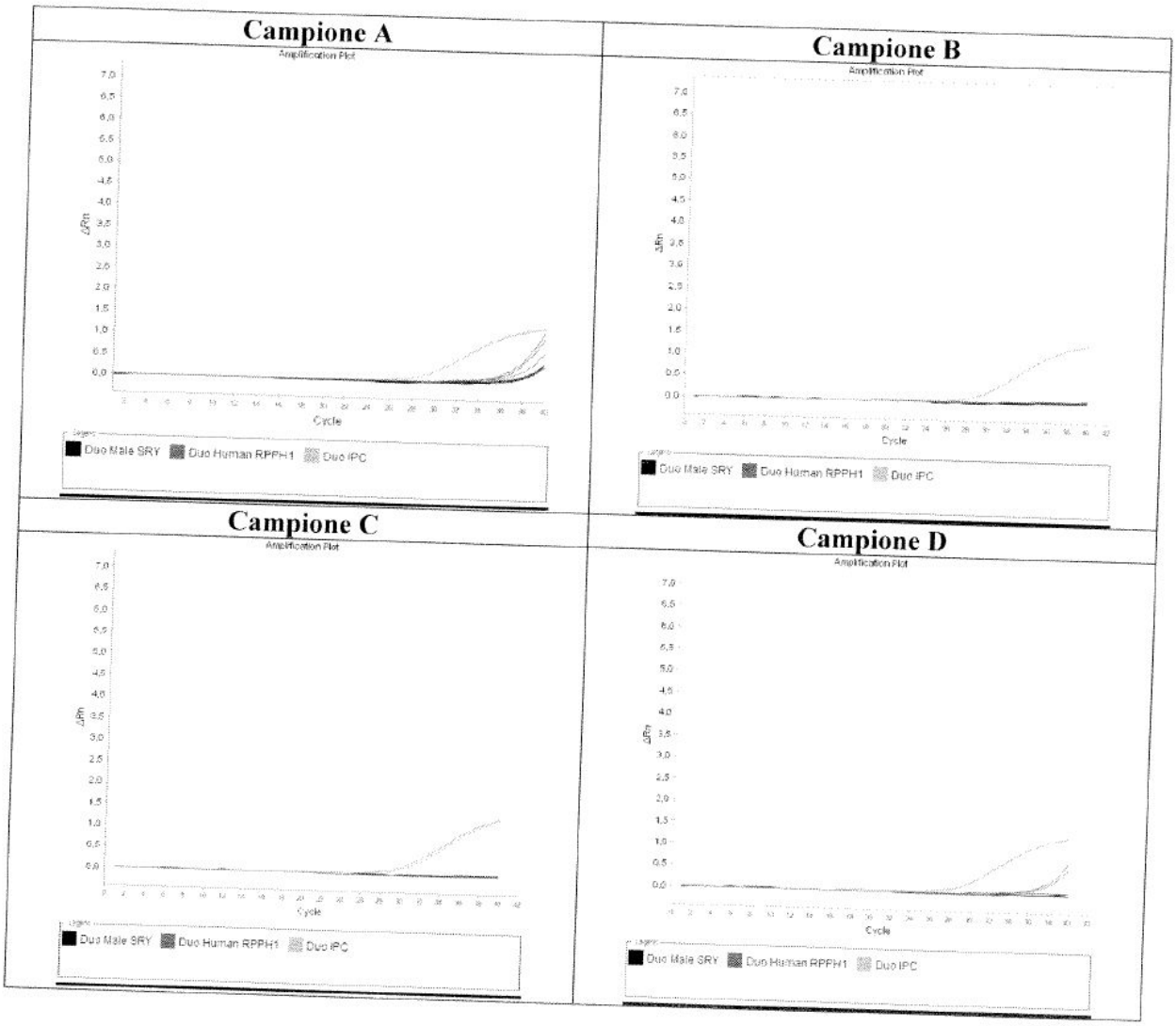
Well	Sample Name	Target Name	Cт	Cт Mean	Cт SD	Quantity (ng/ul)	Quantity Mean
A1	STANDARD	Duo Human RPPH1	23,610	23,499	0,157	**50,00**	
A1	STANDARD	Duo IPC	30,108	30,018	0,127		
A1	STANDARD	Duo Male SRY	24,424	24,362	0,088	**50,00**	
A2	STANDARD	Duo Human RPPH1	23,387	23,499	0,157	**50,00**	
A2	STANDARD	Duo IPC	29,928	30,018	0,127		
A2	STANDARD	Duo Male SRY	24,299	24,362	0,088	**50,00**	
B1	STANDARD	Duo Human RPPH1	24,928	25,296	0,520	**16,70**	
B1	STANDARD	Duo IPC	29,961	29,868	0,131		
B1	STANDARD	Duo Male SRY	25,904	26,258	0,500	**16,70**	
B2	STANDARD	Duo Human RPPH1	25,663	25,296	0,520	**16,70**	
B2	STANDARD	Duo IPC	29,775	29,868	0,131		
B2	STANDARD	Duo Male SRY	26,611	26,258	0,500	**16,70**	
C1	STANDARD	Duo Human RPPH1	26,645	26,627	0,025	**5,560**	
C1	STANDARD	Duo IPC	29,943	29,931	0,016		
C1	STANDARD	Duo Male SRY	27,653	27,590	0,088	**5,560**	
C2	STANDARD	Duo Human RPPH1	26,609	26,627	0,025	**5,560**	
C2	STANDARD	Duo IPC	29,920	29,931	0,016		
C2	STANDARD	Duo Male SRY	27,528	27,590	0,088	**5,560**	
D1	STANDARD	Duo Human RPPH1	28,236	28,211	0,036	**1,850**	
D1	STANDARD	Duo IPC	29,733	29,756	0,033		
D1	STANDARD	Duo Male SRY	29,524	29,480	0,063	**1,850**	
D2	STANDARD	Duo Human RPPH1	28,185	28,211	0,036	**1,850**	
D2	STANDARD	Duo IPC	29,779	29,756	0,033		
D2	STANDARD	Duo Male SRY	29,435	29,480	0,063	**1,850**	
E1	STANDARD	Duo Human RPPH1	29,691	29,648	0,061	**0,620**	

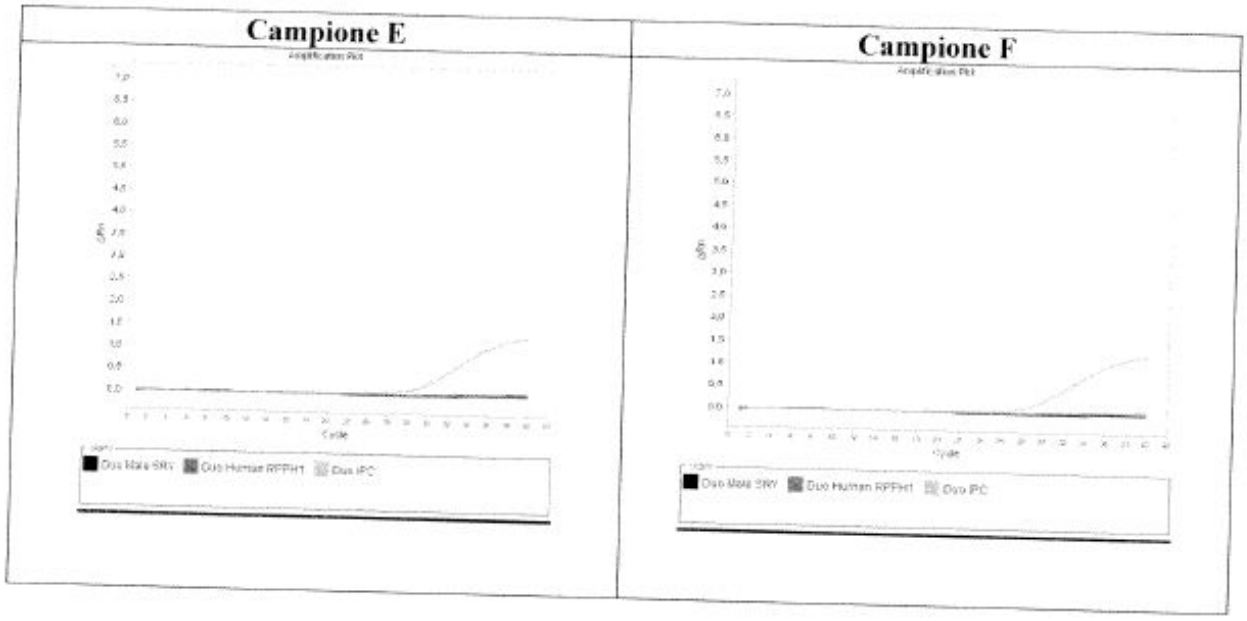
E1	STANDARD	Duo IPC	29,753	29,765	0,017		
E1	STANDARD	Duo Male SRY	30,859	30,861	0,002	0,620	
E2	STANDARD	Duo Human RPPH1	29,604	29,648	0,061	0,620	
E2	STANDARD	Duo IPC	29,777	29,765	0,017		
E2	STANDARD	Duo Male SRY	30,862	30,861	0,002	0,620	
F1	STANDARD	Duo Human RPPH1	31,231	31,233	0,003	0,210	
F1	STANDARD	Duo IPC	29,715	29,721	0,007		
F1	STANDARD	Duo Male SRY	32,178	32,244	0,094	0,210	
F2	STANDARD	Duo Human RPPH1	31,236	31,233	0,003	0,210	
F2	STANDARD	Duo IPC	29,726	29,721	0,007		
F2	STANDARD	Duo Male SRY	32,310	32,244	0,094	0,210	
G1	STANDARD	Duo Human RPPH1	32,437	32,379	0,082	0,068	
G1	STANDARD	Duo IPC	29,669	29,740	0,100		
G1	STANDARD	Duo Male SRY	33,934	33,989	0,078	0,068	
G2	STANDARD	Duo Human RPPH1	32,321	32,379	0,082	0,068	
G2	STANDARD	Duo IPC	29,811	29,740	0,100		
G2	STANDARD	Duo Male SRY	34,044	33,989	0,078	0,068	
H1	STANDARD	Duo Human RPPH1	33,744	33,762	0,026	0,023	
H1	STANDARD	Duo IPC	29,622	29,673	0,073		
H1	STANDARD	Duo Male SRY	34,822	34,997	0,248	0,023	
H2	STANDARD	Duo Human RPPH1	33,781	33,762	0,026	0,023	
H2	STANDARD	Duo IPC	29,725	29,673	0,073		
H2	STANDARD	Duo Male SRY	35,173	34,997	0,248	0,023	
Well	**Sample Name**	**Target Name**	**CT**	**CT Mean**	**CT SD**	**Quantity (ng/ul)**	**Quantity Mean**
A3	A	Duo Human RPPH1	37,367	36,720	0,588	0,002	0,003
A3	A	Duo IPC	29,716	29,735	0,038		
A3	A	Duo Male SRY	38,885	38,616	0,239	0,002	0,002
A4	A	Duo Human RPPH1	36,219	36,720	0,588	0,004	0,003
A4	A	Duo IPC	29,711	29,735	0,038		
A4	A	Duo Male SRY	38,427	38,616	0,239	0,002	0,002
A5	A	Duo Human RPPH1	36,573	36,720	0,588	0,003	0,003
A5	A	Duo IPC	29,779	29,735	0,038		
A5	A	Duo Male SRY	38,537	38,616	0,239	0,002	0,002
B3	B	Duo Human RPPH1	Undetermined				
B3	B	Duo IPC	29,830	29,767	0,055		
B3	B	Duo Male SRY	Undetermined				
B4	B	Duo Human RPPH1	Undetermined				
B4	B	Duo IPC	29,724	29,767	0,055		
B4	B	Duo Male SRY	Undetermined				
B5	B	Duo Human RPPH1	Undetermined				
B5	B	Duo IPC	29,748	29,767	0,055		
B5	B	Duo Male SRY	Undetermined				
C3	C	Duo Human RPPH1	Undetermined				
C3	C	Duo IPC	31,141	30,790	0,311		

C3	C	Duo Male SRY	Undetermined				
C4	C	Duo Human RPPH1	Undetermined				
C4	C	Duo IPC	30,546	30,790	0,311		
C4	C	Duo Male SRY	Undetermined				
C5	C	Duo Human RPPH1	Undetermined				
C5	C	Duo IPC	30,683	30,790	0,311		
C5	C	Duo Male SRY	Undetermined				
D3	D	Duo Human RPPH1	37,911	37,701	0,298	0,001	0,001
D3	D	Duo IPC	29,867	29,730	0,130		
D3	D	Duo Male SRY	Undetermined				
D4	D	Duo Human RPPH1	Undetermined	37,701	0,298		
D4	D	Duo IPC	29,716	29,730	0,130		
D4	D	Duo Male SRY	Undetermined				
D5	D	Duo Human RPPH1	37,490	37,701	0,298	0,002	0,001
D5	D	Duo IPC	29,607	29,730	0,130		
D5	D	Duo Male SRY	Undetermined				
E3	E	Duo Human RPPH1	Undetermined				
E3	E	Duo IPC	29,822	29,661	0,214		
E3	E	Duo Male SRY	Undetermined				
E4	E	Duo Human RPPH1	Undetermined				
E4	E	Duo IPC	29,743	29,661	0,214		
E4	E	Duo Male SRY	Undetermined				
E5	E	Duo Human RPPH1	Undetermined				
E5	E	Duo IPC	29,419	29,661	0,214		
E5	E	Duo Male SRY	Undetermined				
F3	F	Duo Human RPPH1	Undetermined				
F3	F	Duo IPC	29,741	29,637	0,111		
F3	F	Duo Male SRY	Undetermined				
F4	F	Duo Human RPPH1	Undetermined				
F4	F	Duo IPC	29,520	29,637	0,111		
F4	F	Duo Male SRY	Undetermined				
F5	F	Duo Human RPPH1	Undetermined				
F5	F	Duo IPC	29,649	29,637	0,111		
F5	F	Duo Male SRY	Undetermined				
G3	G	Duo Human RPPH1	Undetermined				
G3	G	Duo IPC	29,644	29,752	0,126		
G3	G	Duo Male SRY	Undetermined				
G4	G	Duo Human RPPH1	Undetermined				
G4	G	Duo IPC	29,890	29,752	0,126		
G4	G	Duo Male SRY	Undetermined				
G5	G	Duo Human RPPH1	Undetermined				
G5	G	Duo IPC	29,723	29,752	0,126		
G5	G	Duo Male SRY	Undetermined				
H3	H	Duo Human RPPH1	37,727	37,727		0,001	0,001

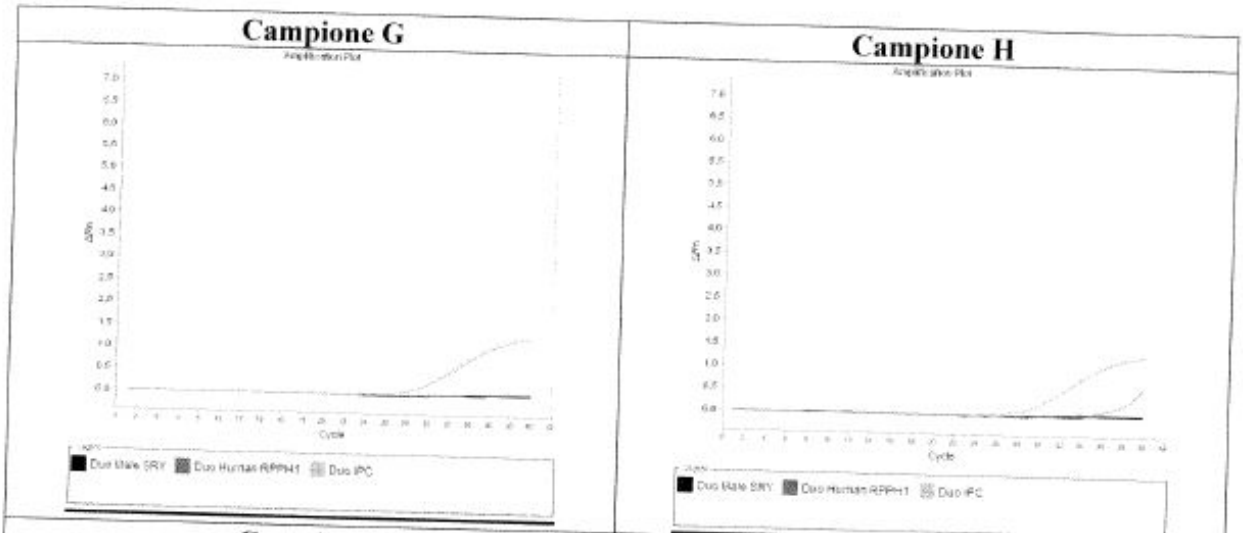
H3	H	Duo IPC	29,775	29,778	0,013		
H3	H	Duo Male SRY	**Undetermined**				
H4	H	Duo Human RPPH1	**Undetermined**	37,727			
H4	H	Duo IPC	29,793	29,778	0,013		
H4	H	Duo Male SRY	**Undetermined**				
H5	H	Duo Human RPPH1	**Undetermined**	37,727			
H5	H	Duo IPC	29,767	29,778	0,013		
H5	H	Duo Male SRY	**Undetermined**				
A6	I	Duo Human RPPH1	35,112	36,645	1,784	**0,009**	**0,005**
A6	I	Duo IPC	29,810	29,800	0,053		
A6	I	Duo Male SRY	**Undetermined**	38,616			
A7	I	Duo Human RPPH1	38,603	36,645	1,784	**0,001**	**0,005**
A7	I	Duo IPC	29,847	29,800	0,053		
A7	I	Duo Male SRY	**Undetermined**	38,616			
A8	I	Duo Human RPPH1	36,221	36,645	1,784	**0,004**	**0,005**
A8	I	Duo IPC	29,743	29,800	0,053		
A8	I	Duo Male SRY	38,616	38,616		**0,002**	**0,002**
B6	L	Duo Human RPPH1	**Undetermined**				
B6	L	Duo IPC	29,697	29,705	0,043		
B6	L	Duo Male SRY	**Undetermined**				
B7	L	Duo Human RPPH1	**Undetermined**				
B7	L	Duo IPC	29,752	29,705	0,043		
B7	L	Duo Male SRY	**Undetermined**				
B8	L	Duo Human RPPH1	**Undetermined**				
B8	L	Duo IPC	29,666	29,705	0,043		
B8	L	Duo Male SRY	**Undetermined**				
C6	M	Duo Human RPPH1	**Undetermined**				
C6	M	Duo IPC	30,345	30,408	0,056		
C6	M	Duo Male SRY	**Undetermined**				
C7	M	Duo Human RPPH1	**Undetermined**				
C7	M	Duo IPC	30,451	30,408	0,056		
C7	M	Duo Male SRY	**Undetermined**				
C8	M	Duo Human RPPH1	**Undetermined**				
C8	M	Duo IPC	30,428	30,408	0,056		
C8	M	Duo Male SRY	**Undetermined**				
D6	CN	Duo Human RPPH1	**Undetermined**				
D6	CN	Duo IPC	29,456	29,529	0,083		
D6	CN	Duo Male SRY	**Undetermined**				
D7	CN	Duo Human RPPH1	**Undetermined**				
D7	CN	Duo IPC	29,511	29,529	0,083		
D7	CN	Duo Male SRY	**Undetermined**				
D8	CN	Duo Human RPPH1	**Undetermined**				
D8	CN	Duo IPC	29,620	29,529	0,083		
D8	CN	Duo Male SRY	**Undetermined**				

[22] Finally, for each DNA sample (A, B, C, D, E, F, G, H, I, L, M, CN, and NTC), we report below the plot [*tracciato*] relative to the PCR reaction. Each plot (Male SRY, Human RPPH1 and IPC) consists of three curves (triplicate), each of which is shown with three different colors as indicated in the tables recorded for each plot:

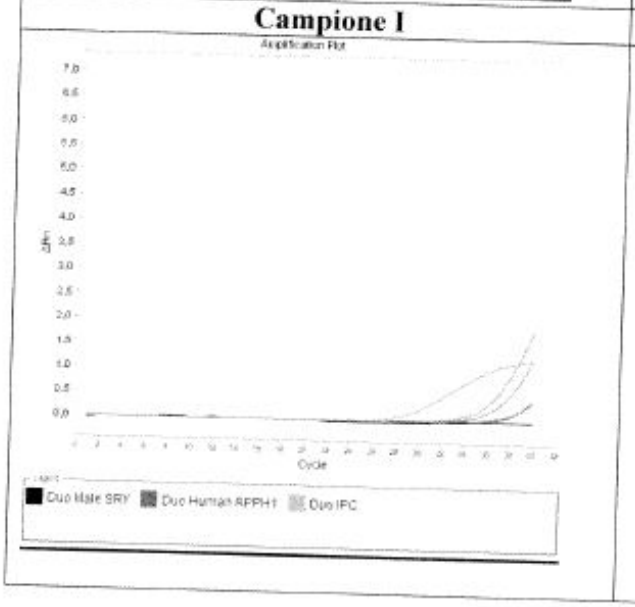


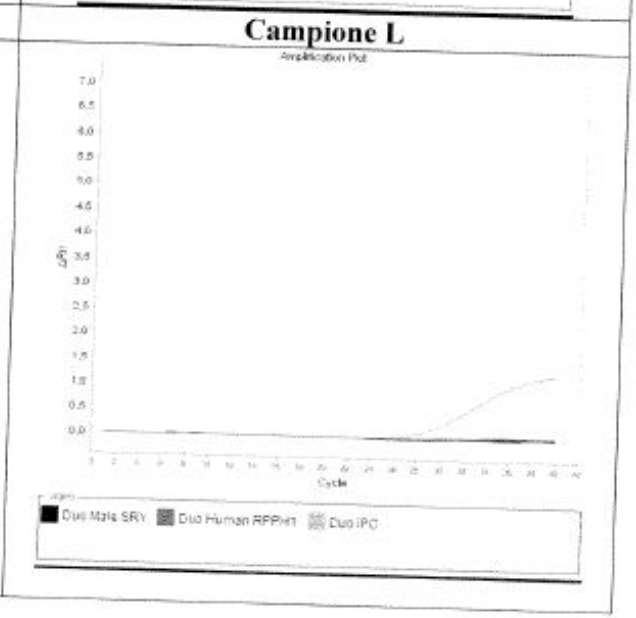

Campione E
Amplification Plot
Cycle
Duo Male SRY
Duo Human RPPH1
Duo IPC

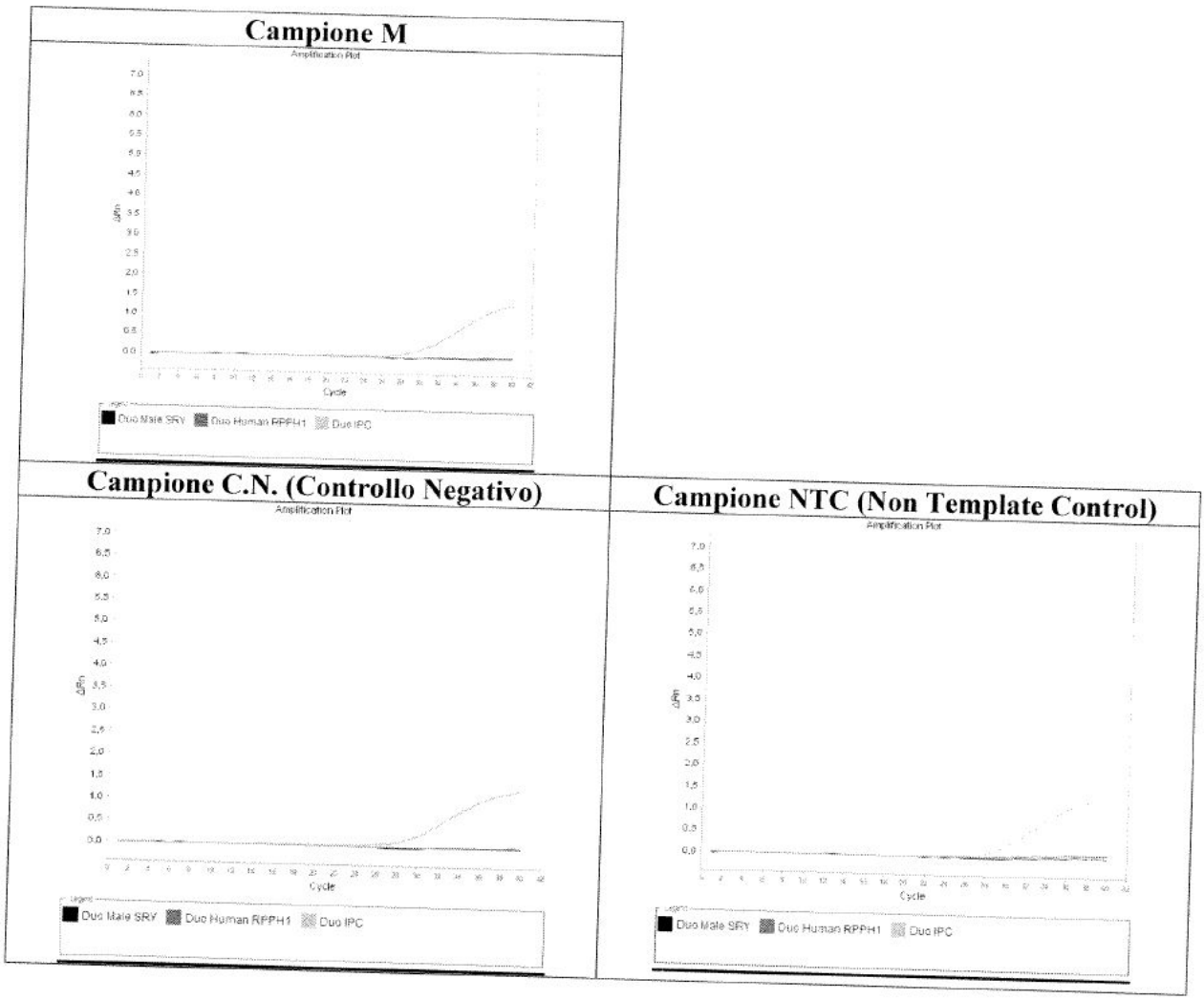
Campione F
Amplification Plot
Cycle
Duo Male SRY
Duo Human RPPH1
Duo IPC


Campione G
Amplification Plot
Cycle
Duo Male SRY
Duo Human RPPH1
Duo IPC

Campione H
Amplification Plot
Cycle
Duo Male SRY
Duo Human RPPH1
Duo IPC


Campione I
Amplification Plot
Cycle
Duo Male SRY
Duo Human RPPH1
Duo IPC


Campione L
Amplification Plot
Cycle
Duo Male SRY
Duo Human RPPH1
Duo IPC


Campione M
Amplification Plot
Cycle
Duo Male SRY
Duo Human RPPH1
Duo IPC
Campione C.N. (Controllo Negativo)
Amplification Plot
Cycle
Duo Male SRY
Duo Human RPPH1
Duo IPC
Campione NTC (Non Template Control)
Amplification Plot
Cycle
Duo Male SRY
Duo Human RPPH1
Duo IPC

CYTOLOGICAL ANALYSIS

[25] Cytological analysis on cotton fragments taken from the swabs performed on 03/22/2011 was carried out, with prior notice to the parties, on 04/05/2011, at the Department of Anatomical, Histological, Forensic, and Locomotive Sciences — Histology and Medical Embryology Section of Sapienza University of Rome, in the presence of the parties.

On April 5, 2011, the following memorandum was drafted:

"On 4/5/2011 at 10:10 am, at the Department of Forensic Medicine of the University of Rome — La Sapienza, Forensic Genetics Laboratory, 336 Viale Regina Elena, as ordered at the hearing of 1/22/2011, testing by the Appointed Experts Prof. Carla Vecchiotti and Prof. Stefano Conti was begun, in the presence of the following consultants for the parties:

- Dr. Patrizia Stefanoni, consultant for the Prosecutor's Office;
- Prof. Giuseppe Novelli, consultant for the Prosecutor's Office;
- Dr. Emiliano Giardina, consultant for the Prosecutor's Office;
- Prof. Francesca Torricelli, consultant for the civil plaintiff;
- Prof. Adriano Tagliabracci, consultant for Raffaele Sollecito;
- Dr. Valerio Onofri, consultant for Raffaele Sollecito;
- Ms. Donatella Donati, attorney for Raffaele Sollecito, acting on behalf of Mr. Luca Maori;
- Prof. Carlo Torre, consultant for Amanda M. Knox;
- Dr. Sarah Gino, consultant for Amanda M. Knox;
- Dr. Walter Patumi, consultant for Amanda M. Knox.

It is noted that Prof. Novelli removed himself at 10:35; subsequently, the Appointed Experts and the consultants for the parties [*i CTP*, presumably *Consulenti Tecnici delle Parti*] reported to the Histology Section of the Appointed Experts' home Department, where the slides [*vetrini*] relative to the Items 36 (knife) and 165b (clasps) were prepared and examined. The Experts and the consultants examined the results with a microscope and agreed to photographically document the item labeled with the letter H (handle-blade contact edge). Prof. Tagliabracci and Dr. Onofri asked that the experts proceed to request the raw data (electronic files generated by the instrument) relative to the quantization and electrophoretic analysis relating to Items

165 B (clasp) and 36 **[26]** (knife). Dr. Gino, Dr. Patumi, and Prof. Torre concurred with the request. The present memorandum ends at 1:50 pm."

CYTOCENTRIFUGATION TO DETECT CELLULAR MATERIAL

After verifying the integrity of the adhesive tape placed around the box containing the tubes in which the fragments of the swabs performed on 03/22/2011 were inserted, we proceeded with further laboratory tests.

Analysis to detect cellular material was conducted via the technique of cytocentrifugation, by means of the *Cytospin 3* apparatus produced by *Shandon*. A cytocentrifuge is an instrument which employs centrifugal force to isolate and prepare a cellular monostrate on appropriate slides, and at the same time, allows cellular integrity to be preserved. From each sample for DNA extraction, a small cotton fragment was removed, on which analysis to detect any cellular material present was performed.

Before proceeding with the analysis of the samples, all the working surfaces were treated with disinfecting and antifungal solutions, left to act for about 30 minutes before cleaning. Furthermore, to limit any contamination as far as possible, an appropriate support was used to prevent contact between the plate containing the samples and the working surfaces.

All the operations were carried out using disposable gloves, which were regularly changed between one sample and the next.

Each sample was washed by means of repeated pipetting, in a 90-µL volume of extraction swab. The entire volume was then loaded onto a sample holder with a centrifuge and a sterile, disposable funnel, and subjected to sedimentation using the following parameters: time 5 minutes, velocity 800 rotations per minute (rpm), average acceleration. The entire operation was performed at room temperature.

After cytocentrifugation, the samples were immediately observed with an optical microscope to determine the presence of material deposited on the slide.

[27] With prior notice to the parties, on May 30, 2011, at 11:30 am, at the Histology and Medical Embryology Section of the University of Rome — La

Sapienza, we proceeded with the coloration of the cytocentrifugation slides, obtained on April 5, 2011, and the acquisition of images of the slides themselves.

On this date, no consultants for the parties were present.

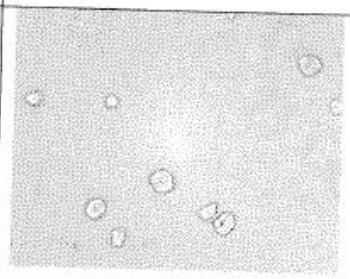
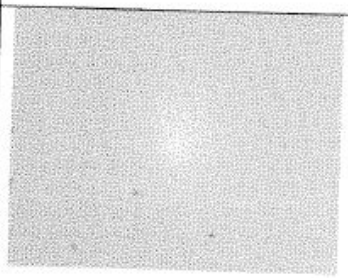
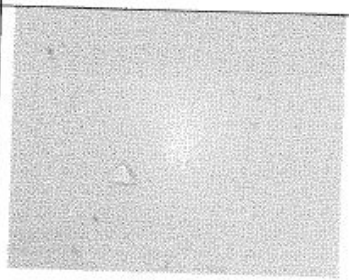
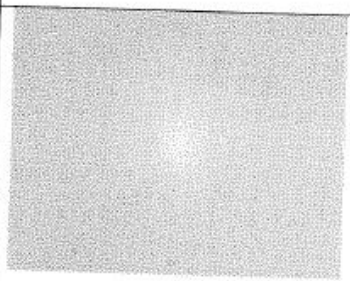
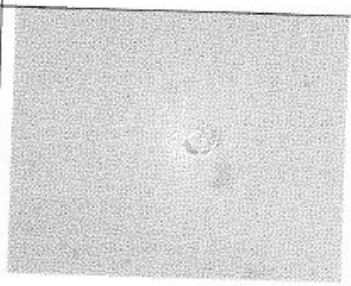
The following memorandum was drafted: “We note that none of the consultants for the parties, regularly informed of the testing session, were present. Consequently, the Appointed Experts performed what was announced (photographic documentation of cytocentrifugation results). The present memorandum ends at 1:30 pm.”

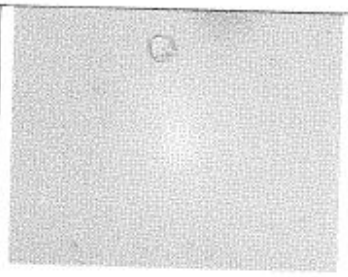
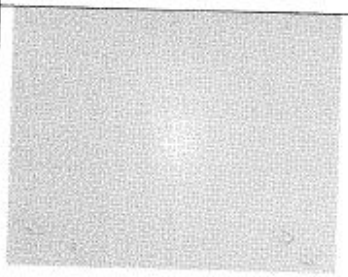
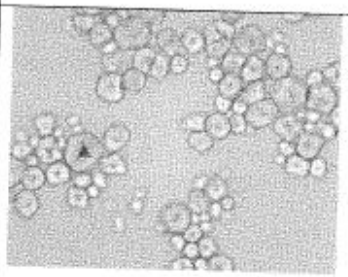
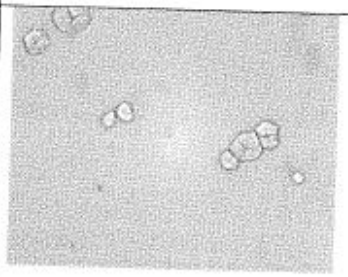
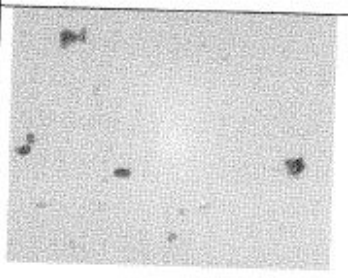
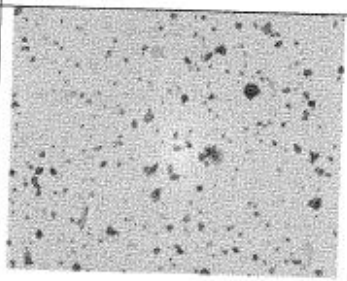
In order to determine the presence of any cellular material, each sample was colored with hematoxylin (*Hematoxylin, Vector Laboratories, CA. Cat. H-3401*). Hematoxylin is a common basic colorant used to highlight acidic cellular structures such as endoplasmic reticulum and nucleic acids contained in the cell nucleus. The structures colored with hematoxylin appear blue in color.

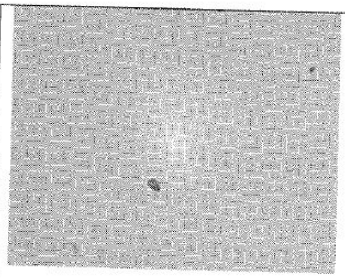
Excess colorant was removed with water and each sample was analyzed with an optical microscope with 20X magnification. A *ZEISS Axioskop 2 plus* microscope was used for this purpose, and the slides were individually photographed.

Descriptions and images of the material present in the individual slides are reported in the following table:

[28]

Sample	Observations	
A	Sample presents various debris of inorganic nature. Granules with circular/hexagonal morphology with a radial and/or linear structure are evident. No cellular material is present.	
B	Sample presents debris of inorganic nature. No cellular material is present.	
C	Sample presents various debris of inorganic nature. No cellular material is present.	
D	Sample presents various debris of inorganic nature. No cellular material is present.	
E	Sample presents a few pieces of debris and inorganic crystals. A very few granules with circular/hexagonal morphology and central linear structure are present. Cellular material is not evident.	

F	Sample presents debris and a very few granules with circular/hexagonal morphology with central radial structure. Cellular material is not present.	
G	Sample presents debris of inorganic nature. Cellular material is not present.	
H	Sample presents numerous granules with circular/hexagonal morphology with central radial structure, identical to those observed in other samples. Sample H, however, displays a decisively higher number of these structures compared to the samples already observed. Cellular material is not present.	
I	Sample presents various debris of inorganic nature. Certain granules with central radial structure identical to those observed in previous samples are present. Cellular material is not present.	
L	Sample presents certain precipitates of intense color but certainly not of cellular nature. Probably oxidized precipitates.	
M	Sample M appears similar to sample L. The density of the fragments is decisively higher. Granules are not in evidence. Cellular material is not present.	

C.N.	Sample presents rare debris of inorganic nature.	

[30] Analysis and photographic examination of various images present in the literature allowed us to establish that the structures present in certain slides (granules with circular/hexagonal morphology with central radial structure) present a morphology attributable to that of granules of amide, a polysaccaridic carbohydrate of vegetable nature. The sample which presents the greatest concentration of these structures is sample H. By means of microscopic analysis, moreover, it was possible to determine that the dimensions of the individual granules are between 3-10 μm approximately.

CONCLUSIONS

In conclusion, **there is no evidence of cellular material in the samples analyzed** with the cytocentrifugation technique and coloration with hematoxylin.

Some samples (A-E-F-H-I) and in particular sample "H", **show granules with a characteristic circular/hexagonal morphology and a radial central structure**. A more in-depth microscopic study, along with a review of the data present in the literature, allowed it to be determined that the structures in question are attributable to starch granules: therefore material of a vegetable nature.

Taking note that no DNA suitable for further laboratory investigations (amplification, electrophoresis) was present either on the swabs [*tamponature*] (A-B-C-D-E-F-G-H-I) taken from Exhibit 36 (knife) or on those (L-M) taken from Exhibit 165B (hooks of the bra), the experts verbally informed the consultants of the parties that they would proceed to a detailed examination of the Technical Report drawn up

by the Scientific Police, as according to the task formulated during the assignment hearing.

[31] Before tackling the second part of the assignment given to us, we consider it appropriate to give an outline [*cenni*] of the forensic methodology applied for investigative purposes, a methodology which can be summarized in the following steps: 1) define the crime scene; 2) reconstruct the way in which the crime happened; 3) collect elements used for the purposes of identification; 4) send the items to the laboratory for expert analysis.

NOTES ON INSPECTION AND COLLECTION TECHNIQUES

By way of introduction, it is appropriate to note what is said in Barry Fischer's ***Techniques of Crime Scene Investigation*** *(CRC ed. 2003*) about the correct approach to the scene of a crime on the part of general non-specialist staff (*Golden Rules*): what to do and what not to do at the crime scene (*Crime Scene Dos and Don'ts*) in order to avoid basic *errors* and to reduce the risk of contamination.

The starting point is always *Locard's Principle*[1], according to which two objects which come into contact with one another exchange material in different forms.

Equally, the same principle scientifically supports the possibility of contamination and alteration [of the scene] on the part of anyone else, investigators included, who comes into contact with the scene.

THINGS TO DO

- Cordon off [*limitare*] the crime scene (primary and secondary);
- Note any alteration of the scene due to your own intervention or that of a third party;
- Avoid introducing contamination (direct or indirect) to the scene;
- Accurately record the position of objects before moving them (attention: do not try to replace objects in their original positions);

1 "Locard's exchange principle states that 'with contact between two items, there will be an exchange' (Thornton, 1927) and is known most commonly as the idiom 'every contact leaves a trace'. Essentially, Locard's principle is applied to crime scenes in which the perpetrator(s) of a crime comes into contact with the scene, so the perpetrator(s) will both bring something into the scene and leave with something from the scene".

- Pay attention to yourself as a source of pollution of the evidence.

THINGS NOT TO DO

- Permit or carry out an indiscriminate access, especially without recording it;
- Alter the condition of the sites;
- Move around without taking precautions (PPE and movement procedures);
- Fail to document access;
- **[32]** Assume others will note original conditions.

It is stressed that the *Crime Scene Manager* is a figure of great importance, whose task is to ensure that the operations of survey and documentation by the various components of the team proceed correctly, especially in complex crimes, and who is able to manage the emergency situation through [ensuring] the correct flow of established procedures (***Crime Scene Management: scene specific methods***, *R. Sutton Wile, ed. 2009*).

The correct procedures for cordoning off the crime scene therefore also fall within this remit, different levels of containment consequently being adopted to avoid conditions which may entail alteration of the evidence, including contamination.

It is thus crucial to mark off a more external area, intended as the 'perimeter of containment', followed by an area of secondary containment within and then a primary area, up to the scene of the crime itself (*Crime Scene*) (***Increasing Crime Scene Integrity by Creating Multiple Security Levels****, Greg Dagnan, Criminal Justice Missouri Southern State, 2006*).

Particular attention must be paid to the floor, in that this is the most common place where evidence collects but at the same time has the biggest potential for contamination (***Protecting the Crime Scene****, G. Schiro – Louisiana State Police Crime Laboratory*).

The Guidelines of the U.S. *Department of Justice – Office of Justice Programs,* ***Crime Scene Investigation – A guide for Law Enforcement****, January 2000)* appear extremely conclusive:

2. Contamination Control:

- Contamination control and the prevention of cross-contamination in single or multiple scenes is essential to maintain the safety of staff and the integrity of the evidence;
- Limit access to the scene;
- Use set routes to enter and exit the scene;
- **[33]** Designate a secure area for refuse and equipment;
- Use personal protective equipment (PPE) to prevent contamination of staff and to minimize contamination of the scene;
- Clean/Sanitize or dispose of instruments/equipment and protective personal equipment between evidence collections and/or between the various scenes;
- Use disposable equipment when performing direct collection of biological samples;
- Maintain scene security throughout processing, up until the final exit from the crime scene;
- Package items in order to avoid contamination and cross-contamination;
- Maintain the evidence at the scene in an appropriate way in order to prevent its degradation or loss.

The update to the aforementioned *Guidelines of the U.S. Department of Justice, **Crime Scene Investigation: a Reference for Law Enforcement Training**, June 2004*, highlights protocols/procedures which are even more stringent:

- Designate a separate area/areas for the refuse produced in the course of the investigation of the scene;
- Establish an area/areas as a location for equipment;
- Nominate one person responsible for the removal of refuse;
- Use appropriate PPE (personal protective equipment);
- Dispose of PPE in biohazard containers;
- Use clean or disposable tools/equipment;
- Throw away disposable tools/equipment in biohazard containers or in a specialized container after use;
- Clean reusable instruments before the collection of each new piece of evidence.

The manual ***Handbook of Forensic Services*** for the *Laboratory Division of the Federal Bureau of Investigation (2007)* reports the following with regard to the protocols of collection, packaging and storage of DNA evidence:

- If the DNA evidence is not properly documented, its origin may be contested;
- **[34]** If the item is not properly collected, biological activity may be lost;
- If it is incorrectly packaged, contamination may occur;
- If it is incorrectly stored, decomposition or deterioration may occur.

Blood on surfaces

- Absorb the suspected blood stain on a clean cotton swab. Dry the swab and package in clean paper or in an envelope with sealed corners. Do not use plastic containers;

Blood stains

- Allow clothing with suspected blood stains to dry. Wrap dried bloodstained clothing in clean paper. Do not put damp or dry clothing in plastic or airtight containers. Put all debris or residues from the clothing in clean paper or in an envelope with sealed corners;
- Where dried blood stains are present on immovable objects, absorb the stains on cotton swabs dampened with distilled water. Dry the swab and pack it in clean paper or in an envelope with sealed corners. Do not use plastic containers.

Storage of DNA evidence

- Store in a refrigerator, freezer (if dried), [or] at room temperature, away from light and humidity.

These recommendations on collection procedures and protocols are accepted and clearly stated in all investigative manuals:

- ***Evidence Field Manual****, Office of Forensic Sciences, New Jersey State Police, Rel 1/08*:… "Biological evidence … Blood … Thoroughly dry the stains and package in a sealed paper container, a paper bag, or wrap in clean paper … Do not use plastic containers and staples … Knives with traces of blood … (ditto) … traces of saliva … (ditto) … Fingernail swabs … dampen

the swab with distilled water and swab under the nails (one swab for each hand). Air dry, package, label and send to the laboratory".

- **[35]** ***Forensic Evidence Handbook****, Missouri State Highway Patrol, Forensic Laboratory*

- *North Carolina State Bureau of Investigation,* ***Evidence Guide****, January 2010*: "Collection, packaging and storage of evidence:…Avoid excessive heat, humidity, and temperature fluctuations, maintaining the evidence in environmentally-controlled conditions… Allow a damp specimen or one constituted of body fluids to dry before storing. Store the evidence in appropriate containers (paper, envelopes, boxes, but not in plastic) in order to avoid the formation of condensation… Always package items in paper. Never use plastic bags… Safety considerations for biological evidence… Always follow universal precautions… Use clean gloves… Do not agitate the stain and avoid spreading fine particles which may float in the air".

These collection methods are succinctly summarized in ***Protecting the Crime Scene****, G. Schiro – Louisiana State Police Crime Laboratory:*

Particular attention must be paid to the floor, in that this is the most common place where evidence collects but at the same time has the biggest potential for contamination.

Blood and traces of bodily fluid can be collected in the following ways: if the stained object can be transported to the laboratory, wrap it in a piece of paper or in an envelope and send it to the laboratory; if it cannot be transported, absorb the stain on material dampened with distilled water, dry it, before permanently packaging it. For transportation purposes and in order to prevent cross-contamination, the material may be put in a plastic container for no longer than 2 hours. Once arrived in the laboratory, the material must be removed from the plastic and allowed to dry. At this point, package it in a paper container and put it in a paper envelope…

Damp traces of blood and bodily fluids can be collected in the following ways: all items must be packaged separately in order to prevent cross-contamination. If the item can be transported to the laboratory, package it in a paper container (or a plastic container if the **[36] transport** time is less than 2 hours), take it to a secure place and

allow it to dry completely, then repackage it in a paper container. If it cannot be transported to the laboratory, then absorb the stain on a small fragment of sterile cotton. Package it in paper (or plastic if the transport time is less than 2 hours), put it in a secure place and let it dry completely; then repackage it in a paper container. ***Under no circumstances can wet or damp items be left in plastic or paper containers for more than 2 hours…***

This last direction must be absolutely respected, and all the protocols and procedures give specific warnings about it: as, for example, in the ***Physical Evidence Handbook****, Department of Justice of the State of Wisconsin, State Criminal Laboratory (7th edition)*:

- …it needs to be ensured that the item is not altered or contaminated between the time of its collection and the time of its examination…
- items for DNA examination must ***always be packaged in paper*** or in a cardboard box, even if they appear dry…

And again, in ***Understanding DNA Evidence: A Guide for Victim Service Providers,*** *Department of Justice*:

…investigators and laboratory staff must always wear disposable gloves, use clean instruments, and avoid touching other objects, including their own bodies, when handling evidence. Environmental factors, like heat and humidity, can also accelerate the degradation of DNA. For example, wet or damp items which are packaged in plastic will create a growth environment for bacteria which can destroy DNA evidence. Consequently, biological evidence must be completely air-dried, packaged in paper, and correctly labelled. Treated in such a way, DNA can be stored for years without risk of degradation, even at room temperature…

The adoption of the protocols and procedures hitherto explained are universally applied not only on other continents, but also at a European level, as in the ***Interpol Handbook on DNA Data Exchange and Practice – Recommendations from the Interpol DNA Monitoring Expert Group*** *– second edition 2009:*

…liquid samples… If blood, semen or saliva are present as a liquid or a damp stain, they must be sampled using a dry swab or pipette. Sterile cotton swabs are available to sample stains from the crime scene. The stain must be sampled on one

area of the swab, and not wiped over the entire surface of the **[37]** swab tip… *dry stains*…: use a lightly dampened swab to sample the material for DNA, concentrating the stain in the smallest possible area [of the swab]… In all circumstances, it is important to also take a control sample… If at any point during the sampling, the sample is dropped or enters into contact with any other surface, ideally the procedure should be halted and a new disposable DNA kit used… collect clothing and gloves and dispose of them in appropriate containers…

Anti-contamination guidelines:

- Due to the sensitivity of current DNA techniques, extreme caution (such as wearing a mask) must be taken if the person carrying out the sampling has a medical condition which causes the loss of bodily fluids or particles, as in the case of colds, coughs or influenza. Other conditions like eczema can require the wearing of additional clothing;
- all containers used for transportation must be cleaned before and after use or, if possible, not reused;
- the work area of crime scene staff must be regularly cleaned with wipes containing chlorohexadine;
- disposable materials should be used wherever DNA may be found;
- disposable gloves must always be worn over cuffs and should be changed for each item/sample. It is necessary to change gloves during the sampling of different items…;
- where possible, take the container to the evidence and not the evidence to the container…'
- contact between the victim and suspect samples must be avoided at all times;
- handling of the objects must be reduced to the minimum possible, and the objects must not be reopened [once packaged], not even for interrogation purposes;
- make sure that any person attending the crime scene has no contact with the suspect and with his/her clothing;
- multiple suspects, the victim and their clothing must be kept separate at all times, and must not come into contact with the same objects.

[38] Given the importance of the correct application of inspection/collection procedures to the success of laboratory investigations, a working group called '*Scene*

of Crime' was promoted within ***ENFSI*** (European Network of Forensic Science Institutes, an organization begun in 1995 and to which 19 European countries belong) with the aim of standardizing the procedures and methods employed; guidelines were published in the Good Practice Manual for Crime Scene Management.

In ***Guidance on the Production of Best Practice Manuals within ENFSI, ref cod. QCC-BPM-008, 01/05/2008***, the following points amongst others are highlighted:

- 4.3.2 The expert must also evaluate the risk of contamination (or any other problem which could affect the integrity of the evidence) [which may have happened] before the items provided for examination are sent to the laboratory to be examined, or before the start of the analysis…
- 5.1.1 Particular emphasis must be given in the manual to procedures for avoiding contamination, and to the advice given to assist individuals in the management of specific risks associated with the analysis;
- 5.1.3 Considerations about appropriate anti-contamination precautions must be based not only on those for the analyses under discussion, but for all types of evidence which could potentially be available. If these include materials which could be required for subsequent DNA analysis, extreme caution must be taken due to the sensitivity of current DNA techniques, by means of the wearing of appropriate clothing including gloves and face masks (see Appendix 2);
- 5.4.1. All items must be packaged and sealed as soon as they are collected, using bags or containers of an appropriate size and made of material which avoids damaging the packaging or breaking the seals;
- 5.4.2 Once sealed, the containers must not be reopened outside of the laboratory environment. If in exceptional circumstances they must be reopened, complete and detailed documentation should be drawn up as to the conditions in which they were opened.

[39] Also from ENFSI – to which the Scientific Police Service of Rome and the Carabinieri of Rome belong – precise working directions are given in the document ***European Crime Scene Management: Good Practice Manual***. In particular:

- …it is essential that all agents are aware of the importance of preserving the scene;
- Avoid the temptation to examine. Consider and record all contamination risks. Take down the names of everyone at the scene;
- Protect the scene: identify the extent of the scene and cordon it off. Prevent access by other people;
- Refine the most appropriate cordoned-off area. It is better that too large an area is cordoned off rather than one which is too restricted – it can always be reduced later on;
- Protect the scene;
- Establish a meeting point outside the cordoned-off area;
- Concrete and efficient channels of communication amongst those examining the scene and the team of investigators are essential in every case;
- Crime Scene Manager: Make sure that everyone who enters the scene wears protective clothing, shoe covers, masks and gloves;
- Provide advice and quality assurance on all scientific questions including the storage and collection of evidence, and withdrawal from the scene;
- Contamination: it is essential that all steps are followed to ensure there is no contamination of the collected evidence. If contamination is discovered, the results of any scientific analysis could be invalidated. Protection from contamination must always start at the crime scene and continue until the sample is deposited at the Forensic Science Laboratory;
- It is essential that each action performed in the collection of evidence is documented.

NOTES ON FORENSIC LABORATORY TESTS

[40] Once the evidence collected during the inspection has arrived in the laboratory, it is subjected to a series of tests according to a standardized protocol in forensic laboratories, which can be summarized in the illustrative passages which follow.

In order to establish the presumed biological nature of samples taken during an inspection or present on items under examination, *presumptive tests* are carried out, which allow a **generic identification** to be made of the presumed biological material (e.g. blood, seminal fluid, saliva, etc). However, these tests must be confirmed by other methods which are able to establish the nature of the material under examination. The presumptive reactions for the **generic identification of blood** are based on the properties some substances have, when reduced to a colourless state (leuco base[2]) to turn a particular colour when in the presence of a peroxide (e.g. H^2O^2) and a peroxidase (e.g. haemoglobin) due to oxidation. In current use is *Adler's reaction*, which consists of testing the item under examination with a saturated solution of benzidine in acetic acid, subsequently adding drops of H^2O^2. If blood is present, the solution rapidly turns blue. Recently, reagent strips (*Combur Test*) were introduced into laboratory practice: [the strips are] impregnated with organic hydroperoxide and tetramethylbenzidine as a colourmetric indicator; if haemoglobin is present, causing oxidation, [the strips] turn from yellow to green-blue. The test is quick and easy to carry out, and the sensitivity varies, according to the Authors, from 1:300,000 to 1:500,000. False positives are known due to the presence of oxidants (e.g. metals like copper and iron), vegetable or animal peroxidases etc, while false negatives [can occur] due to strong reductive substances (e.g. cyanide) inhibiting the action of the haemoglobin.

If the traces are not visible to the naked eye (e.g. if present on a dark substrate) the Luminol test can be used (composed of an alkaline solution of luminol, sodium carbonate and sodium perborate). The solution is sprayed onto the substrate and, on reacting with haemoglobin, produces a chemiluminescence which is visible for a few

2 'Leuco base': "a colourless compound formed by reducing a dye so that the original dye can be regenerated by oxidation".

seconds. There may also be false positive results with this test if non-haematic peroxidases are present.

In order to confirm that the material under examination is human blood (**species identification**) specific tests are employed based on immunochromatographic reactions which use monoclonal anti-human haemoglobin antibodies combined with a chromogen substance. The extracted material is placed on a **[41]** reagent strip on which anti-human haemoglobin antibodies are immobilized: if human blood is present, the haemoglobin-antibody complex will concentrate the chromogen particles in a blue line. A positive control allows the accuracy of the reaction to be verified.

For the identification of **seminal fluid**, light sources can be used which cause the appearance of a white-blue light (known as 'lunar') if seminal fluid is present.

Among the most widely used ***generic and species-specific*** tests for seminal fluid are the immunochromatographic methods. These are quick and easy to perform, and allow the detection of two of the major components of human sperm: the Prostate Specific Antigen (*PSA*) and Semenogelin (Sg). In addition to this, of course, microscopic identification of the spermatozoa (if present) is carried out, after they have been suitably stained [for viewing].

For the identification of **saliva**, immunochromatographic tests with monoclonal antibodies [specific for] human alpha-amylase are used, an enzyme present in large quantities in human saliva.

Once it has been confirmed that the collected material is a biological substance of human origin, laboratory investigations proceed with the aim of arriving at an individual identification.

For this purpose, [the direction of] the testing turns to the search for a genetic profile through DNA analysis.

Individual identification by DNA analysis: The cells of the human body have a structure constituted of a membrane, cytoplasm and the organelles associated with it, and the nucleus. The genetic material of the cell (DNA) is located inside the nucleus: it forms a complex with proteins, and is organized in linear structures called chromosomes. The human genome is constituted of two types of genetic material: the nuclear DNA (consisting of 23 pairs of chromosomes, 22 of these being autosomal pairs, and 1 pair of sexual chromosomes) and mitochondrial DNA. The nuclear DNA (*deoxyribonucleic acid*) contains all the information necessary to construct an

organism, to make it function and to maintain it, as well as transmitting life from one generation to the next. It is a macromolecule made up of sub units called nucleotides, each of which is composed of a five-carbon sugar (deoxyribose) attached to a nitrous base (adenine, guanine, thymine and cytosine) and a phosphate group. The characteristics of an individual transmitted from one generation **[42]** to the next are controlled by features of DNA called *genes*. The genetic constitution of an organism is called its *genotype*, while the *phenotype* is the physical manifestation of these genetic characteristics. The location in the chromosome of a particular gene is called its *locus*. Genes can exist in different forms, called *alleles*, which may give rise to the expression of different characteristics. An organism which inherits two identical alleles from its parents (for each specific locus, one allele is of paternal origin and one of maternal origin) is defined as *homozygote*, while those which possess two alleles that differ from one another are termed *heterozygote*. It is believed that the human genome contains only 20,000-25,000 genes, and only 1-1.5% of the genome is directly involved in the codification of proteins. About 75% of the genome is termed extragenic and more than 50% of this is composed of DNA repeats; 45% of these are interspersed repeated sequences, and the rest are sequences of DNA in tandem repeats (Lander et al., 2001; Li, 2001). It is these latter, made up of *satellites, minisatellites and microsatellites, which are the regions of the genome most used for individual identification*. At the end of the '80s, in the same period in which minisatellites were discovered, microsatellites were identified [*descritti*] – better known as *Short Tandem Repeats* or STRs. These have a repeated sequence from 2-7 bp, and to this day represent the most commonly used technique in the field of forensics. Tetrameric repetitions (a unit repeated in four bases) were combined in the so-called *multiplex* [technique] in order to obtain 16 loci amplified simultaneously in a single PCR reaction. The STR regions are highly polymorphic[3], and are therefore able to provide a very high power of discrimination (Butler, 2005).

In 1997, the Federal Bureau of Investigation (FBI) laboratory proposed the establishment of a group of STR loci to be used in a national DNA database known as CODIS (Combined DNA Index System) (Budowle, 1999). The thirteen CODIS loci are: CSF1PO, FGA, TH01, TPOX, VWA, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51 and D21S11. A DNA profile obtained with the analysis

3 Polymorphism: "natural variations in a gene, DNA sequence, protein, or chromosome that have no adverse effect on the individual and occur with fairly high frequency in the general population".

of the 13 STR loci provides an average *random match probability* of about 1 in a trillion in randomly chosen individuals in the population (Butler, 2005). These loci are recognized in the United States as the standard for the purposes of human identification.

[43] In the field of forensic DNA analysis within Europe, on the other hand, the *Official Journal of the European Union,* dated 5.12.2009, established that Member States of the European Union would be invited to use at least the markers indicated in the relevant Annex – which form the *European Standard Set (ESS)* – in order to facilitate the exchange of DNA analysis results between laboratories of the Member States. The *European Standard Set* comprises the following markers: *D3S1358, VWA, D8S1179, D21S11, D18S51, HUMTH01, FGA, D1S1656, D2S441, D10S1248, D12S391* and *D22S1045*.

DNA extraction: a biological sample taken from a crime scene (e.g. blood, saliva, seminal fluid etc), as with a blood or saliva sample taken from a suspect or for a paternity case, contains many substances as well as DNA. The DNA must therefore be separated from the remaining cellular material before it can be examined. The cellular proteins which encircle and protect DNA in the cell can, in fact, inhibit DNA analysis. Many extraction techniques were developed in order to purify DNA molecules of proteins and other cellular material, but the fundamental principles of this technique can be summarized in three main phases: 1) fragmentation and break-down of cell membranes to allow the release of nucleic acids; 2) denaturation of proteins; 3) separation of DNA from the proteins and removal of contaminants.

The specific method of extraction applied in each individual case depends on the type of biological sample to be examined: for instance, whole blood must be treated differently to a haematic trace or a bone fragment. Organic extraction in **phenol-chloroform** was the extraction method commonly used in the past, and it is characterized by the use of different chemical substances through successive passages. If it is true that this method functions well for DNA to a high molecular weight, it also takes a long time, requires the use of toxic chemical substances, and requires transferring the sample between different test-tubes, therefore increasing the risk of error and contamination.

Extraction with ***Chelex 100*** resin is characteristically much quicker than the organic technique, taking only a few passages inside a single test-tube, and therefore offers minor possibilities of contamination inside the laboratory. Introduced into the

forensic DNA community in 1991, Chelex 100 (*Bio-Rad Laboratories, Hercules, CA*) is an ion exchange resin, which is made into a suspension [with distilled water] (usually at 5%), and added to the samples to be **[44]** examined (Walsh et al, 1991). The samples are then subject to boiling for a few minutes in order to break down the cells and release DNA. The Chelex denatures the double-stranded DNA, converting it to single-stranded DNA.

Solid phase DNA extraction methods were developed in recent years in order to allow high yield DNA extraction. One very popular technique uses spin-columns, in which the nucleic acids selectively bind to a silica surface (e.g. in the form of small beads) in the presence of high concentrations of chaotropic salts; these disrupt the hydrogen bonds in water, denaturing the proteins. In a pH solution of less than 7.5, the adsorption of DNA to the silica is around 95%, and the impurities can then be removed by washing. Subsequently, the DNA can be easily eluted from the silica material using a low salt alkaline solution. This method can be carried out with the aid of centrifugation or vacuum aspiration.

A further solid phase extraction approach uses a silica-coated paramagnetic resin (Tereba et al., 2004); [with this method] isolation of the DNA can happen in a single test-tube, simply by adding and removing solutions. The DNA molecules bind reversibly to the magnetic particles, and a magnet is used to isolate these particles on one side of the test-tube, thus holding the impurities (proteins, cellular debris) in solution, to be removed through subsequent washing. Finally, the DNA is released from the magnetic particles by a phase of heating for a few minutes.

The **FTA card** contains chemical substances able to protect DNA from degradation by nucleases, and from bacterial growth (Burgoyne, 1996): the DNA therefore remains stable at room temperature for a period of several years. Use of the FTA card simply entails adding a drop of blood onto the card and drying it. The cells are lysed in contact with the card, and the DNA contained in the white blood cells is trapped in the matrix of the card. A small fragment of card is punched out at the area of the stain, and placed inside a test-tube for subsequent washing with solvents able to remove the heme and other PCR inhibitors.

Special methods were developed for isolating male DNA (sperm heads) from female epithelial cells (Gill et al., 1985) in sexual violence cases, thus enabling interpretation of the data by removing most of the female contributor from the resulting profile. This procedure, known as ***differential extraction***, can be carried out

through the selective digestion of epithelial cells, followed by isolation of the undigested sperm heads **[45]** through centrifugation. The spermatic fraction is then further digested [i.e. to release the DNA] and extracted using dithiothreitol (DTT).

Automation of the DNA extraction process: many different instruments were produced with different properties and capacities to process larger or smaller numbers of samples, but the fundamental mechanism, implementation procedures and simplicity of use are similar. The most popular automated method uses magnetic particles. This procedure allows the simultaneous extraction of DNA from a large number of samples (up to 96) with the guarantee of maximum reproducibility, quality and productivity.

DNA Quantification: determining the quantity of DNA present in a sample is important for the majority of PCR-based analyses, because an excessive quantity can cause the appearance of extra peaks, or peaks which are outside the limits of the measurement technique, while too scarce a template quantity can cause allele drop-out in which the PCR reaction is affected by stochastic phenomena. PCR amplification can also fail due to the presence of inhibitors extracted along with the DNA from the sample, DNA degradation, an insufficient quantity of DNA, or a combination of all these factors. Many methods were developed for DNA quantification, including the slot-blot procedure and the analysis of fluorescence using a microtiter plate.

A relatively recent innovative method in the field of forensic DNA typing is ***quantitative PCR (qPCR)***, also known as ***Real-Time PCR***. This technique requires the use of a machine able to measure DNA concentration during the PCR phases while the template is amplified. Quantitative PCR includes the same phases as traditional PCR (denaturation, annealing and extension); however a fluorescent marker sensitive to the formation of dsDNA is introduced into the reaction, and so allows the amplification product to be measured as it accumulates. The principle advantages of qPCR quantification include a vast dynamic range, high yield capacity, high sensitivity and target-specific quantification (Butler, 2005).

Commercially available kits, today in widespread use in forensics, allow the simultaneous calculation of the total quantity of human DNA and of male [DNA], using a primer sequence targeted at a specific region of the Y chromosome. Cycle by cycle, Real Time PCR analyzes variations in fluorescence from the amplification of a target sequence during PCR. There are three different phases which define the PCR process: 1) **[46]** exponential (or geometric) amplification; 2) linear amplification; and

3) the plateau phase. The best point to measure fluorescence (through a comparison with samples of known concentration) in terms of the number of cycles is the exponential PCR phase, in which the ratio between the quantity of product and the starting amount of DNA is more reliable. The qPCR instruments use the 'cycle threshold' (Ct) for the calculations. The Ct value is the point at which the fluorescence of the signal exceeds an arbitrary threshold level: the fewer cycles required to exceed this threshold, the greater the quantity of DNA template molecule placed in the reaction.

Another DNA quantification method, less sensitive than qPCR and not specific to humans, is the ***Qubit Fluorometer 2.0***, which utilizes a fluorometric technology using *Molecular Probes* markers; these emit fluorescent signals only when they are bound to specific target molecules, even in the presence of free nucleotides or degraded nucleic acids. With this method, samples with concentrations as low as 10pg/ul of DNA can be quantified (the analysis range of the Qubit dsDNA HS Assay kit is 0.2-100 ng, corresponding to a starting sample concentration in the range of 10pg/µl-100ng/µl).

DNA Amplification: The development of the Polymerase Chain Reaction (***PCR***) in 1985 by Kary Mullis gave a big boost to scientific progress in the field of DNA analysis, particularly in the forensic community. This technique allows the production of multiple copies of a nucleic acid sequence *in vitro* (Mullis, 1990). During the PCR reaction, the double-stranded DNA (dsDNA) is subject to a three-phase process: denaturation, annealing and extension. The strands of each template molecule are denatured through heating (~95ºC) and subsequent cooling (~55ºC), allowing the primers (small oligonucleotide sequences) to bind to the single strands. The temperature is then raised again (~72ºC) and a DNA polymerase enzyme extends the primers, absorbing the dinucleotide triphosphates (dNTPs). In this way, the synthesis of target sequences of dsDNA happens in a rapid cyclical process. At each cycle, the PCR generates a 2^n increase of the target DNA (in theory, the quantity of DNA doubles). The PCR requires a mix of reagents and amplification parameters which must be optimized to produce sufficient amplification product. The major components included in the "master mix", which must be aliquoted into each PCR test-tube, are the following: buffer, dNTPs, polymerases, primers and magnesium. PCR **[47]** has become an important analytical tool in the forensic field due to its sensitivity, specificity, speed of analysis and ease of automation. The PCR amplification

technique allows the analysis of low quantity (>1ng) forensic samples of extracted DNA, as well as [being] a valid and reliable approach to the analysis of biological samples recovered from a crime scene.

Capillary Electrophoresis: the PCR kits commonly used in forensic practice allow the simultaneous amplification of numerous DNA fragments, which, as STRs, are constituted by different numbers of repeating units: therefore different alleles in the generated amplicons present different lengths. In order to be analyzed, they must be separated using an appropriate technique with a high resolution [i.e. separation] capacity, so that even those alleles which differ from each other by a single base can be analyzed, in a range from 100 to 500 bp. Moreover, the method used must be reproducible to allow the results from different laboratories to be compared. To separate the various molecules in the amplicon mixture produced by the PCR reaction, the negatively charged phosphate groups in the DNA backbone are used: in an electrical field, the ions are attracted by the pole of the opposite charge; therefore in the case of nucleic acids, by the positive pole. This process is called electrophoresis, and it refers to the migration of electrical charges in a separation medium, to each end of which a potential difference is applied. The capillary electrophoresis technique (*CE*) was introduced in the early ’80s, and with subsequent development of the instrumentation, quickly gained in popularity in the fields of molecular biology and forensics. This instrumentation is completely automated, and allows the examination of multiple wavelengths simultaneously and therefore a high number of loci which overlap in length, with minimum sample consumption subject to the run. The signal emitted by fluorochromes, excited by a laser in proximity to the anodic extreme, is recorded by a detector consisting of a *Charged-Coupled Device* (*CCD*): the greater the number of photons hitting the surface of the silicone matrix, the greater the accumulation of electrons and consequently the height of the digital signal into which it is converted. The data is finally sent to a computer which, correlating the fluorescence peak with the migration time, translates the fluorescence signal into a length measurement expressed in bp or in nucleotide sequence. The sizes of the PCR products of each sample are compared with the fragments contained in the **[48]** allelic ladder. The *ladder* is constituted of a mixture of alleles of known length (these are the most common alleles of a particular STR present in the population, and therefore representative of the variability of a given STR) and is used to correlate the size of the amplification product with the number of replicates by which it is formed;

in this way, the genotype of the sample can be established. Each peak of the sample must not differ in length by more than 0.5 bp from the corresponding ladder peak, or the allele is not assigned and the peak is defined *off-ladder* (OL). At the end of the electrophoretic run of each sample, a specific software produces a file called *raw data*: a graph which correlates the *Relative Unit of Fluorescence* (RFU) on the Y axis with the number of *data points* on the X axis.

Interpretation of the Results: the conversion of an electropherogram into a genetic profile is carried out through software, but the profiles generated from the samples must be interpreted by expert staff. Guidelines were developed for the interpretation of genetic profiles to ensure that the results obtained are reliable: this aspect is of crucial importance, especially when the samples to be analyzed contain very low quantities of DNA, degraded DNA, or mixed profiles. Each laboratory must develop a proper interpretative strategy based on internal validation studies, and on the results reported in the literature (*Scientific Working Group on DNA Analysis Methods*, SWGDAM, 2010). Some of the most important guidelines for the correct interpretation of electropherograms are the following:

- it is necessary to establish a minimum value for the heights of peaks to be considered alleles; all peaks below this value are considered background noise;
- the electropherogram must show balanced peaks – that is, peaks of a comparable height. In particular, individual heterozygous loci must have peaks of about the same height. To evaluate the peak height balance of a locus, the ratio between the height of the smallest allele and that of the largest allele is calculated: usually this ratio is always greater than 90% (>0.90) but the threshold value is placed at >0.60.
- it is necessary to consider the maximum percentage of *stutter* produced at each locus. Stutter are aspecific peaks due to [strand] slippage and to the erroneous pairing of the repeating regions of the two DNA strands during the PCR reaction. The peaks are usually shorter by one repetition than the nearest allelic peak. The presence of stutter is of great importance in the interpretation of mixed traces, in that it can be difficult to establish if a peak is stutter or a true allele. It should be recalled **[49]** that the maximum *stutter* percentage observed in any individual locus is stated in the user manual of the specific amplification kit. Stutter is identified by the ratio between the height or area of

the minor peak, and the height or area of the major peak: this ratio is generally below 15%.

EXAMINATION OF THE TECHNICAL REPORT ON THE FORENSIC GENETIC TESTS BY DR. PATRIZIA STEFANONI

[50] Examination of the Technical Report on the Forensic Genetic Tests (**RTIGF**) drafted on June 12, 2008 by Dr. Patrizia Stefanoni, Principal Technical Director [and] Biologist of the State Police, reveals the following data regarding the extraction of biological traces, laboratory analyses performed, and interpretations of the electropherograms relative to Item 36.

LABORATORY ANALYSES REPORTED IN THE RTIGF REGARDING ITEM 36 (KNIFE)

Exhibit 36 is described as follows on page 5 of the Technical Report: "Large knife with an overall length of 31 cm, a 17 cm long blade, and a black handle".

SAMPLES FROM ITEM 36 (KNIFE)

In the G.U.P hearing (04.10.2008) and in the Court of Assizes (hearing on 23.05.2009), Dr. Stefanoni explained that no traces visible to the naked eye were present on the knife under examination, and that the samples were taken from parts of the [knife] where, based on her own experience, it might have been possible to find biological material. (G.U.P hearing on 04.10.08, pages 17-18: "*with regard to sample A, nothing was visible; however, our experience and common sense led us to collect [a sample] there…the [location] of sample B was not just chance…the sample was taken at that point because, again only from a visual inspection, that is, with no instrument…streaks were visible to the naked eye"*. Court of Assizes (record of the 23.05.09 hearing, page 81): "*these scratches were the only element which could, so to speak, guide me in a sample which otherwise was completely random, as happened subsequently; the other samples which were taken from the knife, therefore I'm*

referring to…also to C, which in fact was sampled at the same time as A and B, but also in particular those which followed, E and G, were done **[51]** *more or less in a random manner because there was no fact, no element which would have led me to take a sample at this point, say, rather than another"*.

Court of Assizes (record of the hearing on 23.05.09), page 94): "*sample B was taken at this point on the basis that no significant biological trace was visible, so to speak, to the naked eye. [Sample] A was taken at that point, naturally on the handle, as also with D and F, with the intention of possibly finding DNA from the person who had handled that weapon [arma]"*.

It is not explicitly indicated in the report whether the environment where the sampling was carried out (particularly the work bench surfaces as well as all the equipment present) was preemptively decontaminated with suitable substances (e.g. sodium hypochlorite or similar), whether sterilized instruments were used, and the method by which these were sterilized. With regard to the samples taken from the exhibit, it is not specified whether these were performed using sterile swabs, with changes of gloves for each individual sample, and with the wearing of lab coats and masks by the operatives.

On page 77 of the Technical Report on the Forensic Genetic Tests (RTIGF) four photos are shown of the exhibit, bearing letters from A to G on the handle and the blade, which are indicative of the points from which the samples of presumed biological material were taken.

In particular, it is apparent that three samples were taken from the **handle** of the knife (samples indicated with the letters **A-D-F**) and four samples from the **blade** (samples indicated with the letters **B-C-E-G**) for a total of seven samples.

The **Work Status Report** cards (**SAL**) show that each individual sample was given an identification code (*Sample ID Code*), reported below:

Sample A ("presumed exfoliated cells, letter A") = **47329**

Sample B ("presumed haematic substance, letter B") = **47330**

Sample C ("presumed haematic trace, letter C") = **47331**

Sample D ("presumed exfoliated cells, letter D") = **48649**

Sample E ("presumed haematic substance, letter E") = **48651**

Sample F ("presumed exfoliated cells, letter F") = **48654**

Sample G ("presumed haematic substance, letter G") = **48655**

[52] In addition, the following information is reported (c.f. SAL): *Type of Sample, Sample Description, Quantity extracted, Location extracted, Test Performed, Date of extraction*.

The SAL shows that the **generic test for blood** was performed on the samples indicated with the letters **B-C-E-G** (samples taken from the knife blade), using the tetramethylbenzidine test (TMB).

The aforementioned method, widely recognized in forensics, is numbered amongst the *preliminary methods* for the identification of blood; if positive, further tests (known as 'confirmatory') are needed in order to correctly identify blood.

The summary tables of the tests carried out on the samples and the results obtained are reproduced below, as shown on pages 77-78 of the **RTIGF**:

Analisi eseguite traccia B presunta sostanza ematica	diagnosi generica	diagnosi specie-specifica	**Analisi eseguite traccia C** presunta sostanza ematica	diagnosi generica	diagnosi specie-specifica
Tetrametilbenzidina (TMB)	⊗	//	Tetrametilbenzidina (TMB)	⊗	//
Anticorpo anti-uomo	//	⊗	Anticorpo anti-uomo	//	⊗

⊗= negativa

Analisi eseguite traccia E presunta sostanza ematica	diagnosi generica	diagnosi specie-specifica	**Analisi eseguite traccia G** presunta sostanza ematica	diagnosi generica	diagnosi specie-specifica
Tetrametilbenzidina (TMB)	⊗	//	Tetrametilbenzidina (TMB)	⊗	//
Anticorpo anti-uomo	//	⊗	Anticorpo anti-uomo	//	⊗

From the above tables, it is clear that the blood tests carried out on the samples indicated by the letters **B-C-E-G** (knife blade) were **negative for the presence of blood**.

The **"species-specific" test** was performed on the aforementioned samples, and also tested **negative for the human species**.

Conversely, the tetramethylbenzidine test was not performed on the samples indicated by the letters **A-D-F** (samples taken from the knife handle).

[53] It must be emphasized that **no laboratory tests were performed** to detect biological material of a non-haematic nature on any of the samples analyzed.

It is considered that it would have been necessary to proceed to morphological tests to identify cells which might have been present, by means of colouration with one of the reagents commonly employed in histology (hematoxylin). Such a test, quick and easy to carry out, would have required a minimum quantity of material and would not in any way have compromised further laboratory testing, but could have clarified the nature of the material removed from the item under examination.

Despite the negative result of the blood identification test and the failure to carry out cell identification tests (and therefore the failure to identify the material removed from Exhibit 36) ***the Technical Consultant hypothesized the presence of "presumed exfoliated cells" on the material sampled from the knife handle*** (traces A-D-F) ***and of "presumed biological material"*** of another nature (presumably blood, considering the tests aimed at the detection of haematic peroxidases) ***on the material sampled from the blade*** (samples B-C-E-G).

The hypotheses formulated by the Technical Consultant about the nature of the material analyzed are wholly arbitrary in that they are not supported by any *scientifically* objective confirmation.

EXTRACTION OF DNA

DNA extraction (pages 77-78) was carried out on all the samples (samples A-B-C-D-E-F-G) using the automated extractor BioRobot "EZI" (Qiagen), as reported on page 78 of the RTIGF.

The Work Status Report (SAL) shows that:

- the extraction of DNA from samples A-B-C was performed on 13.11.07;
- the extraction of DNA from samples D-E-F-G was performed on 17.12.07.

The "*Quantity of extract*" was 50 µl for each sample (c.f. SAL).

QUANTIFICATION OF DNA

[54] Quantification of the DNA extracted from the aforementioned samples was then performed.

On page 78 of the **RTIGF**, the following tables are shown:

Estrazione DNA BioRobot "EZ1" (QIAGEN)	**traccia A** presunte cellule di sfaldamento eseguita	**traccia B** presunta sostanza biologica eseguita	**traccia C** presunta sostanza biologica eseguita
Quantificazione 7700 Sequence Detector *ABI PRISM*™ della *Applied Biosystems*	☑	☑	⊗

☑= positiva

Estrazione DNA BioRobot "EZ1" (QIAGEN)	**traccia D** presunte cellule di sfaldamento eseguita	**traccia E** presunta sostanza biologica eseguita
Quantificazione 7700 Sequence Detector *ABI PRISM*™ della *Applied Biosystems*	⊗	⊗

Estrazione DNA BioRobot "EZ1" (QIAGEN)	**traccia F** presunte cellule di sfaldamento eseguita	**traccia G** presunta sostanza biologica eseguita
Quantificazione 7700 Sequence Detector *ABI PRISM*™ della *Applied Biosystems*	⊗	⊗

The above tables show that DNA quantification for all samples was performed with Real Time PCR, using the *7700 Sequence Detector ABI PRISM*™ apparatus from the company *Applied Biosystems*.

On the other hand, no information is given about the kit used for the DNA quantification.

Regarding the quantification result for the samples indicated by the ***letters A and B***, the expression "***positive***" is reported, but no numerical value is given for the DNA detected; conversely, the quantification result was "***negative***" for the samples indicated with the ***letters C-D-E-F-G***.

[55] It should be emphasized that from an examination of the ***Real Time PCR reports exhibited, it appears that quantification using this method was only carried out***, on 18 December 2007, ***on the DNA extracts indicated with the following numbers***:

48649 = sample D

48651 = sample E

48654 = sample F

48655 = sample G

These samples all tested negative for the presence of DNA (c.f. Real Time Report DNA=0.00).

A report is also attached relating to quantification of the extracts from the [following] samples, dated 13 November 2007:

1. ***A (sample code ID 47329)***
2. ***B (sample code ID 47330)***
3. ***C (sample code ID 47331)***

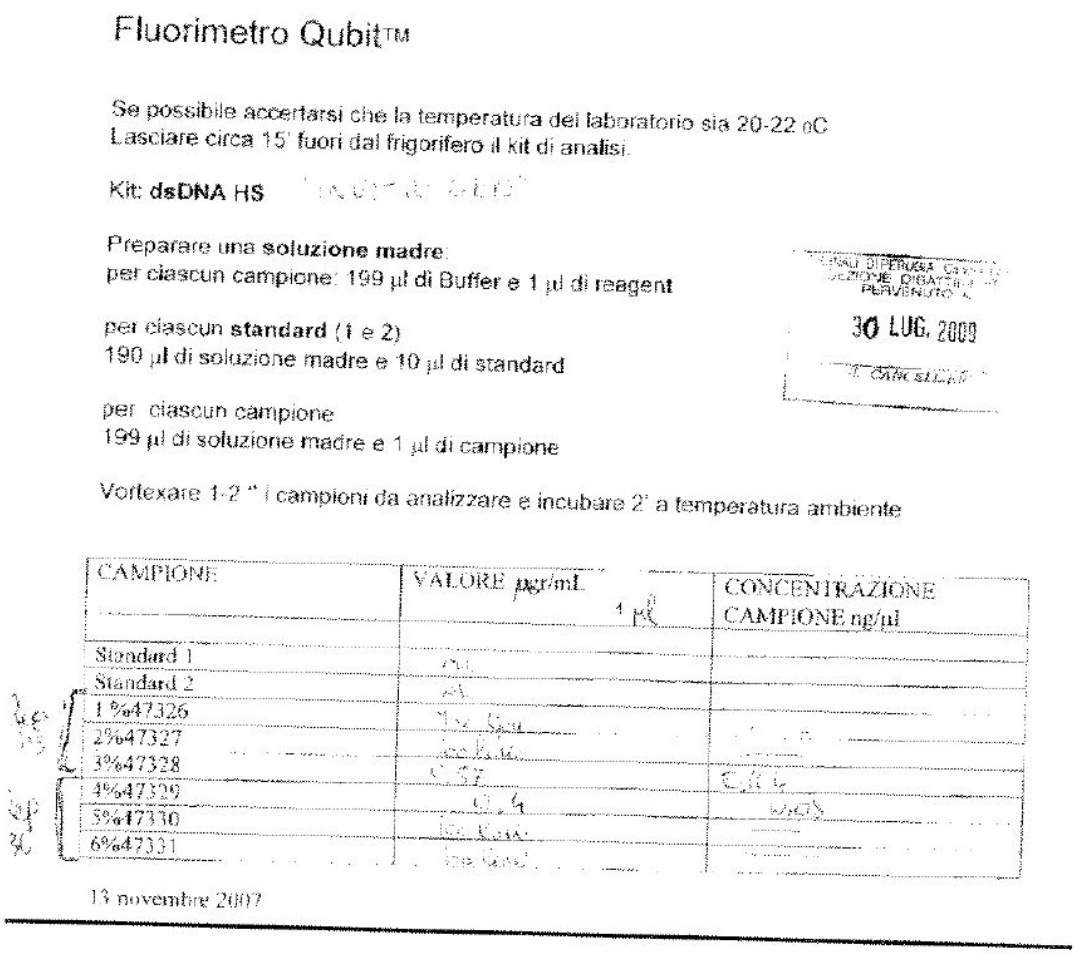
[This was] performed using the ***Qubit Fluorometer***™ from the company *Invitrogen*, and using the *dsDNA HS Kit*. This is a selective test for double-stranded DNA, but not specific for human DNA. Samples with a DNA concentration range between 0.2-100 ng can be accurately and easily quantified.

The attached card shows that the following reagents were prepared:

- Stock solution: for each sample 199 µl of Buffer and 1 µl of reagent;
- Standard (1 and 2): 190 µl of stock solution and 10 µl of standard;
- For each sample: 199 µl of stock solution and 1 µl of sample.

Using the Qubit Fluorometer™, the following concentration values for the samples were obtained:

- **Sample A (sample code ID 47329): 0.08 ng/µl**
- **Sample B (sample code ID 47330): too low**
- **Sample C (sample code ID 47331): too low**



Fluorimetro Qubit™

Se possibile accertarsi che la temperatura del laboratorio sia 20-22 oC
Lasciare circa 15' fuori dal frigorifero il kit di analisi.

Kit: **dsDNA HS**

Preparare una **soluzione madre**:
per ciascun campione: 199 µl di Buffer e 1 µl di reagent

per ciascun **standard** (1 e 2)
190 µl di soluzione madre e 10 µl di standard

per ciascun campione
199 µl di soluzione madre e 1 µl di campione

Vortexare 1-2 " i campioni da analizzare e incubare 2' a temperatura ambiente

30 LUG. 2009

CAMPIONE	VALORE µgr/mL	CONCENTRAZIONE CAMPIONE ng/µl
Standard 1		
Standard 2		
1 %47326		
2%47327		
3%47328		
4%47329		
5%47330		
6%47331		

13 novembre 2007

[56] From this, it can be inferred that ***what is reported on page 78 of the RTIGF*** (and confirmed in the GUP questioning, page 178, where it is claimed that quantification was performed using Real Time and that quantification of the Y [chromosome] was not carried out) ***is not consistent with what was performed in reality***. In fact, the tables show that ***quantification of the DNA extracted from all the samples taken from Exhibit 36 was carried out using the 770 Sequence Detector ABI PRISM™ equipment*** (Applied Biosystems); in contrast, it is apparent from the attached cards that ***the aforementioned method was only performed on samples D-E-F-G. A different method was employed for samples A-B-C using the Qubit Fluorometer™, and not mentioned in the final Technical Report, as would have been due and correct.***

Regarding the ***interpretation of the quantification***, it should be noted that on page 78, the following is stated: "*the samples testing <u>positive</u> to quantification*

(samples A and B) were subjected to amplification and subsequent capillary electrophoresis…".

As regards the ***quantification of sample A*** (knife handle) the results obtained with the *Qubit Fluorimeter*™ show that the concentration of DNA in this sample was equal to 0.08 ng/µl. Taking into account that the "*quantity of extract*" was 50 µl (c.f. SAL), and multiplying 0.08 ng/µl x 50 µl, the total [amount] of DNA was equal to 4 ng: certainly a significant quantity, which allowed sample A to be considered ***positive to quantification.***

[57] On the other hand, it is not possible to comprehend the criteria adopted in the ***assessment of the positive quantification result for sample B and the negative result for sample C***, given that ***the same result, "too low", was obtained for both samples***: that is, a value which must be considered not only below the sensitivity threshold of the Fluorimeter indicated by the manual (DNA concentrations equal to 0.2 ng/µl), but below 0.08 ng/µl, a value which the Fluorimeter detected for sample A.

Nor is it comprehensible, considering the negative results on sample B, what Dr. Stefanoni reported during the GUP questioning (page 178) where she stated that ***the DNA in sample B, quantified with Real Time PCR*** (it is recalled that such quantification as confirmed during the hearing was never carried out or, at least, no documentation was provided to support this claim), ***was "in the order of some hundreds of picograms", a value which does not appear in any of the documents provided to us (SAL, Fluorimeter report, Real Time report, RTIGF).***

AMPLIFICATION

With regard to the subsequent amplification of the extracts, the following is reported on pages 78-79 of the Technical Report:

"*The amplification of the autosomal STRs was performed according to the methods already reported on page 31; the samples which tested positive to quantification (samples A and B) were subjected to amplification and subsequent capillary electrophoresis. The samples which tested negative to quantification*

(samples C, D, E, F, G) were analyzed following concentration using the Speed-Vac SC110 instrument, 'Savant' brand".

On page 31 of the Technical Report, the "*PCR amplification*" methodologies are reported:

"*with the aim of obtaining a DNA profile from the genetic material extracted from the samples tested, the following genetic regions (loci) were amplified using pairs of oligo-nucleotide primers specific for the regions flanking the various polymorphisms of interest:* ***D3S1358, HumvWA31, D16S539, HumFGA, HumTH01, TPOX, CSF1PO, D8S1179, D21S11, D18S51, D5S818, D13S317, D7S820, D19S433, D2S1338, amelogenin (sex test)****, using the commercial kit "****AmpFISTRIdentifiler****" from Applied Biosystem (Foster City, CA) according to the "User Manual"*.

[58] It is recalled that the directions for a correct amplification provided by the manufacturer of the *AmpFISTR Identifiler* kit are the following:

- *No. samples x 10.5 µl of AmpFISTR PCR Reaction Mix*
- *No. samples x 0.5 µl of AmpliTaq Gold DNA Polymerase*
- *No. samples x 5.5 µl of AmpFISTR Identifiler Primer Set*
- *10 µl of DNA with concentration ± 0.125 ng/µl*
- *Final volume of the reaction 25 µl*

The DNA concentration range advised by the manufacturer is 0.5-1.25 ng/µl.

Alongside the samples under examination, ***controls must be prepared*** in order to monitor the effectiveness of the selected amplification conditions and/or the existence of contamination.

The controls typically include a "negative control" and a "positive control".

The "***negative control***" is constituted of: ***PCR amplification Mix (without any template DNA) + water or buffer*** (used in the preceding phases of extraction and dilution), in the same volumes used to amplify the samples under examination.

The reason for the inclusion of a negative control in the amplification phase is to determine whether the reagents used for the extraction and dilution of the DNA, obtained from the samples under examination, are free of DNA: that is, **it allows it to be determined whether contamination from extraneous DNA is present.**

The "***positive control***" is constituted of: "***PCR amplification Mix + standard template DNA of known sequence*** (provided by the manufacturer of the kit), in the same volumes used for the amplification of the samples under examination.

The purpose of the positive control is to ensure that the components and parameters of the reaction are correct, and therefore suitable to amplify the DNA regions of interest: in short, ***to allow the effectiveness of the selected experimental conditions to be monitored***.

[59] Taking into account what was reported in the RTIGF (page 78) and the absence of specific notes in the SAL, it must be considered that no modification was made to the directions provided by the manufacturer of the kit about the volumes to employ for the amplification reaction.

In actuality, from an examination of the documentation in the case file, numerous gaps emerge about the amplification methods.

In particular:

1. the ***volumes of the Mix*** are never indicated: it is recognized, in fact, that the equilibrium of a PCR reaction depends on the concentration of the individual reagents used;
2. the ***quantity of DNA*** used for each PCR reaction is never indicated: it is equally recognized that best results are obtained when the quantity of template DNA added corresponds to the range indicated by the kit.

Hence the need to preemptively quantify the extract.

Regarding ***point 2***, further clarifications are necessary:

- on page 79 of the RTIGF, it is noted that the samples indicated with the letters C-D-E-F-G (samples, however, which tested negative to DNA quantification) were concentrated using *Speed-Vac SC110*, but it does not appear that quantification of the concentrated extracts was repeated. It is noted in the RTIGF that no amplification product was obtained from the aforementioned samples;
- in the absence of any notes about modifications made to the amplification protocols, it must be considered, as noted previously, that the protocols associated with the kit were followed for ***samples A and B***: that is, total volumes of 25 µl were used, constituted respectively of:

- 15 µl of amplification Mix+10 µl of DNA extracted from sample A
- 15 µl of amplification Mix+10 µl of DNA extracted from sample B

With regard to the extract from ***sample A***, if the volumes indicated in the Identifiler Kit were used, it should be assumed that the quantity of DNA used for the PCR reaction was a total of 0.8 ng (0.08 ng/µl = 0.8 ng): therefore a quantity of DNA which falls within the range suggested by the kit (0.5-1.25 ng/µl of template DNA) and that, as we will see, provided an electrophoretic trace in accordance with the quantity of DNA used for the reaction.

[60] On the other hand, considerable perplexity arises about the quantity of DNA extracted from ***sample B*** which would have been added to the reaction mix. The conditional[4] is necessary, in that the extract from sample B was quantified using the *Qubit Fluorometer*™ and although having given an uninterpretable result ("***too low"***) was considered "positive", in contrast to ***sample C*** which, although being "***too low"***, was considered negative!

We learn from the transcript of the GUP questioning that Dr. Stefanoni concentrated the volume of the extract from sample B several times.

In particular, she stated having first concentrated the extract from an initial volume of 50 microlitres "***to around 20, 22, 23 microlitres***" (GUP page 178), and of having subsequently carried out Real Time quantification of the total [amount of] DNA, and not of the DNA of masculine origin.

Since from the Real Time quantification (***never carried out!***) she obtained a concentration of "***a few hundred picograms of DNA***" (GUP, page 178) she took steps to further concentrate the extract in order to obtain a ***final volume of 10 µl*** which she would have used for the PCR reaction.

We consider that quantification was not even performed on the final volume, since there is no confirmation either in the documentation in the case file (SAL, Real Time report, RTIGF) nor was such a circumstance ever reported by Dr. Stefanoni in the course of her questioning.

In practical terms, an amplification was performed without knowledge of one of the basic parameters: that is, the concentration of the DNA possibly extracted from sample B.

4 i.e. the conditional tense, presumably! ("sarebbe stato").

What is surprising is that, given the delicate nature of the testing, quantification of samples B and C was not carried out with Real Time – as happened for samples D-E-F-G – to ascertain if a Low Copy Number (LCN) sample was being dealt with, for which a different and more complex procedure should have been adopted.

The theory that this was a sample which could have been considered LCN was proposed by Dr. Gino in the course of the GUP hearing, and was endorsed by Dr. Stefanoni ("***Yes it is possible, certainly!"***, page 178) who, although conscious of the problems associated with samples of low DNA quantity (Low Copy Number), did not consider it appropriate to apply any of the precautions recommended by the scientific community in the case of LCN.

[61] ***It is emphasized that the amplification was performed only once*** (pages 21-22, transcript of the GUP questioning. To the question, "…***the testing of a trace of this sort should be repeated several times to be considered reliable?"*** The Technical Consultant replies: "***In theory yes"***. To the question, "***How many times did you do it?"*** she responds, "***In this case only once"***.

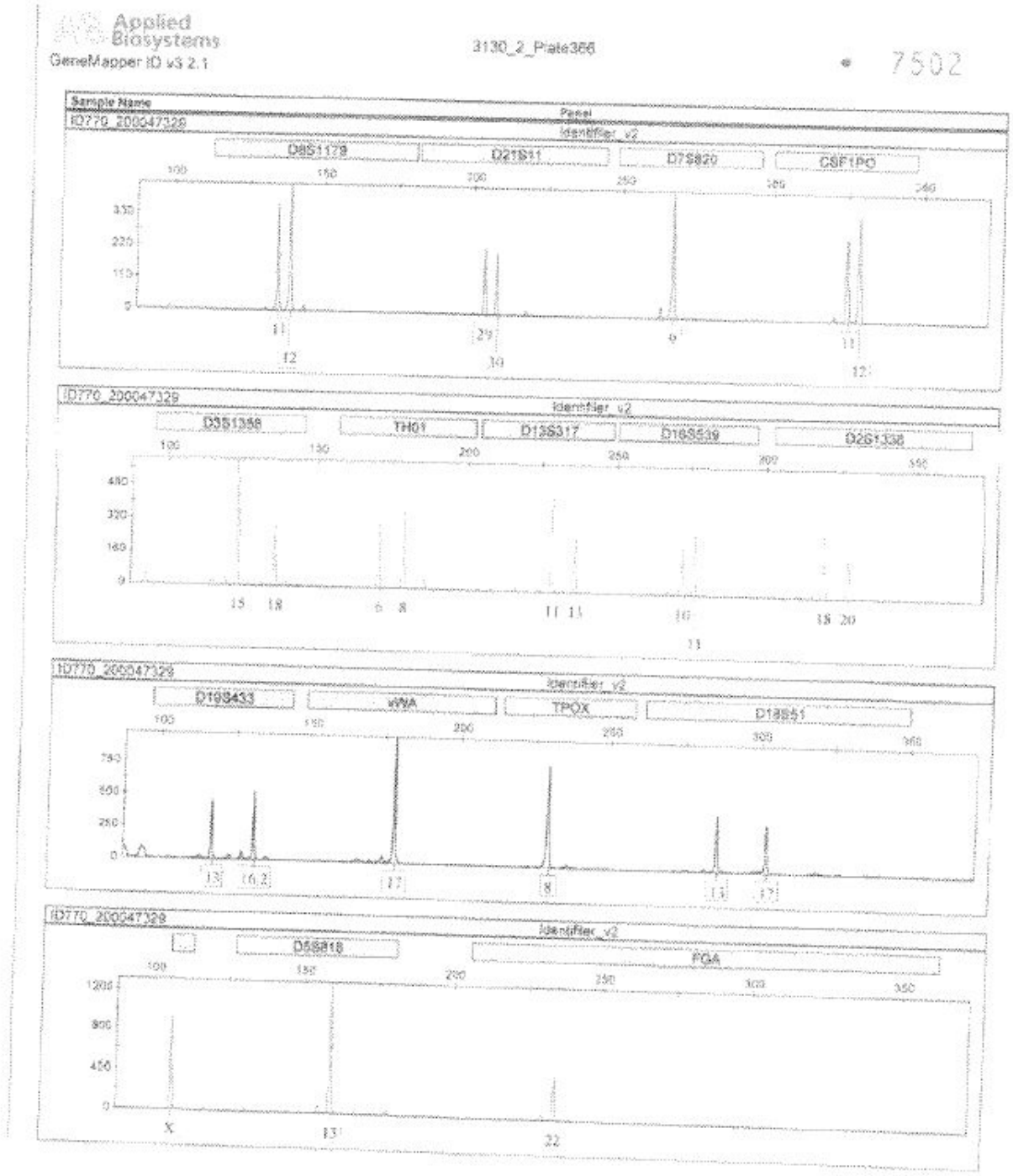
CAPILLARY ELECTROPHORESIS

The amplification product obtained from samples A and B was subjected to capillary electrophoresis using the *ABIPRISM 3130 Genetic Analyzer* instrument, and the analysis software *"Gene Mapper"*.

Since it does not appear from the documents that any modification was made to the electrophoresis protocol, it is inferred that the run conditions were the standard ones indicated by the kit.

The **electropherogram** relating to the electrophoretic run of the amplified DNA from **sample A** (knife handle), attached to the RTIGF, is shown below:

[62]


Applied Biosystems
GeneMapper ID v3.2.1
3130_2_Plate366
7502
Sample Name
ID770_200047328
Panel
Identifier v2
D8S1179
D21S11
D7S820
CSF1PO
ID770_200047328
Identifier v2
D3S1358
TH01
D13S317
D16S539
D2S1338
ID770_200047328
Identifier v2
D19S433
vWA
TPOX
D18S51
ID770_200047328
Identifier v2
D5S818
FGA
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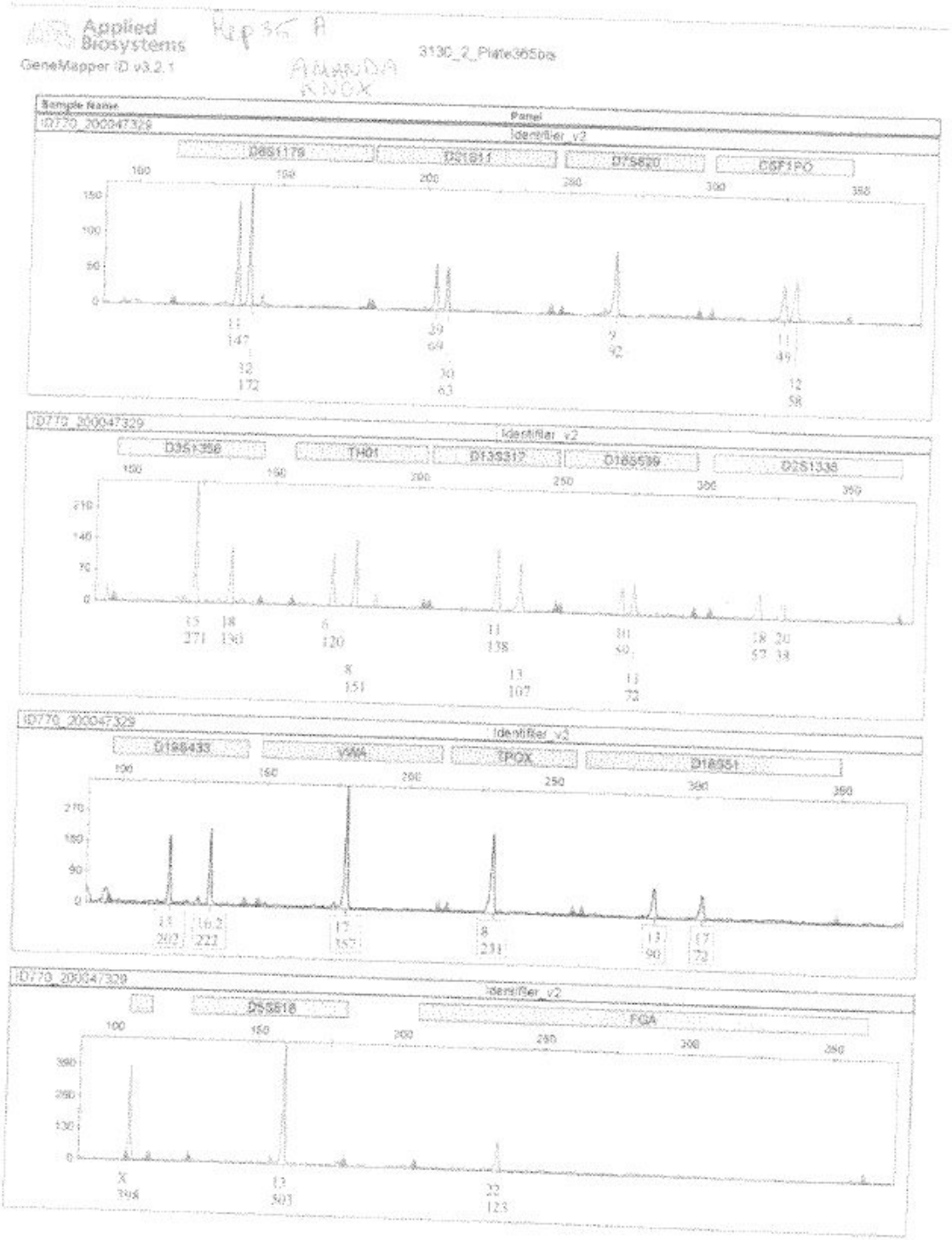
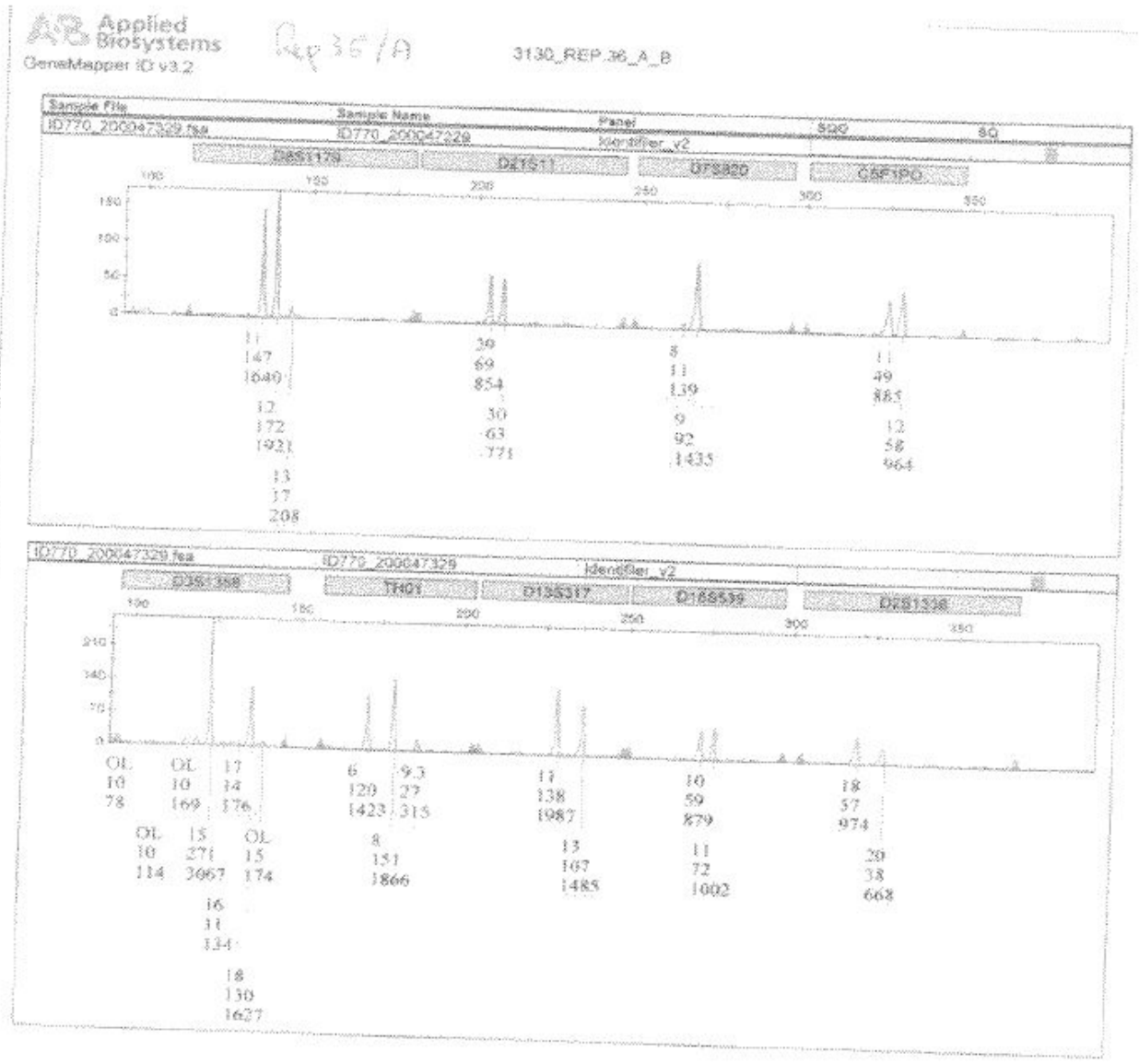
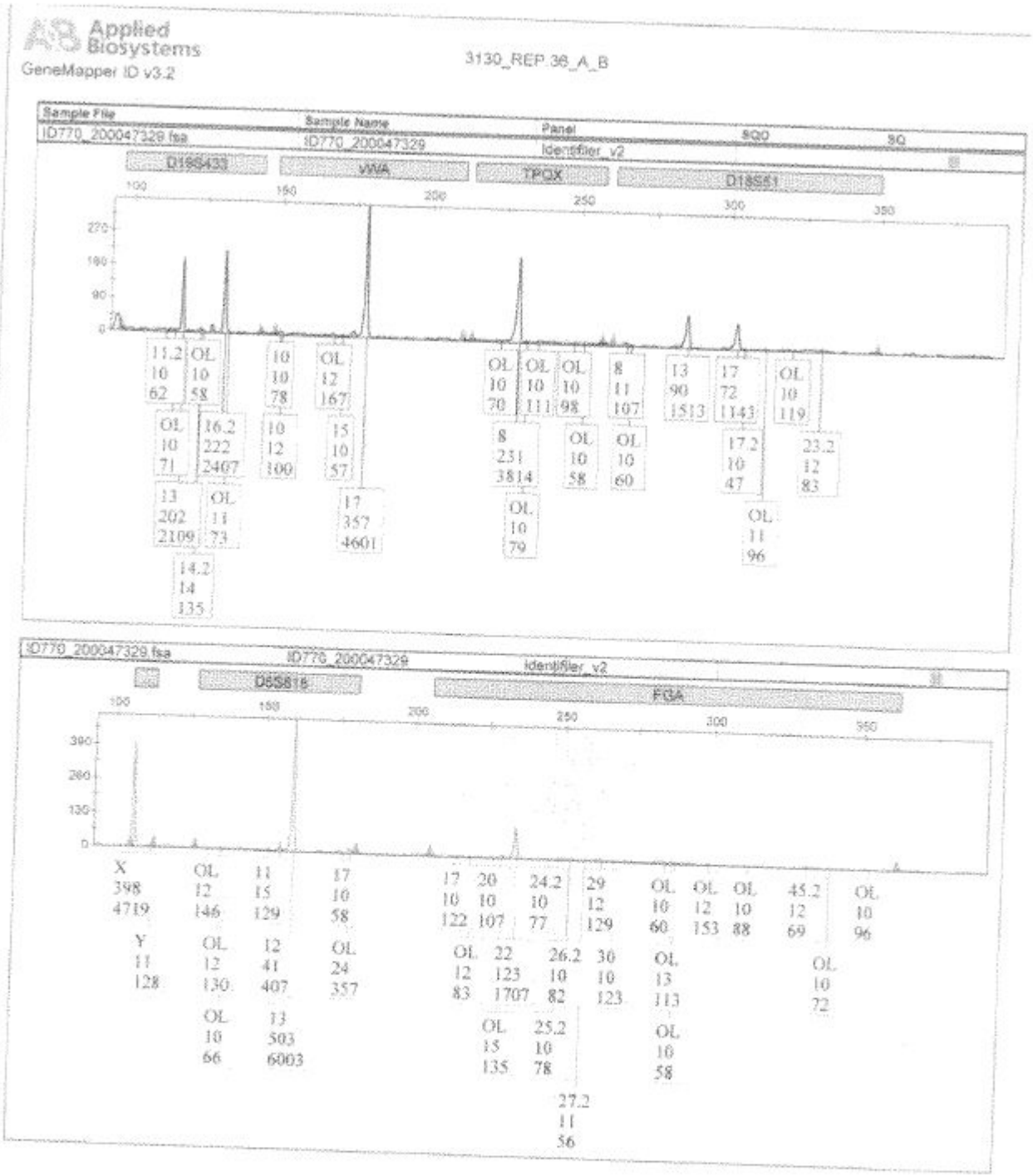
[63] On 29 April 2011, the electropherogram relating to the electrophoretic run of the amplified DNA from sample A was sent to us on CD-rom, on which the heights of the allele peaks are indicated:



[64] And on 11 May 2011, the electropherogram relating to the electrophoretic run of the amplified DNA from sample A was sent to us via e-mail, on which the heights and areas of the peaks are indicated:



[65]



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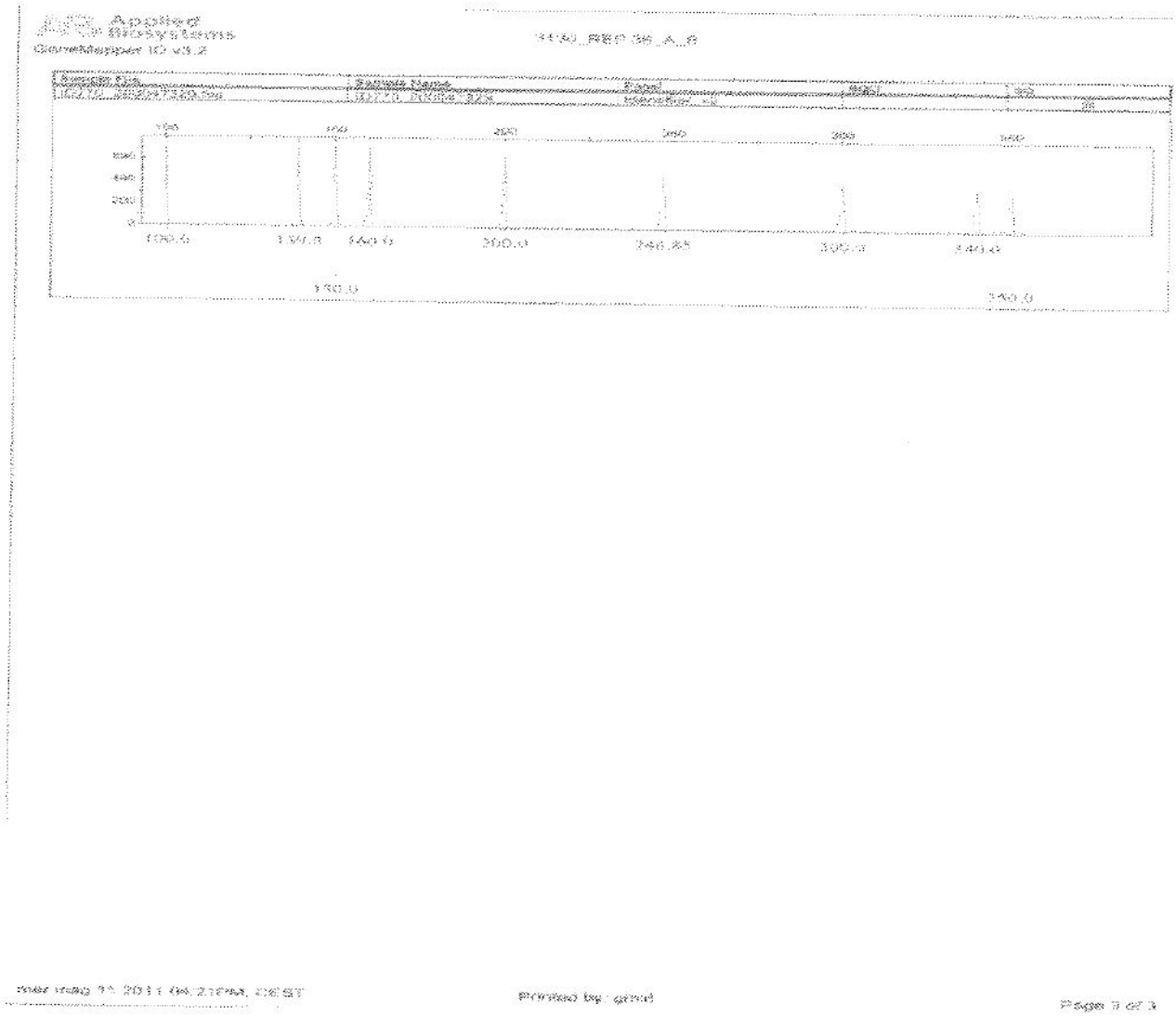
[66]

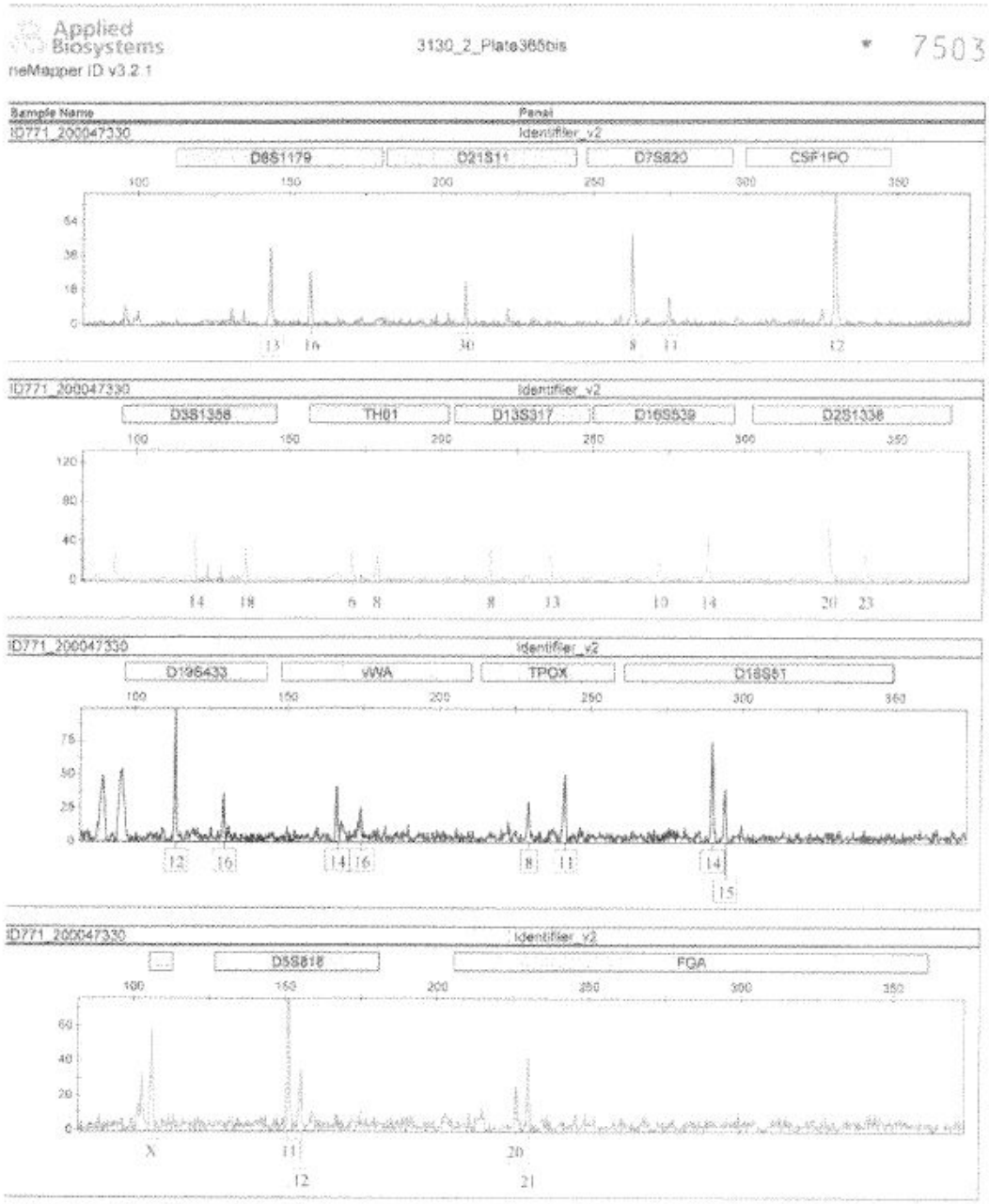


[67] With respect to the electrophoretic run of the amplified DNA from **sample A**, we note from all the electropherograms that: a) *peaks which exceed the 50 RFU threshold are present* (it is emphasized that 50 RFU is the threshold recommended by the kit manual, and it is not advised to go below this point); b) *the alleles are balanced* in that the ratio between the peaks is >0.60 (*Gill. P et al., 2006*), in accordance with the presumed quantity of DNA used for the reaction (0.8 ng).

In the following table, the alleles with the associated peak heights and the calculations of heterozygous balance are shown:

Locus	*Campionatura A*	
	Altezza picchi	*Bilanciamento degli eterozigoti* $Hb=\varphi_d/\varphi_b$
D8S1179	allele 11 ↑ 147 allele 12 ↑ 172	0.85
D21S11	allele 29 ↑ 69 allele 30 ↑ 63	0.91
D7S820	allele 9 ↑ 92	-
CSF1PO	allele 11 ↑ 49 allele 12 ↑ 58	0.84
D3S1358	allele 15 ↑ 271 allele 18 ↑ 130	0.47
TH01	allele 6 ↑ 120 allele 8 ↑ 151	0.79
D13S317	allele 11 ↑ 138 allele 13 ↑ 107	0.77
D16S539	allele 10 ↑ 59 allele 11 ↑ 72	0.81
D2S1338	allele 18 ↑ 57 allele 20 ↑ 38	0.66
D19S433	allele 13 ↑ 202 allele 16.2 ↑ 222	0.90
VWA	allele 17 ↑ 357	-
TPOX	allele 8 ↑ 231	-
D18S51	allele 13 ↑ 90 allele 17 ↑ 72	0.80
D5S818	allele 13 ↑ 503	-
FGA	allele 22 ↑ 123	-

[68] With regard to **sample B** (knife blade), the electropherogram relating to the electrophoretic run of the amplified DNA from the aforementioned sample, attached to the RTIGF, is shown below:



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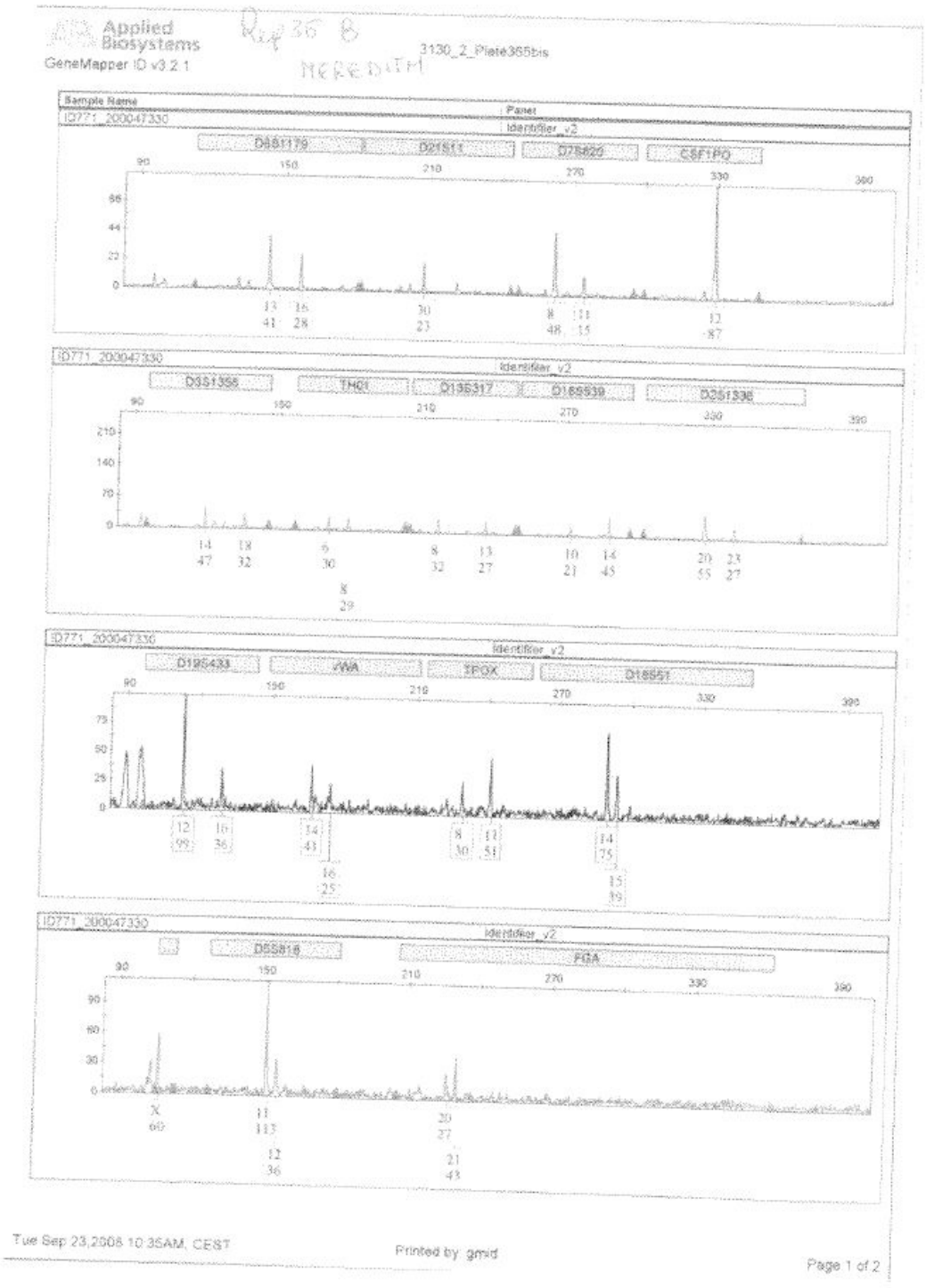
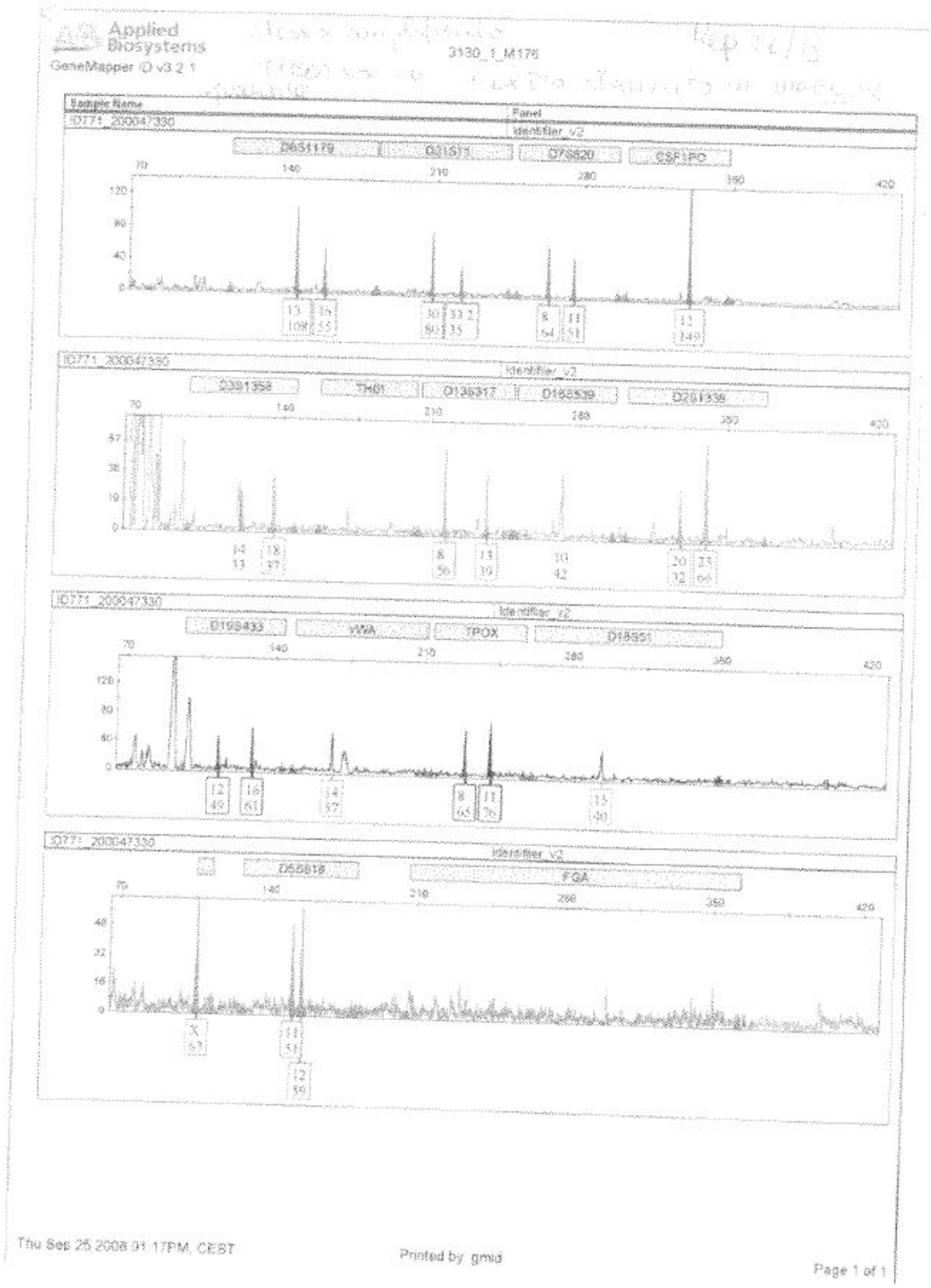
[69] On 29 April, the electropherograms relating to the two electrophoretic runs of the amplified DNA from sample B were sent to us on CD-ROM, on which the allele peak heights are indicated (electropherograms dated respectively *Run 1: Sept. 23 2008, 10:35 AM; Run 2: Sept. 25 2008, 01:17 PM).*

ELECTROPHORETIC RUN 1 (*Sept. 23 2008, 10:35 AM):*

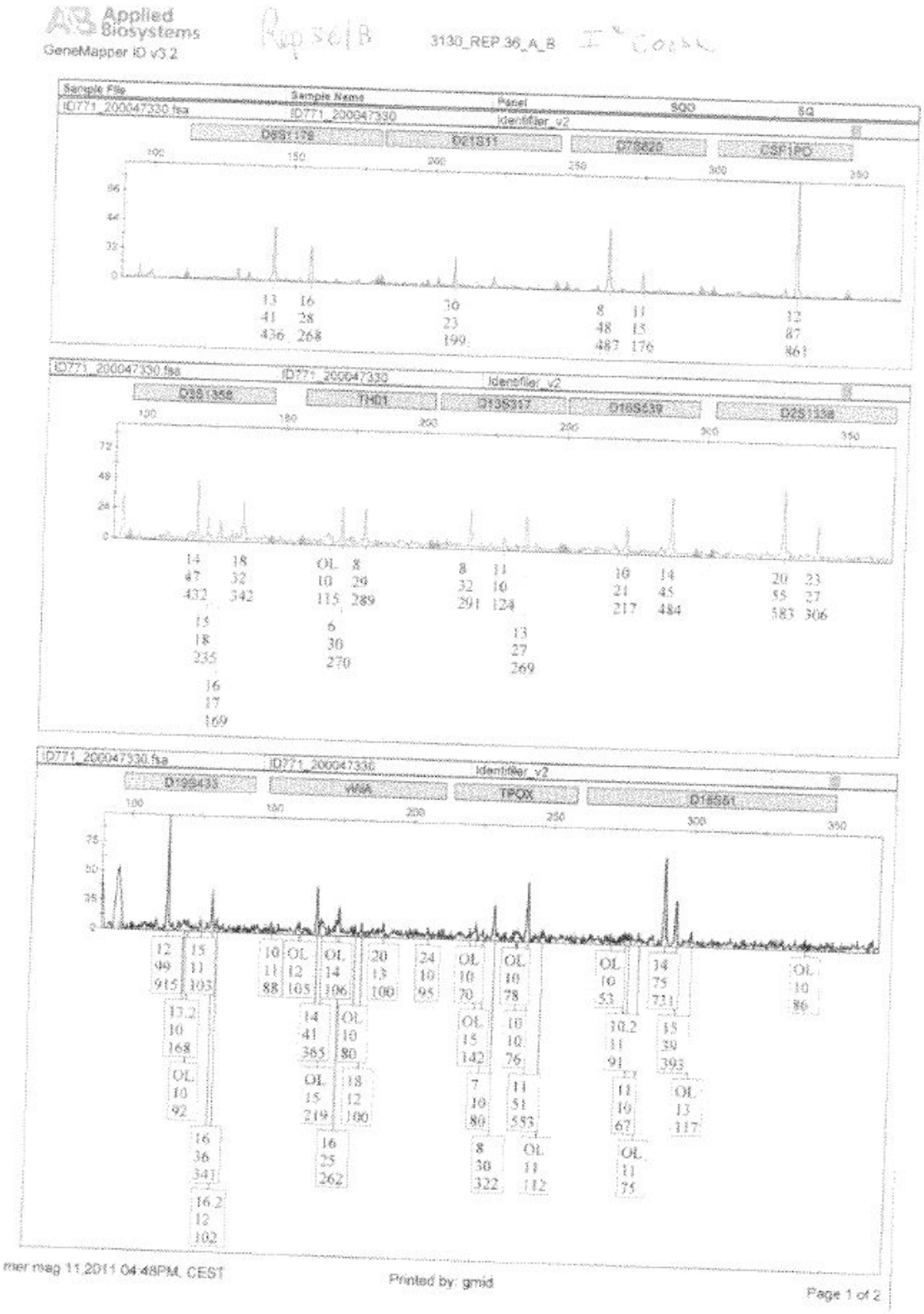


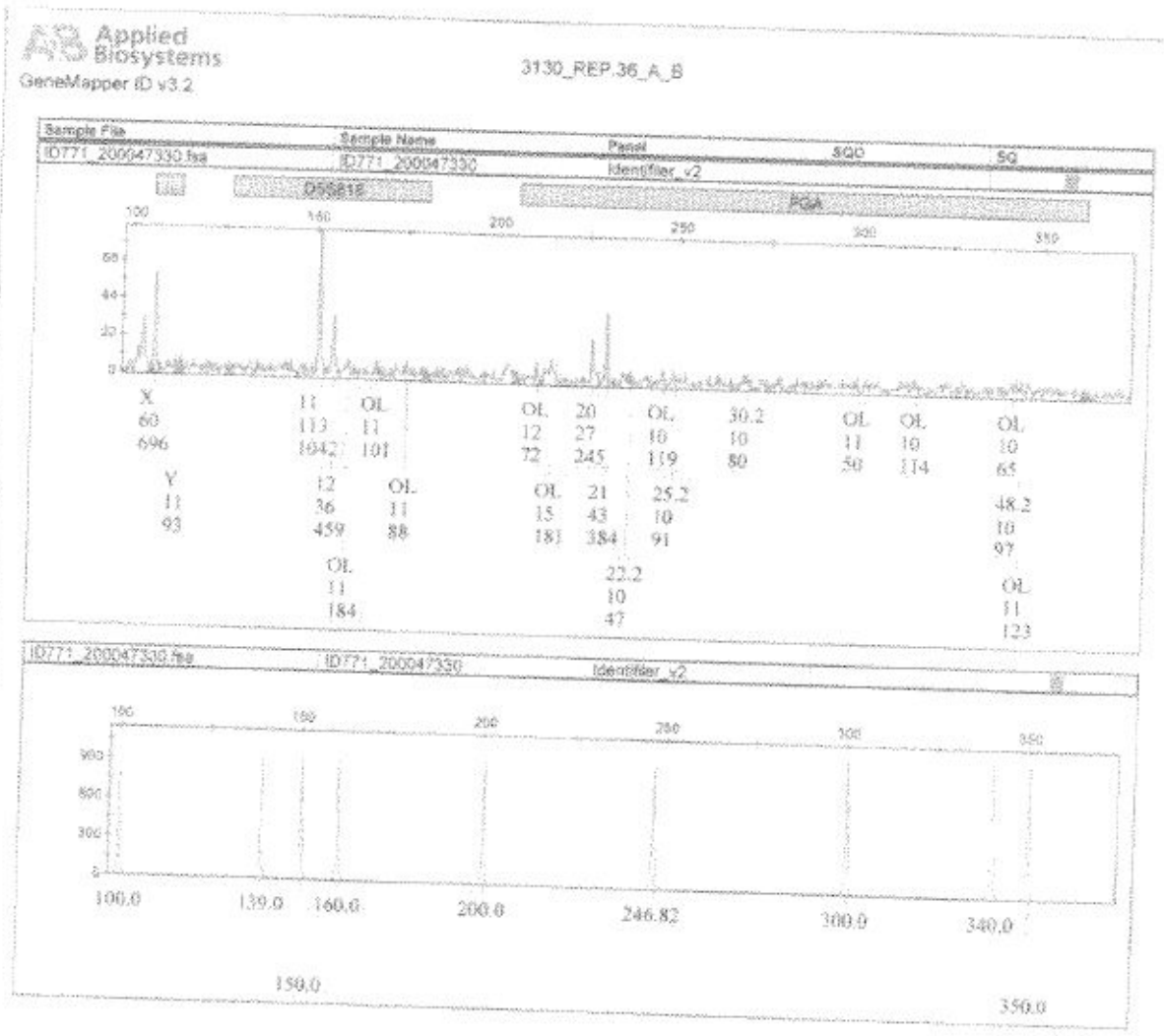
[70] ELECTROPHORETIC RUN 2 (*Sept. 25 2008, 01:17 PM):*



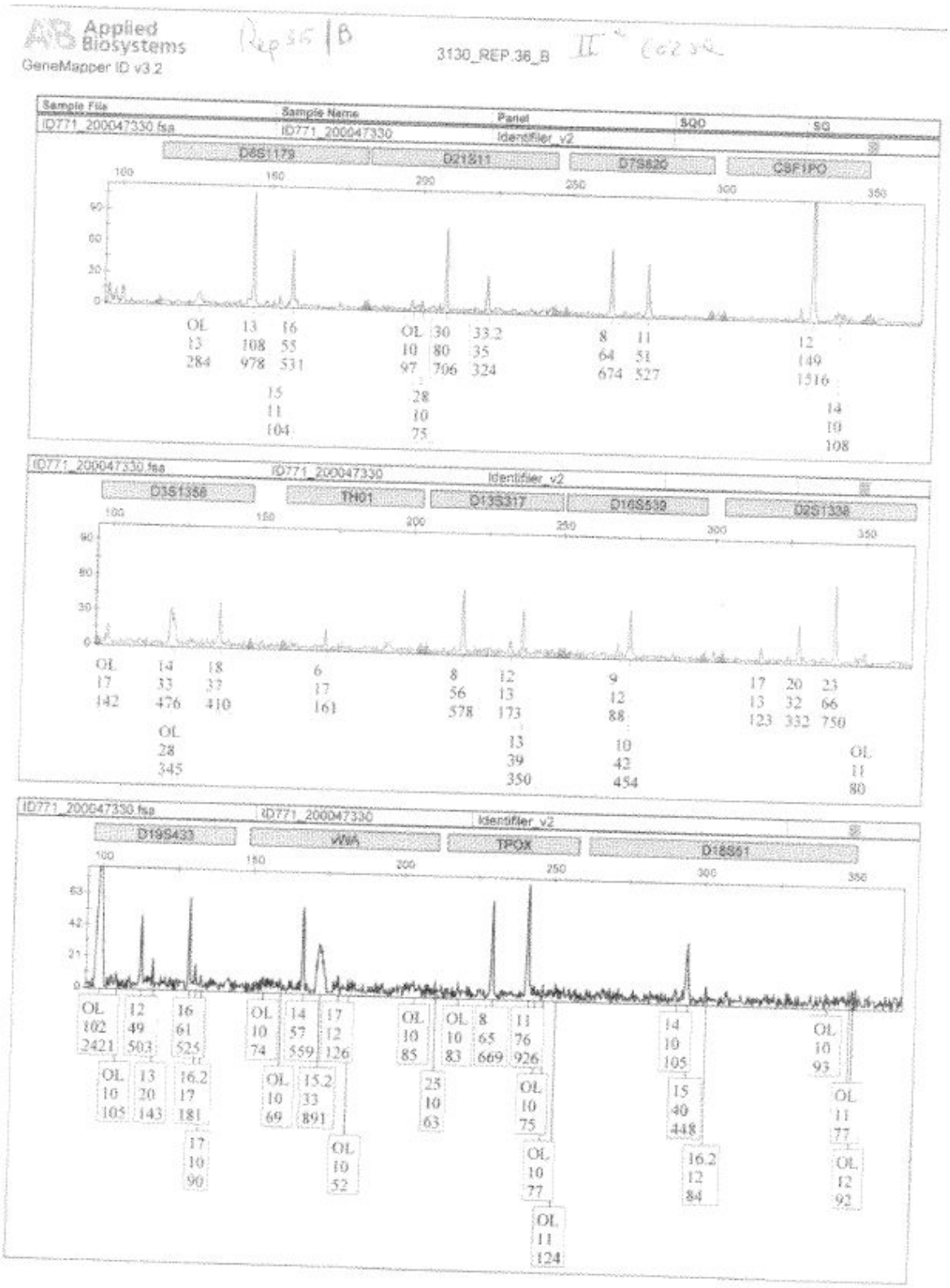
[71] On 11 May 2011, the electropherograms relating to the above runs were sent to us via e-mail, but with the peak heights and areas indicated; these are shown below.

ELECTROPHORETIC RUN 1 (*May 11 2011, 04:48 PM*):




Applied Biosystems
GeneMapper ID v3.2
3130_REP.36_A_B
Sample File
ID771_200047330.fsa
Sample Name
ID771_200047330
Panel
Identifier_v2
SQO
SQ
D5S818
FGA
ID771_200047330.fsa
ID771_200047330
Identifier_v2
100.0
139.0
160.0
200.0
246.82
300.0
340.0
150.0
350.0

[73] ELECTROPHORETIC RUN 2 (*May 11 2011, 04:36 PM)*



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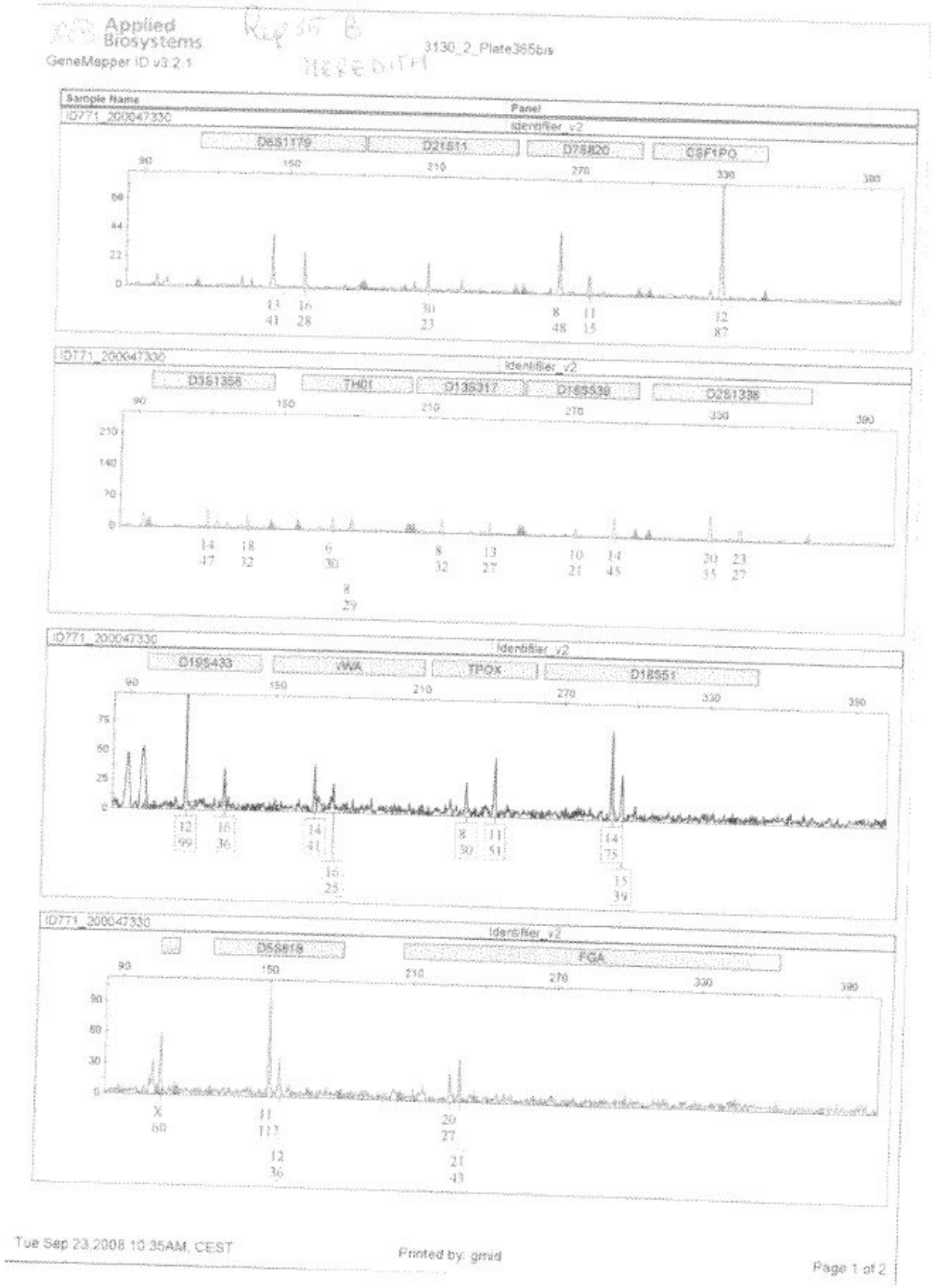
[74] Since the electrophoretic graphs, produced both in CD-ROM and e-mail format, do not differ from each other in any significant way (c.f. peak heights), for ease of exposition our comments here relate to the electrophoretic runs dated *Sept. 23 2008, 10:35 AM and Sept. 25 2008, 01:17 PM*, but are equally intended to apply to the electropherograms dated *May 11 2011, 04:48 PM and May 11 2011, 04:36 PM*.

From an examination of Electrophoretic Run 1, dated *"Sept. 23 2008, 10:35 AM*, it must be noted that: 1) this graph shows *peaks which are clearly below the 50 RFU threshold* (it is emphasized that 50 RFU is the threshold recommended by the kit manual, and it is not advised to go below this point); b) *the alleles are imbalanced* in that the ratio between many peaks is <0.60 (Gill P. et al., 2006).

[75] ELECTROPHORETIC RUN 1 (*Sept. 23 2008, 10:35 AM*).

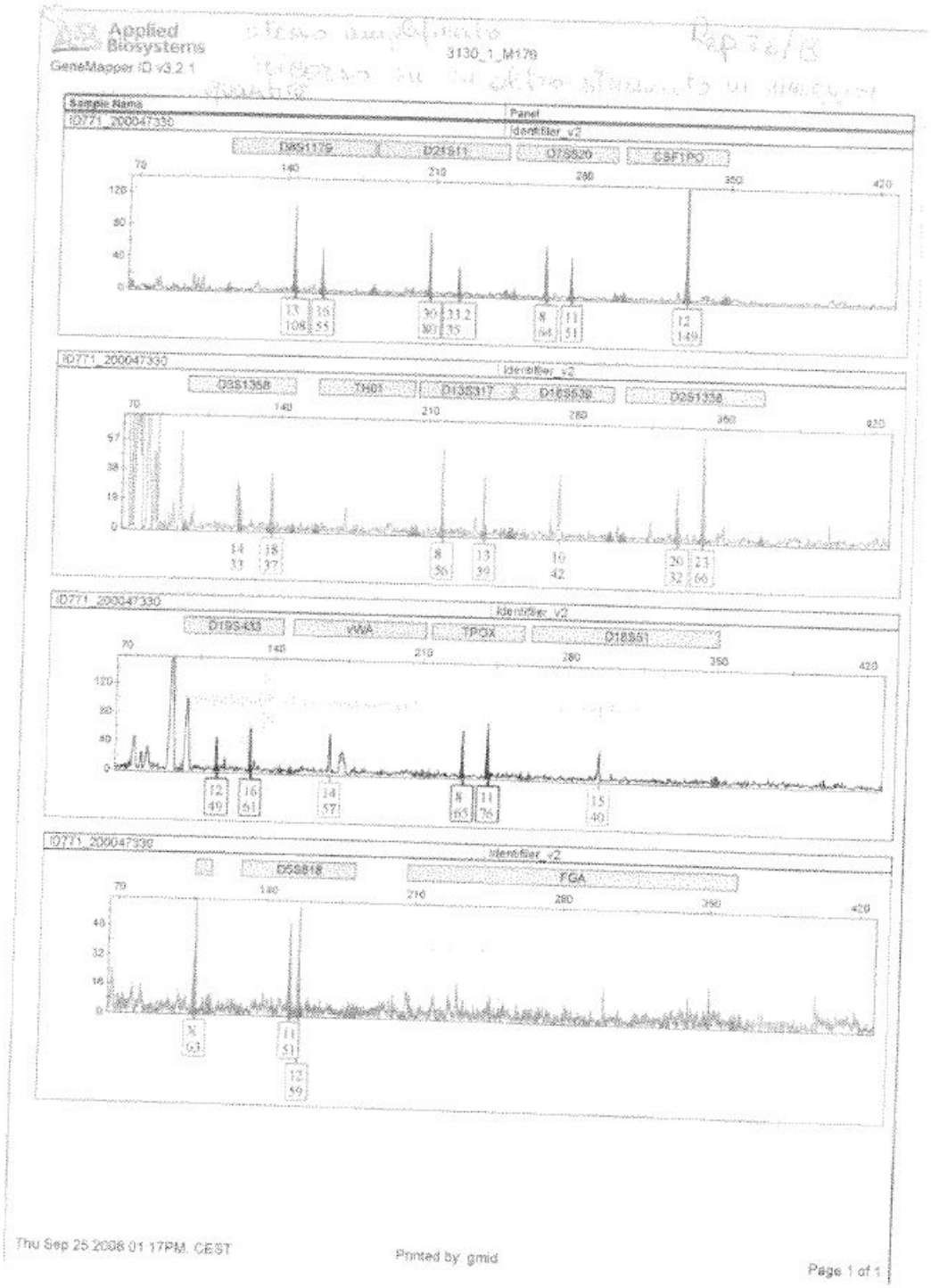


[76] In the following table, the alleles with the associated peak heights and the calculations of heterozygous balance are shown:

Locus	*Campionatura B I corsa elettroforetica*	
	Altezza picchi	*Bilanciamento degli eterozigoti* $Hb=\varphi_a/\varphi_b$
D8S1179	allele 13 ↑ 41 allele 16 ↑ 28	0.68
D21S11	allele 30 ↑ 23	-
D7S820	allele 8 ↑ 48 allele 11 ↑ 15	**0.31**
CSF1PO	allele 12 ↑ 87	-
D3S1358	allele 14 ↑ 47 allele 18 ↑ 32	0.68
TH01	allele 6 ↑ 30 allele 8 ↑ 29	0.96
D13S317	allele 8 ↑ 32 allele 13 ↑ 27	0.84
D16S539	allele 10 ↑ 21 allele 14 ↑ 45	**0.46**
D2S1338	allele 20 ↑ 55 allele 23 ↑ 27	**0.49**
D19S433	allele 12 ↑ 99 allele 16 ↑ 36	**0.36**
VWA	allele 14 ↑ 41 allele 16 ↑ 25	0.60
TPOX	allele 8 ↑ 30 allele 11 ↑ 51	**0.58**
D18S51	allele 14 ↑ 75 allele 15 ↑ 39	**0.52**
D5S818	allele 11 ↑ 113 allele 12 ↑ 36	**0.31**
FGA	allele 20 ↑ 27 allele 21 ↑ 43	0.62

[77] From an examination of electrophoretic run 2, dated *Sept. 25 2008, 01:17 PM*, the following are noted: the loss of alleles for some markers (TH01, D16S539, vWA, D18S51, FGA); a peak is present for one marker which is not present in run 1 (D21S11: presence of allele 33.2); and there is an inversion of peak heights for other markers (D3S1358, D2S1338, D19S433, D5S818).

ELECTROPHORETIC RUN 2 (*Sept. 23 2008, 01:17 PM*).



[78] Regarding the assessment of the peak heights in sample B, the Technical Consultant was specifically asked (GUP, page 20) "*When in your experience do you define an RFU value as rather low?*" and answered: "***below 50, I start to take greater care in the assessment***". Responding to the explanations requested by the GUP (page 21) about the sample with identification code 47330 (sample B, Exhibit 36) – "*Perfect! What do we have here?"* – the Technical Consultant replies: "*Well, here we have a genetic profile which certainly has a lower R.S.U. [RFU?] intensity than the previous one*". Q: "*In the order of...?"* A: "***Let's say we're in the order of, let me see, again the same genetic point that I defined earlier, at which we see a 41 and a 28***". Q: ***Therefore for you there would already be a bit of a risk, as we said before a little bit low?***" A: "***Yes, yes, yes***".

Again, responding to a question from Dr. Biondo (transcript of the GUP hearing, page 72): "*There was another aspect for which, when one does a DNA analysis where the peaks, the heights reported in R.S.U. are low, you said there was a risk. Can you explain this risk?*" The Technical Consultant replies: ***"Well there's a risk in the sense that in practice when I evaluate a genetic profile which has rather low peak heights, roughly below 50 RFU, 50, 60, I risk performing an operation which is something, so to speak, that no forensic geneticist would wish to do, and that is to poorly interpret that profile…that is in the sense that I might make a mistake in the assessment, assess a peak in an erroneous manner because perhaps it is too low, and therefore perhaps run the risk of excluding a person who might be the victim from the framework of the investigations…"***

The following question is reported on pages 21-22 of the GUP questioning: "***… the testing of a sample of this sort should be repeated several times to be considered reliable?"*** The Technical Consultant replies: "***In theory yes***". To the question, "***How many times did you do it?"*** she responds: "***In this case only once***". Q: "*Just once, and therefore in theory why ought it to be considered more reliable if it is done several times?*" A: "***Because reproducibility of the result is****, let's say, a good standard in any scientific experiment quite apart from forensic genetics,* ***obviously to be considered valid a result must be repeatable".***

[79] ***In fact, the Technical Consultant did not repeat the amplification of the extract but performed two electrophoretic runs of the same amplification***, and what

is immediately obvious from a comparison of the two separate runs is the existence of peak imbalance and inversion, to the point where in some cases there is allele loss or the presence of an additional peak (c.f. electrophoretic graph, runs 1-2, *Sept. 2008*).

Moreover, it must be noted that in the electropherograms produced, ***neither the negative control*** *– which, as previously mentioned, could have indicated the presence of possible contamination –* ***nor the positive control****, which would have allowed the effectiveness of the preselected experimental conditions to be monitored, are present.*

From what has previously been stated about the analysis of the electropherograms relating to sample B, it is inferred that the quantity of extracted and amplified DNA must have been very low, falling within the definition of ***Low Copy Number (LCN) or Low-Template DNA (LT-DNA)***.

It is considered appropriate at this point to provide some clarifications about the definition of the term Low Copy Number, and to outline the experiences, suggestions and recommendations of the International Scientific Community aimed at trying to resolve some of the most frequent problems associated with biological samples of low DNA quantity (LCN).

LOW COPY NUMBER (LCN) OR LOW TEMPLATE DNA (LT-DNA)

About a decade ago, when the technique known as LCN typing was developed, it referred to the analysis of samples containing less than 100 pg of DNA (*Gill P. et al., 2000; Gill P., 2001; Budowle B. et al., 2009)*. More recently, LCN has been defined as a template value of less than 200 pg, a value largely associated with the quantity of DNA described by different authors (*Caddy B., Taylor D.R., Limcare A.M., 2009)* as the stochastic threshold for conventional STR typing (*Moretti T.R. et al., 2001 (a); Moretti et al., 2001 (b)*).

These DNA threshold quantities are based on an amount of template DNA which shows an exaggerated peak height imbalance, drop-out, and an increase in laboratory contamination (*Gill P. et al., 2006)*.

[80] Numerous validation studies have been conducted on commercial kits for DNA typing, and the conditions in which these kits produce reliable results have been clearly defined (*Micka K.A. et al., 1999; Cotton E. A. et al., 2000; Krenke B.E. et al., 2002; Holt C.L. et al., 2002; Collins P.J. et al., 2004; Mulero J.J. et al., 2008)*. The optimal quantities of template are thus well-specified, and the range usually varies between 0.5-2 ng of template DNA (1 ng is usually considered the optimal quantity by most commercial kits).

In what follows, we report the scientific contributions from preeminent scientists on the subject of Low Copy Number, as reported in the international literature.

The tragic terrorist attack in Omagh, Northern Ireland, in 1998 (the bombing of a market, with a death toll of 29 victims and 200 injured) raised the question of the validity of LCN typing and its use in legal cases for the first time. The alleged perpetrator of the attack was initially arrested on the basis of LCN typing results, but later released due to the alleged lack of adequate security measures in the collection, transportation and handling of the seized items, as well as [the absence of] adequate laboratory precautions for LCN typing (***The Queen v Sean Hoey. Neutral Citation Number [*2007] NICC 49**).

The criticisms raised by this judicial case led to the creation of a Commission to review LCN typing technologies: this Commission went on to state that LCN typing, as practiced specifically in the United Kingdom, is a "robust" method and "fit for purpose" (*Caddy B., Taylor D.R., Lincare A.M., 2009* - known also as the *"Caddy Report"*). However, at the same time it offered a series of useful recommendations to improve the reliability of the methodology and emphasizing, amongst other things, the following problems associated with LCN typing:

- ***greater potential risk of error in comparison with conventional STR typing protocols;***
- *errors of interpretation due to allele drop-in, allele drop-out, peak height imbalance and large stutter peaks;*
- ***the need for a robust and reliable quantification method in order to determine the amount of DNA available for analysis;***
- **[81]** *LCN profiles are not generally reproducible and, due to the potential for error, the probative value of the results may not be evaluated correctly;*

- *the interpretation of profiles derived from mixtures using LCN typing is problematic, and at the moment there are no interpretation guidelines based on reliable validation studies;*
- ***due to the sensitivity of the method and the types of samples analyzed (for example, "touch" samples), the LCN profile may not be relevant to the specific case;***
- *the evidence cannot be used for exculpatory purposes;*
- *instructions about the proper collection of items and protocols regarding their handling have not been clearly established or communicated;*
- *reagents, consumables and laboratory instrumentation can contain low levels of extraneous DNA which may complicate the interpretation of LCN typing results.*

1. Methods of LCN Typing

There are a number of alternative ways to increase the sensitivity of the analysis to enable LCN typing. These include: increasing PCR cycle number (*Gill P. et al., 2000; Whitaker J.P. et al., 2001; Gill P., 2001; Kloosterman A.D. et al., 2003; Budowle B. et al., 2009)*; Nested PCR (*Strom C.M., Rechitsky S., 1998)*; reducing the volume of the PCR (*Gaines M.L. et al., 2002; Leclair B. et al., 2003; Budowle B. et al., 2009)*; Whole Genome Amplification prior to the PCR (*Hanson E.K., Ballantyne J., 2005)*; strengthening the fluorescent dye signal; use of higher purity formamide in sample preparation for capillary electrophoresis (*Budowle B. et al., 2009)*; post-PCR clean-up to remove ions which compete with DNA during electrokinetic injection (*Smith P.J., Ballantyne J., 2007; Foster L. et al., 2008; Budowle B. et al., 2009);* increasing injection time (*Budowle B. et al., 2009*).

Due to the important limitations in typing samples containing low concentrations of DNA, many scientists urge caution in the practice and interpretation of LCN (*Gill P. et al., 2000; Gill P., 2001; Kloosterman A.D. et al., 2003; Budowle B. et al., 2009*).

[82] Budowle B. et al. (*2009*) call for caution, and suggest the use of LCN exclusively for identifying missing persons (including the victims of mass disasters) and for research purposes. On the other hand, the aforementioned authors advise against the use of current LCN methods in criminal proceedings, since current

methods, technologies and recommendations still do not allow the problems which characterize LCN typing to be overcome.

In particular, since by definition LCN typing cannot provide reproducible results, and therefore the same result may not be expected if the same sample is analyzed twice, the method cannot be considered robust according to conventional standards (*Budowle B. et al., 2009*).

2. Laboratory Procedures

All the practices designed to ***minimize laboratory contamination*** are recognized as important and effective: for example, the use of pressurized facilities, suitable laboratory clothing, the analysis of one sample at a time, the use of instruments and materials free of DNA, as well as decontamination practices (exposure to UV rays and/or ethylene oxide) (*Shaw K. et al., 2008*). Unfortunately, these security measures have still not been officially introduced into the protocols for the collection and handling of items (*Caddy B. et al., 2009: The Queen v Sean Hoey. Neutral Citation Number [2007] NICC 49)*; in fact, this signals the urgent necessity for specific training in sampling procedures for the first rescuers and investigators on the crime scene.

3. Problems associated with low template quantities

The problems which arise from the analysis of sub-optimal quantities of template DNA in a PCR are numerous, and these problems become even more apparent when the template quantity is reduced. Moreover, the interpretation of mixtures has yet to be well-defined.

The fundamental issues of the low template quantity problem are the following: stochastic effects, the detection threshold, profile interpretation, allele drop-out and heterozygous peak imbalance, stutter, contamination, replicate analysis, appropriate controls, and limitations of use.

[83] ***3.1 Stochastic effects:*** Due to the kinetics of the PCR process, a low quantity of starting template will be subject to stochastic effects. In fact, during the first cycles, the primer may not bind in the same way for each allele at a given locus, and therefore a significant imbalance between allelic products may be noted or, in some cases, the total loss of one or both alleles. In other words, an LCN DNA template in a PCR may show stochastic amplification phenomena, visible both as a substantial imbalance of two alleles at a given heterozygous locus, and as allelic drop-

out or an increase in stutter (*Gill P. et al., 2000; Whitaker J.P. et al., 2001; Kloosterman A.D. et al., 2003; Smith P.J., Ballantyne J., 2007; Forster L. et al., 2008; Budowle B. et al., 2009*).

3.2 Detection threshold: Usually, it is recommended that quantities of DNA able to reduce stochastic effects to manageable levels are used for the PCR. However, since differences in the quantification of the template and possible inaccuracies in the pipetted volume may influence the amount of DNA actually present in the PCR, a stochastic interpretation threshold must be used for STR typing results (*Minimum Interpretation Threshold "MIT", Budowle B. et al., 2009)*.

A minimum peak height (or area), established by internal validation studies at the individual laboratory, therefore serves as stochastic control. ***The peaks which appear below this threshold must not be interpreted or are interpreted with extreme caution and for very limited purposes.*** With LCN typing, it needs to be emphasized that peak height is substantially increased due to additional PCR cycles, or to post-PCR clean-up procedures. Therefore, since the LCN situation refers to the interpretation of results which would ordinarily be below the stochastic interpretation threshold, minimum peak height criteria such as that used for standard STR typing (with samples containing from 250 pg to 1 ng of DNA) can not exist. Currently therefore, there is no valid method to establish a threshold for LCN typing, and this will continue to be one of the major weak points of the application.

3.3 Profile Interpretation: The two factors which influence the robustness of LCN typing are stochastic effects and the sensitivity of detection. The protocols proposed for the interpretation of profiles generated by LCN therefore consider these characteristic elements (*Gill P. et al., 2000).* However, while these interpretation guidelines are based on studies of single source profiles using **[84]** near optimal samples, the poor quality which often characterizes samples of forensic interest and mixtures increases the problems of interpreting LCN data.

To date, well-developed guidelines for LCN interpretation of mixtures have still not been described. Since many touch samples are mixtures (*Gill P., 2001; Caddy B. et al., 2009)*, this lack in validation studies and interpretation guidelines must be considered a serious deficiency.

3.4 Allele Drop-out: Allele drop-out is the phenomenon associated with LCN typing which is simplest to describe. For example, if an allele 15 is observed in the LCN evidence, then on the basis of that evidence any individual [with] a homozygote

for allele 15 or rather heterozygote 15, X (where X could be any allele) cannot be excluded as a potential contributor to that sample. In order to support the evidence, the '2p rule' can be used for any individual allele at a particular locus (*Budowle B. et al., 1991*) and this calculation will be of a conservative type. The observation of 2 alleles at a locus may be considered to represent a heterozygous profile and therefore suggests that allele drop-out did not occur. The *Likelihood Ratio* for a single source sample can be calculated using 1/2*fa* for the homozygotes and 1/2*fafb* for the heterozygotes, where *fa* is the frequency of allele *a* and *fb* the frequency of allele *b* (*Gill P. et al., 2000).* In addition, Gill et al. (2000) recommend modifying the calculation by considering the probability of allele drop-out *(p(D)).* The *p(D)* is based on experimental observation. However, it is difficult to justify *p(D)* based exclusively on experimental studies using optimal samples and applying this calculation to any individual real case. Drop-out is, in fact, related both to the quantity and the quality of the sample under examination. These parameters are often undefined in LCN samples and are specific to each individual sample. Allele drop-out cannot be identified as such based only on controlled validation studies conducted in the laboratory, and therefore further research is needed before the values of *p(D)* can be provided.

3.5 Stutter: During the generation of LCN STR profiles, the percentage value of stutter is variable, and absolutely not informative in that a stutter peak may exceed the height or area of the associated allele peak (*Gill P. et al., 2000, Gill P., 2001)*. Although some researchers (*Gill P. et al., 2000)* have tried to define the probability of stutter through statistical calculations, the probability and percentage value of stutter with respect to the true allele cannot be predicted. In fact, it is possible that a stutter peak may show twice in repeated analyses and therefore be interpreted as a true allele. The probability of detecting stutter twice in **[85]** repeated analyses has not yet been studied in depth, and we also await recommendations on the interpretation of stutter in mixed samples.

3.6 Contamination: in its recommendations for the interpretation of mixtures, the DNA Commission of the ISFG gives the following definition of contamination: *"DNA introduced after the crime and originating from a source not related to the crime scene: investigators, laboratory technicians, laboratory instrumentation"* (*Howitt T., 2003; Gill P., Kirkham A., 2004*).

This is a very restricted definition of the source of contamination; in fact, low-level contaminant DNA may derive from reagents and other laboratory consumables, from laboratory staff, and ***from cross-contamination from sample to sample***.

Many LCN samples are 'touch samples', and therefore low levels of DNA originating from environmental contamination at the crime scene may, for example, be identified on the item . In addition, contamination can happen during the collection and handling of samples. Therefore, the appearance of allele drop-in can be both intrinsic to the samples and induced during collection at the crime scene. Predicting the probability of drop-in based exclusively on experimental data may thus not be useful in simulating the circumstances in which drop-in may have occurred. A further difficulty, particularly apparent in mixtures, lies in determining which allele constitutes a drop-in. In fact, these difficulties tend to create bias in deciding whether or not there is support for contamination.

Statements about a profile obtained from the sample under examination regarding the determination as to which is a true allele and which a drop-in, must necessarily be made without knowledge of the suspect's profile; only in such a way, in fact, can a qualitatively unimpeachable and unbiased approach to the interpretation of the profile from the sample be guaranteed. Interpreting a profile from a sample with the suspect's reference profile to hand is indicative of a biased approach, and is in total contrast to the absolutely objective nature of forensic science.

Due to the limitations of LCN typing, extreme care needs to be taken not to overstep quality interpretation practices, in order to ensure that interpretive error is minimized.

[86] ***As well as the risks caused by handling of the samples***, the analysis of such a small amount of starting material inevitably means an exaggeration of stochastic phenomena in those samples. ***Increasing the sensitivity of the system will also increase the risk of contamination in the samples being examined*** *(Budowle B. et al., 2009).*

Gill P. and Buckleton J.S. (2010) agree with Budowle B. et al. (2009) on the issues raised about the contamination problem. In addition, regarding the criticism of the former made by the latter, with respect to their restricted definition of drop-in to laboratory processing, Gill emphasizes that their definitions and analyses are not only

limited to the phases of laboratory processing, but include ***handover phases from sources at the crime scene, at the Evidence Collection Unit and at the DNA Unit*** *(Gill P., Kirkham A., 2004).*

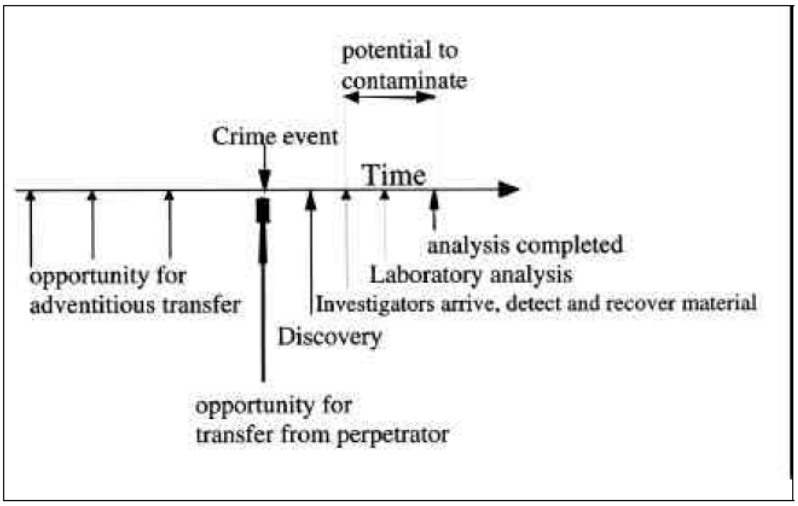


Figure 1: generalized time sequence illustrating the possible methods by which a DNA profile may be propagated.

Regarding the problem of ***contamination***, the same authors emphasize the substantial difference between the phenomenon of ***drop-in*** and what is known as ***"gross contamination":*** while the first refers to the appearance of one or two alleles in a sample originating from unrelated sources, the second refers instead to multiple alleles originating from a single unknown source (and therefore these alleles **[87]** are dependent events). In this respect, the authors emphasize that the principle risk of (random) contamination is erroneous exclusion, particularly if the contaminant profile masks that of the perpetrator.

The authors state that in their opinion, the limitations described regarding Low Template – DNA (LT-DNA) must be applied to all methods of DNA typing, since all DNA profiles may potentially be subject to the same phenomena originally attributed to LT-DNA. They also hold that ***if the due precautions are taken and the Judge is informed about the limitations of the technique used, it is the task of the latter to assess the weight of the evidence. It is not up to the scientist to decide, based on purely arbitrary criteria, whether or not the evidence being examined should be brought to the attention of the Court.***

The aforementioned authors stress that the ***weight of the evidence*** is a fundamental issue***. The evidence can arise in three principal ways: a) in an 'innocent' way; b) as a result of the criminal event itself; c) as a result of contamination or involuntary transfer*** *(Gill P., 2002).* The widespread public opinion exists that ***"if the DNA evidence matches the suspect then s/he must be guilty of the crime"***, and this opinion unfortunately extends to some scientists, judges and lawyers. In fact there exists a perception that the failure to convict signifies a failure of science: this is extremely dangerous, and it is therefore ***important to defend the idea that whether or not a suspect is convicted is an irrelevant question for the scientist, whose responsibility must only be to correctly explain the evidence in the context of the specific case in question.***

The question as to which was the actual method of transfer of the suspect's DNA onto the item must therefore be assessed by the judge and not by the scientist, whose principal role is to explain the various possible methods of transfer which exist as well as the relative probabilities associated with each of these *(Gill P., 2002).*

[88] We report below the ***"Precautions against contamination"*** suggested by J.M. Butler, in cases of samples of biological material containing low amounts of DNA.

"The sensitivity of the PCR requires constant attention from the laboratory staff to ensure that contamination does not effect DNA typing. ***Contamination of the PCR reaction is always a problem, because the technique is very sensitive due to the low amounts of DNA. If not careful, an operative preparing the PCR reaction may inadvertently add his/her own DNA to the reaction. In the same way, the police officer or technician at the crime scene when the evidence is collected may contaminate the sample if adequate precautions are not taken.*** *For this reason, each evidence sample should be taken with clean tweezers or handled with disposable gloves which must be changed frequently.*

In order to detect ***laboratory-induced contamination,*** *everyone in a forensic DNA laboratory must be typed, so that there is a record of all possible contaminant DNA profiles (staff elimination database).*

Laboratory staff should be appropriately covered during contact with samples prior to the PCR amplification. Appropriate protection includes lab coats and gloves

as well as masks and caps to prevent skin cells or hairs falling into the amplification tubes.

These precautions are particularly important when working with very small quantities of sample or with degraded samples.

Some of the ***useful precautions for avoiding contamination in the PCR reaction in the laboratory setting are****:*

- *The pre and post-PCR processing areas should be physically separate;*
- *The instrumentation as well as the tubes and reagents for the PCR should be kept separate from other laboratory instruments, particularly those used for analysis of the PCR product;*
- *Disposable gloves should be worn and changed frequently;*
- *The reactions should be prepared under a fume hood, if available;*
- *Aerosol-resistant tips should be used, and should be changed for each sample in order to prevent cross-contamination during transfer of the liquids;*
- **[89]** *The reagents should be prepared carefully to avoid the presence of any contaminant DNA or nucleases;*
- *Ultra-violet irradiation of the PCR preparation area when the area is not in use, as well as the cleaning of the work area and instrumentation with isopropanol and/or bleach solutions at 10%, help to ensure that extraneous DNA molecules are destroyed before DNA extraction or PCR preparation.*

The PCR product may show results which originate from amplified DNA contaminating a sample which has not yet been amplified. Since the amplified DNA is much more concentrated than the unamplified template DNA, the first will be preferentially copied during the PCR, and therefore the unamplified sample will be hidden.

The inadvertent transfer of even a very small volume of PCR amplicon to an unamplified DNA sample may result in the 'contaminant' sequence being amplified and detected.

For this reason, ***the samples being examined are usually worked on in the laboratory before the reference samples of the suspect, in order to avoid any possibility of the evidence being contaminated with the already amplified DNA from the suspect.***

3.7 Replicate analyses

The most commonly used approach for the designation of an allele in an LCN sample requires the subdivision of the sample into two or more aliquots, and recording only those alleles which are common to at least two replicates (*Gill P. et al., 2000; Gill P., 2001; Gill P. et al., 2007)*. The advantage of this approach is that if drop-in occurs randomly and infrequently, observing an allele several times increases the reliability that it actually derives from the sample being examined (***assuming that no contamination happened during the sampling phase****).*

Most scientists who work with LCN stress the need to perform 2-3 replications and state that an allele must be observed at least twice to be denominated as such (Taberlet P. et al., 1996, even invoke up to 7 replications to increase the reliability of allele denomination): allele redundancy in replicates is therefore the cornerstone of reliable LCN typing.

[90] However, the exact number of replications, the number of times an allele is observed, and the degree of reliability (quantitatively or qualitatively) need to be better defined.

In practice, it needs to be considered that performing more than 2-3 replications is often not possible; therefore most interpretation guidelines and degree of reliability assertions must be applied and declared based on the analysis of 2-3 replicates.

Common sense would suggest that dividing a sample into multiple aliquots increases the limitations of LCN typing, (*Budowle B. et al., 2009)* and that every possible effort should be made to concentrate as much template as possible into a single reaction: however, ***to date, allele redundancy is the only accepted approach.***

Studies on dilutions and redundancy have, up to now, been based on laboratory control samples: [these are] completely different to [samples] originating from items from real cases, possessing undetermined (and often degraded) quantities of DNA, and which may contain PCR inhibitors also able to affect allele drop-out.

Therefore, based on these numerous uncertainties, the prompt definition of ***the data to include in the final report concerning the number of replicates, the degree of reliability related to the uncertainty of the nature of the sample being examined, and its quality, would be desirable.***

3.8 Controls

A further issue characteristic of LCN typing is the number and type of control samples which should be used. For example, the number of negative controls which

should be analyzed to verify that drop-in is sporadic has not yet been discussed. In addition, what constitutes a positive control needs to be better specified: it should be reasonable that positive controls have the same quantity of DNA as is present in the sample being examined, but in practice this is very difficult [to achieve] since the amount of DNA in a sample is unknown and difficult to approximate in mixed samples. Perhaps a range of template quantity should be proposed (for instance, from 20 pg to 200 pg). If the quantity in a positive control approximates that of an LCN sample, allele drop-in and probably also drop-out will happen. Therefore, if a positive control does not provide a sure result with reliability, the control loses **[91]** its function. These problems relating to what constitutes an appropriate positive control must be addressed.

3.9 Limitations of LCN Typing

Some of the differences of LCN typing compared to conventional STR typing can affect its utility (*Gill P., 2001; Budowle B. et al., 2009)*.

Due to the small quantities of DNA contained in LCN samples and the extreme sensitivity of the method (due essentially to the "optimization" of the PCR and capillary electrophoresis protocol) ***levels of "background" DNA as well as DNA from casual contact may be detected; thus profiles possibly emerging from these analyses may not relate to the specific case being examined.***

In addition, **Budowle B. et al.** (2009) assert the need to inform the International Scientific Community of the limitations and problems associated with LCN techniques, so that all those involved in a specific investigative or legal case are aware of the risks and limitations which can affect the results of the investigations. ***Publicizing the potential of the application of LCN typing without describing its limitations is not a responsible role for the forensic geneticist to take.***

In this regard, the essential aspects to remember are the following:

1. *LCN typing is not a reproducible technique, and this limitation must always be well-emphasized in the reports;*
2. ***contamination from handling is possible, and this possibility must always be considered;***
3. *a concentrated sample can provide better results in an analysis than replicates which use allele redundancy for interpretation;*

4. *the number and type of controls used needs to be clearly defined, and to these should be attributed a quantitative or qualitative reliability;*
5. *to date, interpretation guidelines have not been well-defined, and those which exist only relate to samples from a single source; mixture interpretation has not yet been validated;*
6. ***contamination or allele drop-in may have different sources;***
7. **[92]** ***due to the increased sensitivity of the method, secondary transfer cannot be excluded as a possible explanation for the results obtained from LCN typing;***
8. *STR kits, reagents and other materials may not have been subjected to effective quality controls to detect possible contamination from extraneous DNA;*
9. *statistical interpretations and supporting data for the calculation of probabilities need to be better defined and developed to address the uncertainty associated with LCN typing;*
10. *as the analysis provides results from very small samples, the material origin of the DNA cannot, to date, be inferred.*

According to **Budowle B. et al.** (*2009*), ***LCN typing by its nature cannot be considered a robust method.*** While efforts regarding the implementation of the use of this method have principally focused on reducing laboratory contamination and on using redundancy for reliability, a more appropriate approach might now be to improve the phases of collection, extraction and PCR.

The approaches to consider include:

- ***improving collection methodology at the crime scene,*** *and training staff in charge of the investigations there;*
- ***increasing efficiency of recovery and the yield from a collection instrument and/or from the extract to try and increase the amount of template DNA*** *available so that the sample no longer needs to be classified as LCN but can then be analyzed by conventional methods (Budimlija Z.M. et al., 2005; Schiffner L.A. et al., 2005);*
- *improving the PCR so that stochastic effects are less important with limited quantities of template DNA;*

- *considering SNPs as genetic markers of primary use for DNA of low and degraded quantity (Budowle B., van Daal A., 2008; Sanchez J.J. et al., 2006);*
- *improve the quality of the DNA sample (and the quantity of template available) using DNA repair and/or Whole Genome Amplification methods (Smith P.J., Ballantyne J., 2007; Nunez A.N. et al., 2008).*

[93] ***Caragine T. et al.*** (2009) describe the modifications made to the protocols commonly used in STR analysis to improve the low rate of success associated with LCN typing.

These modifications substantially consist of:

- *strengthening the PCR through increasing the number of cycles;*
- *reducing the reaction volume and doubling the annealing time;*
- *modifying the capillary electrophoresis, by increasing the injection time and voltage (Bajda*
  E. et al., 2004).

The authors stress that ***intensifying the DNA signal with these methods inevitably increases the risk of detecting contamination.***

The protection measures advised and used by the authors to ***reduce contamination to the minimum possible*** are the following:

- *Laboratory staff – who must always wear double gloves, hair coverings, shoe covers, safety glasses and lab coats – must work exclusively in areas dedicated to the analysis of LT-DNA samples or pre-amplification;*
- *Fume hoods must be set up for each phase of the procedure (Kwok S., Higuchi R., 1989). Disposable gloves must be changed between examining individual items, between one analysis and another, and several times during the preparation of a single sample;*
- *Workbench surfaces, materials and instruments must be washed first with 10% bleach, then with water and finally with 70% ethanol both before and after each procedure, and also between the analysis of one item and another;*
- *A thorough weekly cleaning of the entire laboratory and the facilities must also be carried out. All the instruments and the water must be irradiated in a UV radiation cabinet before use;*
- *Regarding manual processes, it is stressed that only one sample tube must be opened at a time, using a clean tube opener or paper tissue as an alternative;*

- *Before analyzing samples with capillary electrophoresis, the standard (that used by the authors for this validation study is the GeneScan® 500 LIZ® Size Standard) must be run by itself in order to confirm the absence of extraneous DNA in the capillary;*
- **[94]** *All reagents used in the phases of extraction, quantification, amplification and separation must be preemptively tested to confirm the absence of extraneous DNA.*

Both the samples under examination and the negative controls used for the experiments illustrated in this work were amplified in triplicate using the AmpFISTR Identifiler® kit according to the manufacturer's protocol, with the exception of a 2 minute annealing time and a halved reaction volume with 2.5 U of TAQ for 31 PCR cycles. The data was then analyzed with the analysis software *GeneScan*® and *Genotyper*® (*Applied Biosystems*) with a ***peak height threshold of 75 RFU*** (Relative Fluorescence Units).

The consensus profile, also called the composite profile, was then generated including all the alleles identified in at least 2 out of 3 replicates.

The amplification of LT-DNA samples typically produces stochastic phenomena like allele drop-in and drop-out, peak imbalance at the same locus and an increase in stutter. In the study in question, these effects were shown in DNA quantities equal to and less than 100 pg, and in particular for values below 50 pg. Despite the effects of drop-out for smaller DNA quantities, this method which provides for the development of the aforementioned parameters allowed good peak strength to be obtained, often above ***1000 RFU.***

The experiments carried out further show that, despite the strict application of appropriate protocols to minimize possible sources of drop-in within the laboratory, some spurious alleles in negative controls are nonetheless to be expected.

Recently **J.M. Butler** and **C.R. Hill** (2011) also stated that, faced with limited information provided from low amounts of DNA, forensic scientists will continually find themselves needing to address the question of how far to push DNA testing methods. "Low copy number" (LCN) analysis, also known as "low template DNA" (LT-DNA), refers to the enhancing of detection sensitivity, usually through increasing the number of PCR cycles. The stochastic effects associated with this type of analysis

are allele or locus drop-out, while ***increasing the sensitivity of the method increases the*** **[95]** ***potential for contamination*** or allele drop-in. Validation studies conducted with replicate testing of low amounts of DNA were therefore performed to establish the threshold of allele drop-out and drop-in, using 10, 30 and 100 pg with different commercial STR typing kits, using both a standard and increased number of cycles. The results obtained with heterozygous samples demonstrate that a replicate testing approach provides reliable results with single source samples, if a consensus profile has been created. Due to the limited nature of the biological samples that may be recovered from traces present at a crime scene, decisions often need to be made about whether or not to proceed with testing low amounts of DNA. Just as for any scientific measurement, validation helps to define the limits of the techniques used. In the case of the validation of forensic DNA testing, sensitivity studies, in which control DNA samples are diluted to low amounts, allow a laboratory to establish at which point a detection technique is no longer able to provide reliable results. The authors maintain that repeated testing of replicated aliquots of the same diluted control DNA sample allows the reproducibility of the results to be evaluated. However when increasing the sensitivity of the testing method by increasing the number of PCR cycles, the stochastic effects can become more evident.

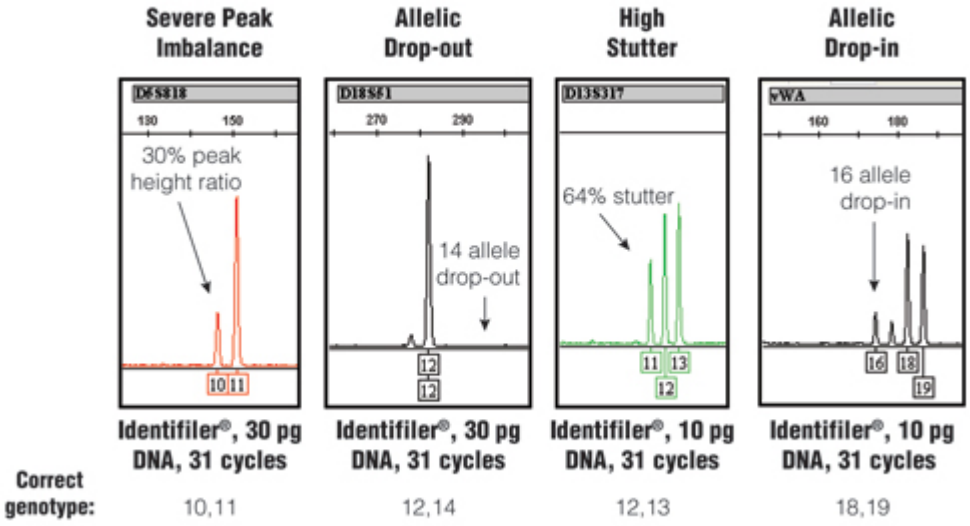
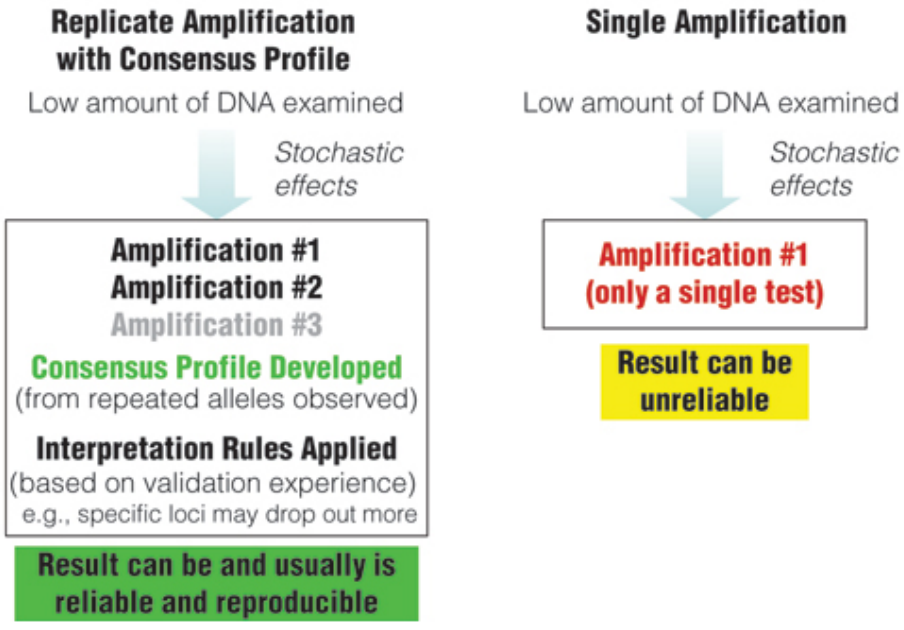


Figure 2. Stochastic effects which happen randomly during the amplification of low amounts of DNA with an increased number of PCR cycles.

[96] While stochastic effects always occur if low quantities of DNA are subjected to PCR (Figure 2), ***a replicate amplification approach with the development of a consensus profile from the repeated alleles can provide reliable results*** (Figure 3). However, amplification results from a single analysis can be unreliable due to allele drop-in or drop-out, as previously noted. Individual results can also vary between replicates, but a combined consensus profile can provide an accurate answer if the repeated alleles are reported.

Every laboratory finds itself needing to confront the analysis of samples with low amounts of DNA. Laboratories and technical staff must decide whether or not to try an "*enhanced interrogation technique"* such as an increase in the number of cycles, desalination of the samples or an increase in the capillary electrophoresis injection. If such an approach is applied, validation studies need to be performed to develop appropriate interpretation guidelines and to establish the degree of variation which can be expected when low amounts of DNA are analyzed. Deciding when to stop the testing or the interpretation of results can be difficult. Some laboratories interrupt the testing based on a certain amount of starting DNA, using the validation data to establish a quantification threshold. Others use stochastic thresholds which are utilized during data interpretation to decide which STR typing results are reliable (for example, where one would not expect to find allele drop-out at that particular locus). The authors conclude by affirming that performing similar experiments to those described in this article can help to determine an appropriate stochastic threshold.



[97] Figure 3. Comparison between different approaches when analyzing low amounts of DNA. Replicate amplification with development of a consensus profile and the application of interpretation rules based on validation experience can produce reliable results.

Only a few months ago, some scientists ("***Publications and letters related to the forensic genetic analysis of low amounts of DNA", Forensic Science International: Genetics 5 (2011) 1-5, Editorial)*** stressed the fact that, particularly over the last two years, numerous publications, communications and letters in numerous scientific journals addressed the subject of the scientific, technical and legal problems associated with the methods and protocols of LCN typing. The sudden increase in publications relating to this subject was triggered by two important legal cases, in the United Kingdom and the United States respectively (*The Queen v Sean Hoey. Neutral Citation Number [2007] NICC 49; The People v. Hemant Megnath, Ind. No. 917/2007, Frye Hearing, 2010)*. These triggered a heated debate on the question of whether the typing of low amounts of DNA using modified laboratory procedures such as increasing the number of PCR cycles, post-PCR purification methods and/or increased injection conditions for capillary electrophoresis have been sufficiently validated by the laboratories which **[98]** apply them in routine cases. Furthermore, additional problems raised concerned the fact that adequate and generally accepted approaches for the biostatistical interpretation of results obtained from such modified protocols are still not available, although the basic idea of using a greater number of PCR cycles for more difficult samples was published more than a decade ago (*Gill P. et al., 2000; Whitaker J.P. et al., 2001)*. The scientific journal in question stated that it had accepted numerous letters to the Editor during the last year with the intention of ensuring a free exchange of opinion, but that it had finally been forced to halt this series of exchanges since, rather than attempting to bring new direction or knowledge to the subject, they were limited to provocative statements and sterile criticisms. However, the Editors of the journal invite the sending of work relating to original scientific research which can provide new information about the validation and interpretation of the results obtained from DNA typing of low quantity and/or quality.

Finally, for completeness, it is considered appropriate to recount the **methods by which Exhibit 36 was acquired.**

From the record of the Court of Assizes hearing on 28/02/2009 (page 175), Inspector Armando Finzi, in service at the Squadra Mobile, was asked about the search of Raffaele Sollecito's house: "*Were you wearing ordinary clothing or did you take precautions?" A: "We were all in ordinary clothes, obviously before entering inside the building all of us put on gloves and footwear".*

It is not clear who collected the knife: "(Court of Assizes hearing on 27/02/2009, page 158) Marco Chiacchiera: "***I took it, put it inside the envelope, closed it, sealed it, and took it back to the police station".*** Armando Finzi: "***It was the first object which I took,*** *I turned and I showed it to Dr. Chiacchiera, saying to him that in my opinion we had to take this knife because after investigative intuition…"* (Court of Assizes, hearing on 28/02/2009, page 178) and it was placed inside a "*new envelope where I keep the gloves, the new gloves" "...I opened the envelope and I put it inside an envelope similar to this one, after which I put it inside the folder and I closed it and I continued the search"* (Armando Finzi, Court of Assizes, hearing on 28/02/2009, page 179); *Q: "Well then we can clarify, you inserted the knife which you found into a paper envelope similar to that one and you sealed it with tape, it's correct to say this?" A: "Yes, not there on the* **[99]** *spot, in the police station, after doing the paperwork I sealed it with a piece of tape,* ***but it wasn't properly sealed, there were two gaps, I closed the two gaps so as not to leave the envelope open"*** (Court of Assizes, hearing on 28/02/2009, page 190).

From the documents, there is a further handover of exhibit 36 in the police station (Court of Assizes hearing on 28/02/2009, page 202): Stefano Gubbiotti: "*I took the knife and I put it inside the…" Q: "You removed it from the envelope with gloves?" A: I removed it from the envelope with gloves because the volume of the envelope did not allow me to put it inside this box". "And I put it in the box and I catalogued it, then I closed it with tape".*

ASSESSMENT OF THE FORENSIC GENETIC TESTS CONDUCTED BY THE SCIENTIFIC POLICE ON ITEM 36 (KNIFE)

From what has been set forth above, we hold that the documentation in the case file reveals inaccuracies and gaps about the analytical procedures performed on Exhibit 36.

In particular:

- regarding the nature of the material collected, **there is no scientifically conclusive evidence to support the possible blood nature of sample B** (knife blade) in that both the generic blood test and the human species test were negative.

The **existence of presumed exfoliated cells** on the samples taken from the knife handle is **equally lacking in scientific basis**.

Therefore we repeat that the theories formulated by the Technical Consultant about the nature of the material removed from Exhibit 36 are wholly arbitrary in that they are not supported by any objective confirmation.

- It should be stressed that the documentation in the case file relating to the **traceability** of the analytical operations performed is completely inadequate.

In particular:

a) regarding the DNA **quantification** tests, both in the Technical Report on the Forensic Genetic Tests (RTIGF) and in the GUP, the Technical Consultant repeatedly claimed to have performed quantification ***with Real Time PCR on all the*** **[100]** ***samples*** taken from the knife, ***but this claim is contradicted by the documentation produced***: in fact, the *Qubit Fluorimeter*™ was used for samples A-B-C.

Regarding ***sample A*** (knife handle) the results obtained (*Qubit Fluorimeter*™) reveal that the DNA concentration in this sample was 0.08 ng/µl.

Taking into account that the *"extract quantity"* was 50 µl (c.f. SAL), multiplying 0.08 ng/µl x 50 µl, the total DNA [obtained] was 4ng: certainly a significant quantity, which allowed sample A to be considered ***positive to quantification.***

The amount of DNA used for the subsequent amplification (0.8 ng) falls within the range suggested by the kit (0.5-1.25 ng/µl of template DNA) and provided an electrophoretic graph of good quality, in accordance with the amount of DNA used for the reaction.

On the other hand, it is not possible to comprehend **the criteria adopted in the assessment of the positive quantification result of sample B and the negative result of sample C**, given that **the same result, "too low", was obtained for both samples**: that is, a value which must be considered not only below the sensitivity threshold of the Fluorimeter indicated by the manual (DNA concentrations of 0.2 ng/µl) but below 0.08 ng/µl, a value which the Fluorimeter detected for sample A.

Neither is it comprehensible, considering the negative results on sample B, what Dr. Stefanoni reported in the GUP questioning (page 178) where she stated that ***the DNA in sample B, quantified with Real Time PCR*** (it is recalled that such *quantification as confirmed during the hearing was never carried out* or, at least, no documentation was provided to support this claim), ***was "in the order of some hundreds of picograms", a value which does not appear in any of the documents provided to us (SAL, Fluorimeter report, Real Time report, RTIGF).***

b) regarding the **extract** obtained from **sample B** (knife blade), the Work Status Report (SAL) shows that this extract was 50 µl. In the **[101]** GUP hearing, the Technical Consultant affirmed having ***concentrated*** sample B to "***20, 22, 23 microliters"***, of having quantified it with Real Time (GUP, page 178: *"Quantification which you did with real time, I imagine?" A: "Eh, yes")* and of subsequently concentrating it again to ***"10 microliters"***, but there is no trace of these operations in the documentation exhibited (c.f. SAL). Neither is it known what the amount of DNA was in the extract concentrated to "20, 22, 23 microliters" and/or the amount of DNA in the extract concentrated to 10 µl.

The quantification problem is of fundamental importance, since a DNA quantity of less than 200 pg/µl falls within the **Low Copy Number** (LCN) definition, for which the International Scientific Community recommends protocols aimed at obtaining scientifically reliable results.

Since the problems arising from the testing of sub-optimal quantities of DNA are numerous (peak imbalance, drop-in, drop-out) different authors have proposed largely interchangeable scientific approaches which can make the interpretation of the data obtained easier.

It should be emphasized that what all the protocols proposed have in common is an awareness that the **main problem of LCN samples** is **contamination** of the item: therefore the authors unanimously agree that suitable ***protocols in the inspection procedures*** must be applied in order to minimize ***environmental contamination*** at the crime scene, and strict ***protocols for the collection and sampling of items*** in order to minimize ***contamination from handling*** at the crime scene.

The procedures recommended by all to reduce ***laboratory contamination*** are equally rigorous (decontamination of the environment, suitable protection of the operatives, control of the reagents employed in procedures, etc.) as it is well-known that contaminant DNA at low levels may derive from reagents and other laboratory consumables, from the staff, and from cross-contamination from sample to sample.

It should also be noted that, according to the International Scientific Community, even if the protocols of a general character indicated above are scrupulously applied, **[102]** ***the increased sensitivity of the PCR method*** (obtained by modifying the standard amplification protocols, increasing the [no. of] PCR cycles, doubling the annealing time, increasing injection time) ***nonetheless constitutes a higher potential for contamination***; therefore, ***secondary transfer cannot be excluded as a possible explanation for the results obtained from LCN typing (****Budowle et al., 2009).*

In addition, the possibility is noted that ***the PCR product of LCN may show results which are due to amplified DNA contaminating an unamplified sample:*** this is because, as previously related, amplified DNA is much more concentrated than un-amplified template DNA, and so the first will be preferentially copied during the PCR.

For this reason, ***the samples being tested should usually be worked on in the laboratory before the reference samples, in order to avoid any possibility of evidence contamination with already amplified DNA.***

In the case in question, it is recalled that Exhibit 36 was placed for testing into a context where a considerable number of samples belonging to the victim had already been examined; therefore, it cannot be excluded that contamination by the aforementioned methods may have occurred - all the more so because the negative controls, which should have been amplified contextually and which could have given an indication as to the absence of contamination, were not produced (Record of the Court of Assizes hearing on 23/05/09, pages 29-30: *"Well, the knife was tested as one item in the course of 50 samples attributed to the victim, some were before the tests on the knife naturally, and others after, so of these 50...I don't know the knife was placed, now I don't know, at a fourth, a third of this series of tests, but in any case even if the knife was analyzed at the end of these 50, 60 samples, in any case this wouldn't affect the validity of the results, because each sample is tested separately, it is absolutely impossible to mix one sample with another, also because the Kercher file is just one of many files we dealt with simultaneously in the laboratory, it's not as if the Scientific Police Service stopped to deal with the Kercher file...").*

[103] Another key aspect concerns the procedure to follow for a reliable ***interpretation of the results***, and for the designation of an allele in an LCN sample. The procedure recognized as valid by the International Scientific Community comes from ***replicate analysis***: that is, it requires the division of the sample into two or more aliquots, with only those peaks recurring in at least two replicates being considered alleles.

The advantage of this approach is that if contamination occurs in a random and infrequent way, observing an allele several times increases the likelihood that it actually derives from the sample being examined, ***assuming that contamination did not happen during the sampling phase.***

Most scientists who work with LCN stress the ***need to perform 2-3 replications, and state that an allele must be observed at least twice to be denominated as such:*** to date, allele redundancy is the recognized and accepted methodology, and is the cornerstone of reliable LCN typing: (GUP hearing on 05.10.08, pages 21-22, to the question "*…the testing of a trace of this type should be repeated several times to be considered reliable?"* the Technical Consultant responds: "*In theory yes*". To the question: "*How many times did you do it*?" she responds: "*In this case only once*". Q: "*Only once, and therefore in theory why ought it be considered more reliable if one*

does it several times?" A: *Because reproducibility of the result is, so to speak, a good standard in any scientific experiment quite apart from forensic genetics, obviously in order to be considered valid a result must be repeatable*").

Therefore, taking into account that in this specific case:

- ***it does not appear that inspection procedures were carried out according to international protocols in order to minimize environmental contamination;***

- ***international protocols of collection and sampling of the item were not applied in order to minimize contamination from handling;***

- ***it is not known whether rigorous decontamination procedures were applied in the laboratory to minimize laboratory contamination;***

- ***a reliable method for quantifying the DNA from samples A-B-C was not employed,*** and the quantification performed with the *Qubit Fluorimeter*™ gave the **[104]** result "too low" for samples B-C, indicating a DNA amount below the sensitivity threshold of the Fluorimeter (>200 pg/µl), and therefore indicative of a probable LCN sample;

- ***the electrophoretic graphs exhibited show that the sample indicated with the letter B*** (knife blade) ***should have been considered an LCN sample*** (peak imbalance, RFU below 50 for most of the alleles) and ***as an LCN sample, all the precautions indicated by the International Scientific Community should have been applied.*** Amongst these we recall:

a) ***rigorous respect for decontamination procedures for the instrumentation, the laboratory and the staff*** (as already mentioned, the procedures adopted to minimize contamination are not reported);

b) ***testing of the item in a laboratory where no items ascribable to the victim were tested,*** to avoid any possibility of evidence contamination with already amplified DNA. ***On the contrary, it was reported that the item was placed for testing into a context where a considerable number of samples belonging to the victim had already been examined*** (Record of the Court of Assizes hearing on 23.05.09, pages 29-30: *"the knife was tested as one item in the course of 50 samples attributed to the victim, some were before the tests on the knife naturally, and others after, so of these 50...I don't know the knife was placed, now I don't know, at a fourth, a third of this series of tests...");*

c) ***performing 2-3 replicate amplifications with the development of a consensus profile. In the case in question, the amplification was only performed once***; *therefore the lack of replicate amplification with the development of a consensus profile provides unreliable results* (GUP hearing on 05.10.08, pages 21-22: to the question, "…*the testing of a trace of this type should be repeated several times to be considered reliable?"* The TC responds: "*In theory yes*". To the question: "*How many times did you do it*?" she responds: "*In this case only once*". Q: "*Only once, and therefore in theory* ***why ought it be considered more reliable if one does it several times?***" A: *Because reproducibility of the result is, so to speak, a good standard in any scientific experiment quite apart from forensic genetics,* ***obviously in order to be considered valid a result must be repeatable***").

d) **[105]** ***employment of negative controls*** *in the amplification procedure to check for the presence of contamination.* ***In the attached eletropherograms, neither negative nor positive controls are reported.***

Thus premised, in this specific case the following conclusions can be drawn:

- in relation to **sample A** (knife handle: identification code 47329), taking into account the considerations previously stated about the electrophoretic graph which shows peaks which exceed the 50 RFU threshold and allele balance ($Hb=\varphi_a/\varphi_b$ >0.60) in accordance with the presumed quantity of DNA used for the reaction (0.8 ng), ***we agree with the conclusion reached by the Technical Consultant about the attribution of the genetic profile obtained from this sample to Amanda Marie Knox;***

- in relation to **sample B** (knife blade: identification code 47330), based on the considerations previously stated about the electrophoretic graph which shows peaks below the 50 RFU threshold and allele imbalance ($Hb=\varphi_a/\varphi_b$ >0.60) indicative of a Low Copy Number sample (LCN), taking into account that in this specific case none of the recommendations from the International Scientific Community relating to the treatment of Low Copy Number samples were followed, ***we do not accept the conclusions about the certain attribution of the profile detected in Sample B to the victim Meredith Susanna Cara Kercher since the genetic profile, as obtained, is unreliable in that it is not supported by scientifically valid analytical procedures.***

Neither, as previously explained, ***can it be excluded that the result obtained from this sample may derive from contamination phenomena occurring at any stage of the collection and/or handling and/or analytical procedures performed.***

LABORATORY ANALYSES REPORTED IN THE RTIGF REGARDING ITEM 165B

[106] Examination of the Technical Report on the Forensic Genetic Tests (RTIGF) drafted on June 12, 2008 by Dr. Patrizia Stefanoni, Principal Technical Director [and] Biologist of the State Police, reveals the following data regarding the extraction of biological traces, laboratory analyses performed, and interpretations of the electropherograms relative to Item 165B.

LABORATORY ANALYSIS

Exhibit 165B is described as follows on p. 13 of the RTIGF: "Clasp of bra with small portion of white-colored fabric attached, stained with presumed blood, found in the room of the victim (formerly Exhibit Y)".

The **Work Status Report (SAL)** [*Stato Avanzamento Lavori*] card [*scheda*] reveals that Exhibit 165B (No. 2 Traces) was given an identifying code (**Sample ID Code**) as recorded below:

Sample ID Code = 48896
Furthermore, the following information is reported (cf. SAL):
Type of Trace: PRESUMED SALIVA
Trace Description: Presumed flaking cells – B (hooks)
Extract quantity: 50
Extract location: 271/F1
Analyses performed
Extraction date: 12-29-07

The SAL shows that the generic test for blood using tetramethylbenzine (TMB) was not performed on Exhibit 165B, and **nor was any laboratory test performed to show the presence of biological material other than blood**.

[107] We maintain that it would have been necessary to proceed to morphological analysis in search of any cells that may have been present by means of coloration with any of the reagents commonly employed in histology (hematoxylin). Such a simple and quick test would have required a minimal amount of material that would not have in any way compromised the subsequent laboratory tests but would have been able to clarify the nature of the material taken from the item under examination.

Notwithstanding the omitted test for cells (and thus the failure to identify the material taken from Exhibit 165B), the Technical Consultant hypothesized the presence of "presumed flaking cells" on the aforementioned item.

The Technical Consultant's hypothesis regarding the nature of the material analyzed (which was confirmed at the preliminary hearing and at the trial), is wholly arbitrary in that it is not supported by any *scientifically* objective finding.

EXTRACTION OF DNA

The **extraction of the DNA** from Exhibit 165B was performed using the *BioRobot "EZJ" (Qiagen)* automatic extractor (p. 201 of the RTIGF).

The Work Status Report (SAL) shows that:
- the extraction of DNA from the traces 165B was performed on 12-29-07;
- the *"Quantity of extract"* was 50 μL (cf. SAL)

QUANTIFICATION OF DNA

Subsequently, the quantification of the DNA extracted from Exhibit 165B was performed.

On p. 201 of the RTIGF, the following table is displayed:

Estrazione DNA BioRobot "EZ1" (QIAGEN)	**traccia A** sostanza ematica eseguita	**traccia B** presunte cellule di sfaldamento eseguita
Quantificazione 7700 Sequence Detector *ABI PRISM™* della *Applied Biosystems*	☑	☑

[108] From what is reported in the above table we see that the quantification of the DNA was performed by means of Real Time PCR, using the *7700 Sequence Detector ABI ORISM*™ apparatus from *Applied Biosystems*.

However, no indication of the kit used for this quantification is given.

Examination of the ***Real Time PCR*** relations exhibited shows that quantification via this method was performed, on January 3, 2008, in two repetitions which yielded the following values:

48896 = 0.14
48896 = 0.09

Hence the average [amount] of DNA present was 0.115 ng/µL.

Given that the "*quantity of extract*" was 50 µL (cf. SAL), multiplying 0.115 ng/µL x 50 µL, the total DNA was 5.775 ng/µL [*recte*: ng], certainly a meaningful amount, permitting the amplification to be repeated.

AMPLIFICATION: AUTOSOMIC STRs

[109] With regard to the subsequent amplification of the extracted DNA, we read as follows on p.201 of the RTIGF:

"*The amplification of the autosomic STRs and of the Y chromosome was performed on the Trace B extract in the manner reported previously on pp. 31, 33 and 34. Trace A however was subjected only to amplification of the autosomic STRs. The results of both amplifications were analyzed via capillary electrophoresis.*"

On page 31 of the CT the procedural details of "*amplification via PCR*" of the autosomic STRs are recorded:

"*in order to obtain the DNA profile from the genetic material extracted from the traces [being] analyzed, the following genetic regions (loci) were amplified via the use of pairs of specific oligonucleotide-primers for the regions supporting [*fiancheggianti*] the various polymorphisms of interest:* ***D3S1358, HumvWA31, D16S539, HumFGA, HumTH01, TPOX, CSF1PO, D8S1179, D21S11, D18S51, D5S818, D13S317, D7S820, D19S433, D2S1338, amelogenin (sex test)*** *using the "AmpFISTRIdentifier" commercial kit of Applied Biosystem[s] (Foster City, CA) according to the "User Manuals".*

We recall that, with regard to the *AmpFISTR Identifiler* kit, the instructions provided by the manufacturer for a correct amplification are as follows:

- *No. samples x 10.5 µL of AmpFISTR PCR Reaction Mix;*
- *No. samples x 0.5 µL of AmpliTaq Gold DNA Polymerase;*
- *No. samples x 5.5 µL of AmpFISTR Identifiler Primer Set;*
- *10µL of DNA with concentration ±0.125 ng/µL;*
- *Final reaction volume 25 µL;*

The range of DNA concentration advised by the manufacturer is 0.5-1.25 ng/µL.

In parallel to the samples under examination, ***controls must be prepared*** in order to monitor the efficacity of the preselected amplification conditions and/or the presence of contamination.

[110] The controls typically include a "negative control" and a "positive control".

The "***negative control***" consists of: ***PCR amplification Mix (without any template DNA) + water or buffer*** (used in the preceding phases of extraction and dilution) in the same volumes used for amplification of the traces under examination.

The purpose of inserting the negative control in the amplification phase is to check whether the reagents used for extracting and diluting the DNA obtained from the samples being examined lack DNA — or, in other words, it ***allows one to verify whether contamination from extraneous DNA is present***.

The "***positive control***" consists of: ***PCR amplification Mix + standard template DNA with known sequence*** (provided by the manufacturer of the kit), in the same volumes used for amplification of the traces under examination.

The purpose of the positive control is to make sure that the components and paramenters of the reaction are correct and thus suitable to amplify the regions of DNA of interest; that is, it ***allows one to monitor the efficacity of the preselected experimental conditions***.

Given what is reported in the RTIGF (p. 202) and the absence of specific annotations in the SAL, it must be concluded that no modification was made to the instructions provided by the kit manufacturer regarding the volumes to be used for the amplification reactions; which is to say that a total volume of 25 µL was used, consisting respectively of

15 µL of amplification Mix + 10 µL of extracted DNA

Hence, the amount of DNA used for the PCR reaction was 1.15 ng (0.115 ng/µL x 10 µL = 1.15 ng).

This is an amount which lies within the range suggested by the kits (0.5-1.25 ng/µL of template DNA).

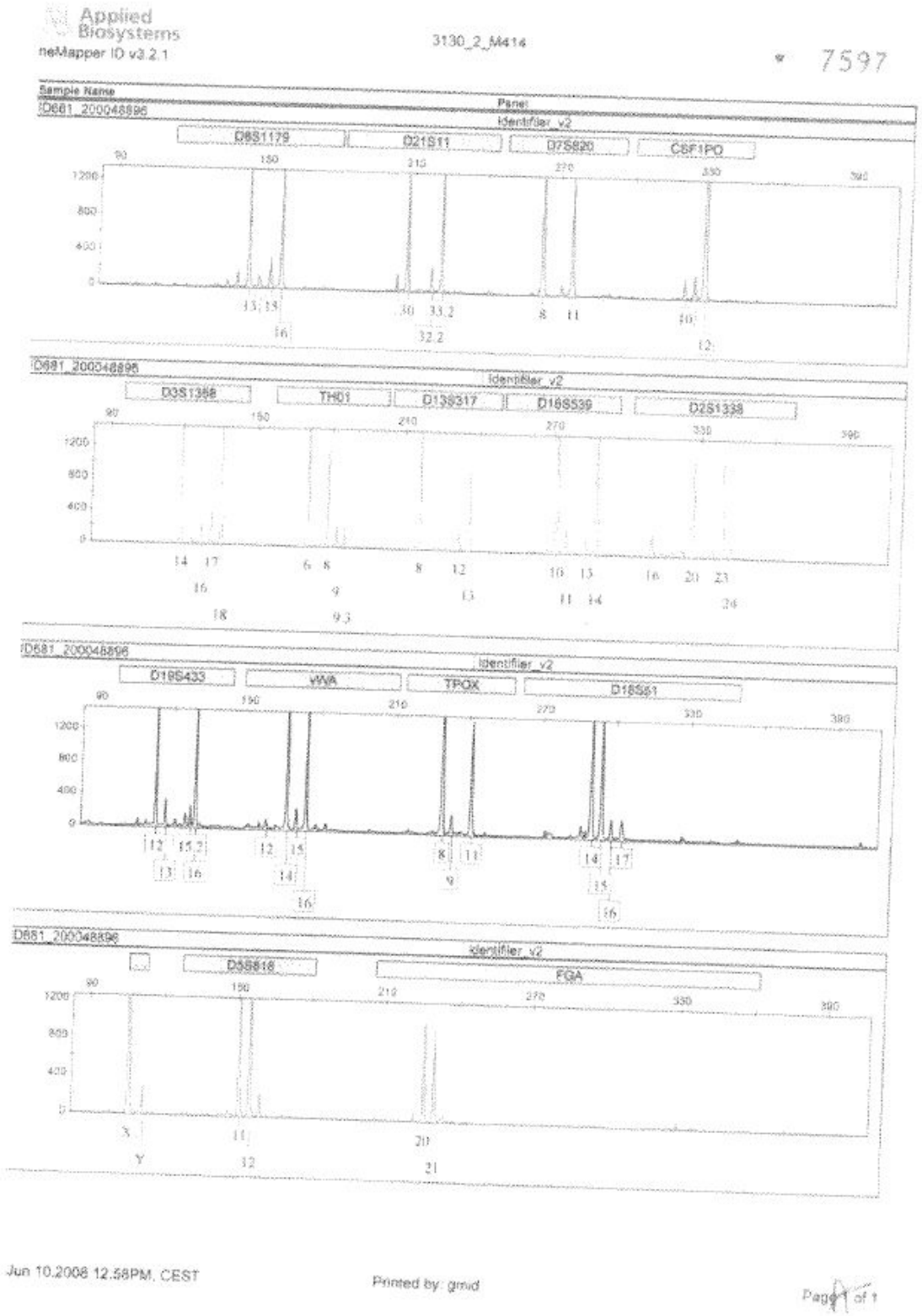
CAPILLARY ELECTROPHORESIS: AUTOSOMIC STRs

[111] The amplification product of the ***autosomic STRs*** obtained from Exhibit 165B was subjected to capillary electrophoresis by means of the *ABIPRISM 3130 Genetic Analyzer* instrument, using the "*Gene Mapper*" analysis software.

Since the record does not suggest that any modification was made to the electrophoresis protocol, we assume that the running conditions were the standard ones indicated by the kit.

We report here the plot relative to the electrophoretic run of the amplified DNA from ***sample 165B*** dated "*Jun 10, 2008*", appended to the RTIGF.

[112]


Applied Biosystems
neMapper ID v3.2.1
3130_2_M414
7597
Sample Name
Panel
Identifier v2
D8S1179
D21S11
D7S820
CSF1PO
TH01
D13S317
D16S539
D2S1338
D3S1358
D16S433
vWA
TPOX
D18S51
D5S818
FGA
Jun 10,2008 12:58PM, CEST
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[113] Since the electrophoretic plot reveals that more than two peaks are visible at several markers, the Technical Consultant correctly hypothesized that this was "*a genetic profile (Table 165-I) deriving from a* ***mixture of biological substances*** *belonging to at least two individuals, at least one of male sex*" (RTIGF, p.202).

The Technical Consultant then concludes that "*Comparison of the genotype deriving from trace B of Exhibit 165 with those belonging to Raffaele SOLLECITO and Meredith Susanna Cara KERCHER (comparisons performed respectively with the genetic profile reported on p.63 Table 30-I, referable to Exhibit 30 and with the genetic profile reported on p.50, referable to Exhibit 21, table 21) yielded a result of* ***compatibility****, i.e. the genetic profile shown in Table 165-I is compatible with the hypothesis of* ***mixture of biological substances*** *(presumably flaking cells) belonging to* ***Raffaele SOLLECITO*** *and to* ***Meredith Susanna Cara KERCHER***".

	KERCHER M.S.C. + SOLLECITO R.
locus genetico	genotipo traccia B
D8S1179:	13, 15, 16
D21S11:	30, 32.2, 33.2
D7S820:	8, 11
CSF1PO:	10, 12
D3S1358:	14, 16, 17, 18
TH01:	6, 8, 9, 9.3
D13S317:	8, 12, 13
D16S539:	10, 11, 14
D2S1338:	16, 20, 23, 24
D19S433:	12, 13, 15.2, 16
HumvWA3:	12, 14, 15, 16
TPOX:	8, 9, 11
D18S51	14, 15, 16, 17
D5S818:	11, 12
HumFGA:	20, 21
sesso	XY=mistura genetica

[114] It should be noted that the interpretation of the alleles, as recorded in the technical report, was contested by Prof. Pascali in the course of the G.U.P. [preliminary] hearing, on 10-04-2008.

The dispute concerned the interpretation of certain peaks held to be alleles by Prof. Pascali and stutter by Dr. Stefanoni.

In particular, on p.100 of the transcript of the interrogation, we read as follows:

Question (Prof. Pascali): "*There a lot of peaks with neither a name nor a number of R.S.U.*[=R.F.U.?]; *can you give us the lot files so we can interpret them?*"

Answer (Dr. Stefanoni) (pp.100-101) "*So as you correctly say, the height, or rather the indication, to put it better, is, as you rightly point out, not recorded for certain peaks appearing in this electropherogram;* ***it's obviously true that we can see that they aren't there, but this is for a simple reason, because since I interpreted this mix, I obviously took it as my responsibility to regard these alleles, so to speak, or these peaks, as insignificant, because from my point of view they're stutter, they're artifacts that are absolutely described and measured, quantified both in the literature and in the kit that I use***."

The Judge asks (p.101) "*So explain to us what you mean by stutter.*"

The Technical Consultant (p.101) states: "*...stutter are extra peaks, so to speak, which show up with a certain frequency according to the genetic point that we go to analyze at the moment in which the PCR has run…*"

She later (p. 102) explains "(omissis) *In making this error it obviously creates artifacts which, however, have a precise characteristic: they are above a certain number of units, which in this case is always four because the units that repeat in these peaks are four; it's always of a smaller unit than the main peak, so we know by looking at the main peak and by looking at the proportion that this extra peak has with respect to the main peak, we can say that this little peak, let's say, is an incorrect addition due to this inherent error[-producing] mechanism of the TAC".*

[115] Judge: "*So you identify it based on characteristics that you read off a diagram, basically?*".

Stefanoni: "*Yes, exactly, from the* ***height*** *which they shouldn't be above* ***according to the international standards*** *that are actually reported in this work that the Professor was citing,* ***which however are recommendations for correct interpretation, so they're guidelines****, for the height and the percentage that this height has with respect to the main peak, this height,* ***this percentage should never be***

above fifteen percent because otherwise there's a risk of mistakenly attributing a peak of this type to stutter".

We agree with Dr. Stefanoni regarding the definition of stutter and we reiterate that **stutter** are non-specific [*aspecifici*] peaks due to the production, during the PCR, of amplification product that is shorter than one repetition with respect to the corresponding allele.

Briefly, the mechanism of stutter formation is as follows: during the replication, the two strands of DNA appear and the polymerasis elongates the one in position 5′→ 3′. It can sometimes happen that in one of the two strands, a repetition remains separated [*spaiata*] and the two strands become staggered. In most cases, the separated repetition lies on the strand that acts as a mold [*stampo*], so that the newly synthesized strand will present one fewer repetition.

The presence of stutter influences the interpretation of genetic profiles, especially in cases where two or more individuals may have contributed to the profile of the trace under examination (mixed trace).

Stutters in fact have the same length as a true allele, and hence it can be difficult to establish whether a peak is actually an allele deriving from a minor contributor, or a stutter.

The behavior of stutters has been amply studied for the STR loci contained in commercial kits. Di- and tri-nucleotide repetitions have a greater tendency to form stutter compared to tetra- and penta- nucleotide repetitions, and this is one of the reasons why the STRs used in forensics have tetra- and penta-nucleotide repetitions.

Furthermore, all loci display a tendency of increased stutter formation for alleles of greater molecular weight.

Stutters are identified by comparing the height of the peak with that of the corresponding allele. This ratio [*rapporto*], for the STR loci used in forensic investigations, is generally less than 15%.

[116] Since, as correctly stated by Dr. Stefanoni, the presence of several peaks at different markers indicates the presence of a mixed profile, and the Technical Consultant herself, regarding the evaluation of stutter, confirms that there exist international standards "***which however are recommendations for correct interpretation, so they're guidelines***", it is necessary to recall what the definition and interpretation of stutter in a mixture reported in the Recommendations of the International Society of Forensic Genetics ("**DNA Commission of the International**

Society of Forensic Genetics: recommendations on the interpretation of mixtures"; *P. Gill et al., For Sci Int., 90-101, 2006*) referred to by the Technical Consultant is.

"***6. Treatment of stutter***

*The characteristics of stutter bands (one repetition fewer compared to the neighboring [*vicino*] allele) were measured in relation to the measurement of the associated neighboring allele.*

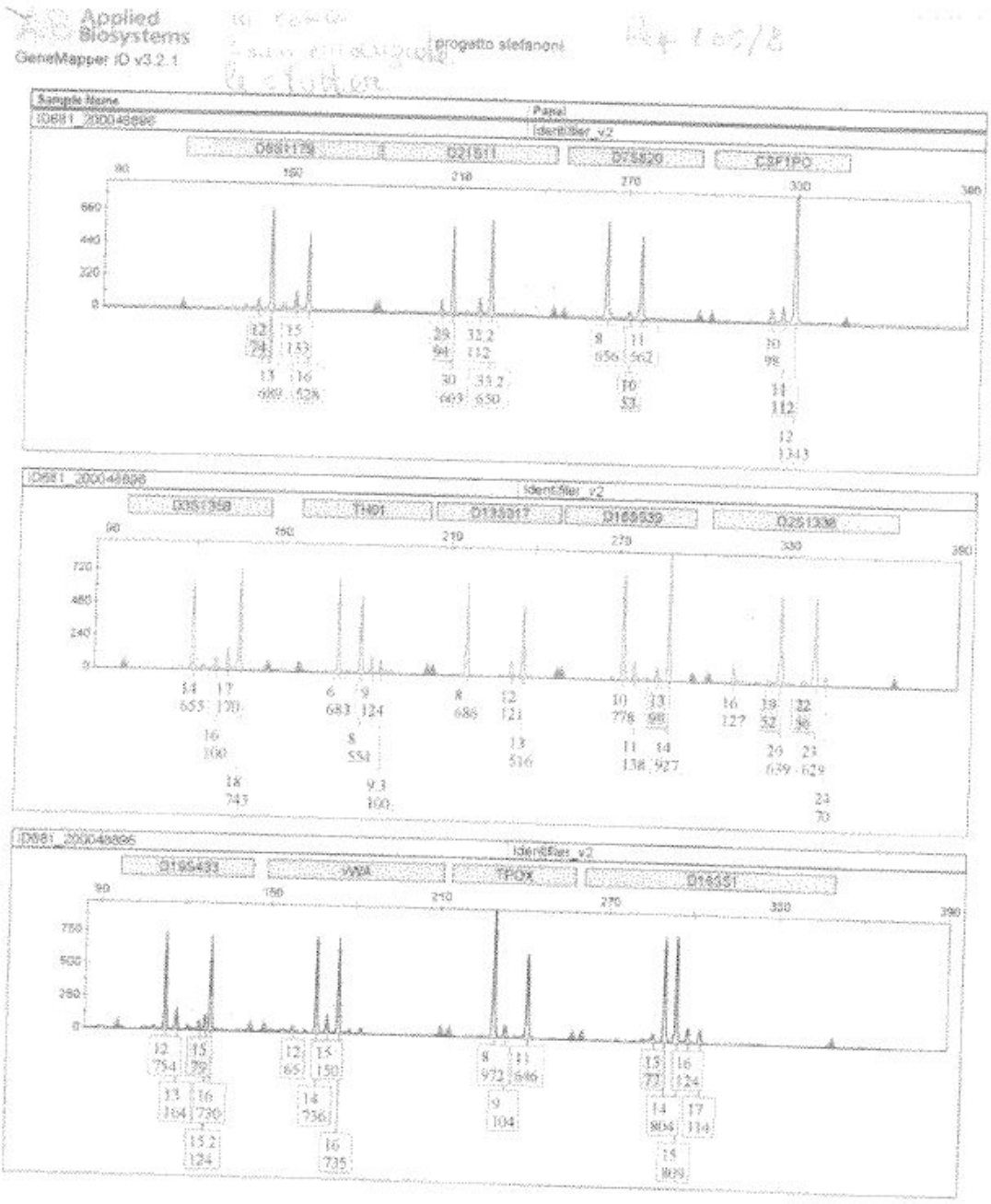
The area or height of the stutter peaks is measured as a proportion (St_p) of the area or height of the neighboring allele.

$St_p = \Phi_{stutter}/\Phi_{allele}$

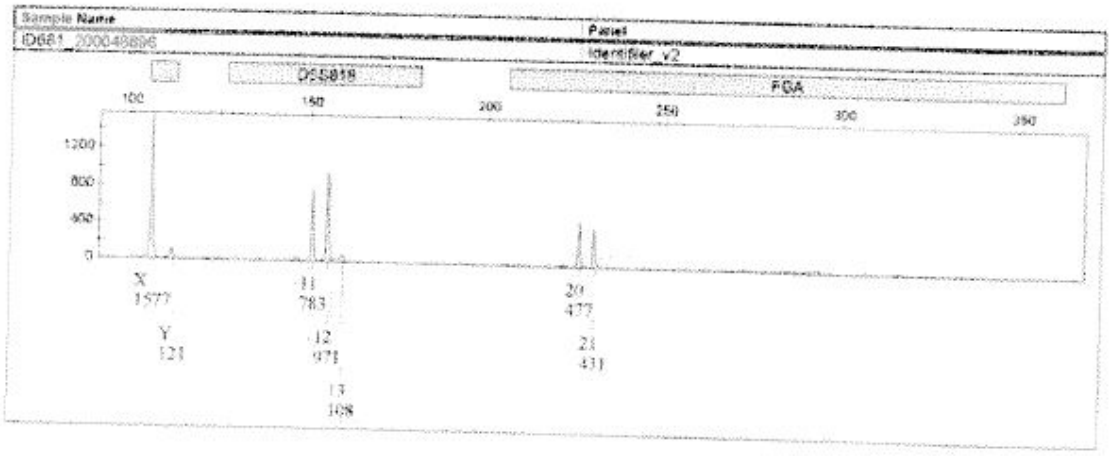
In general, $St_p < 0.15$"

We now report the electropherogram, dated *Sep 25, 2009 10:10 AM*, relating to the interpretation of the stutters performed by Dr. Stefanoni, sent to us by her via CD-ROM, on April 29, 2011.

[117]


Applied Biosystems
GeneMapper ID v3.2.1
progetto stefanoni
Sample Name
Panel
Identifiler v2
D8S1179
D21S11
D7S820
CSF1PO
TH01
D3S1358
D13S317
D16S539
D2S1338
D19S433
vWA
TPOX
D18S51

[118]

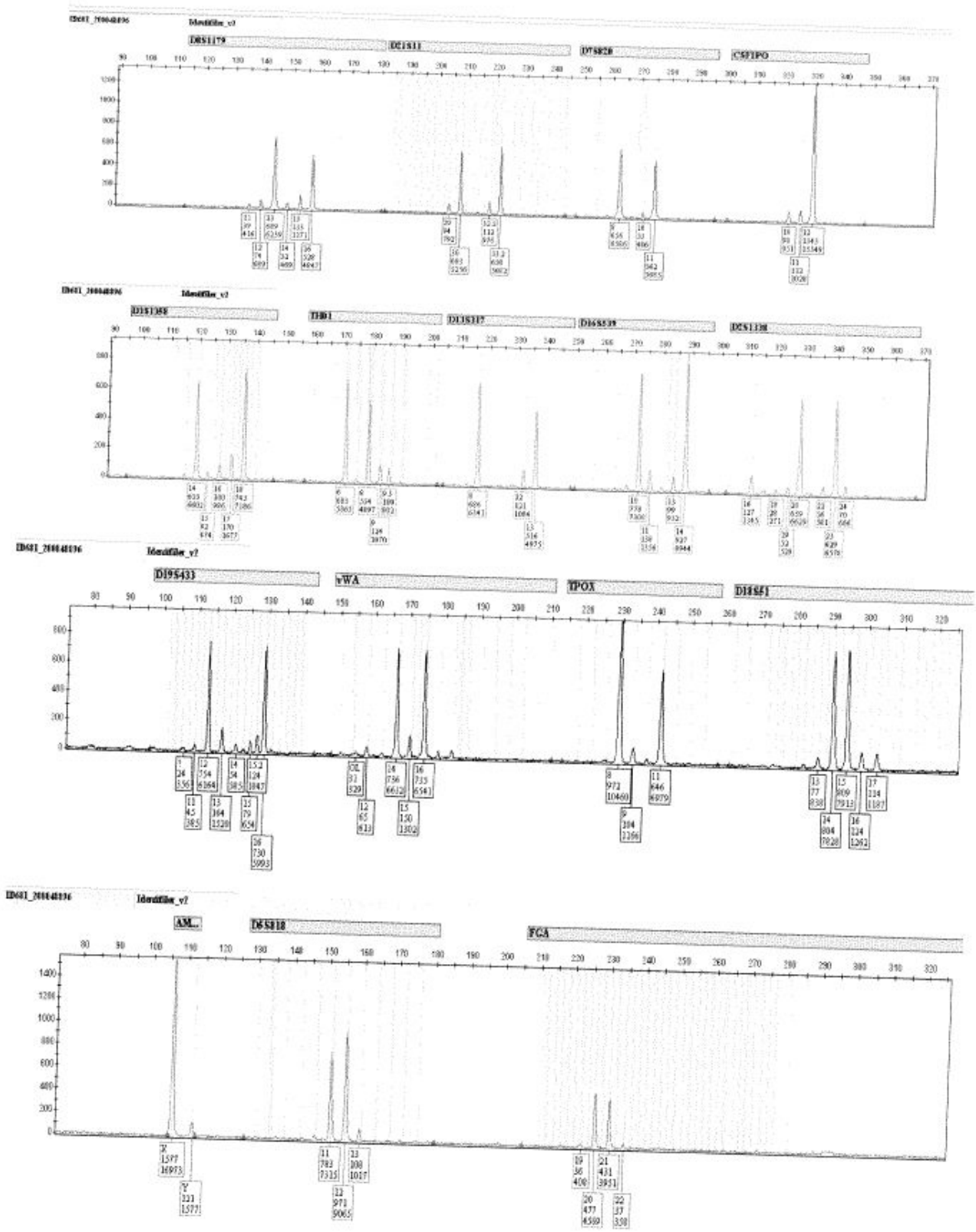


Fri Sep 25,2009 10:12AM, CEST Printed by gmid Page 1 of 1

[119] In order to determine whether the interpretation of the stutters in the electropherogram attached to the RTIGF was performed, as the Technical Consultant claimed, according to the "*international standards*" and according to the recommendations of the ISFG, we examined the electrophoretic graph, sent to us by Dr. Stefanoni via e-mail, on May 10, 2011, with the indications relative to the heights and areas of all the peaks present in the attached graph.

We note that no date of execution of the electrophoretic run is recorded in the graph sent to us on May 10, 2011, but from a comparison between this and the electropherogram dated *Sep 25,2009 10:10 AM*, where the stutters are indicated, we observe that the peaks present the same heights, so we conclude that the graph sent to us on May 10, 2011 refers to the graph dated *Sep 25,2009 10:10 AM*. However, differences in the heights of the peaks expressed in RFU emerge from a comparison of these electropherograms with the electropherogram attached to the RTIGF (dated *June 10, 2008 12:58 PM*) (cf. electropherograms) in that in the graph attached to the RTIGF, the peaks present RFU [levels] above 1200 while in the other electropherograms (electropherogram of May 10, 2011, electropherogram dated *Sep 25, 2009 10:10 AM*) the peaks present RFU [levels] clearly [*nettamente*] below 1200.

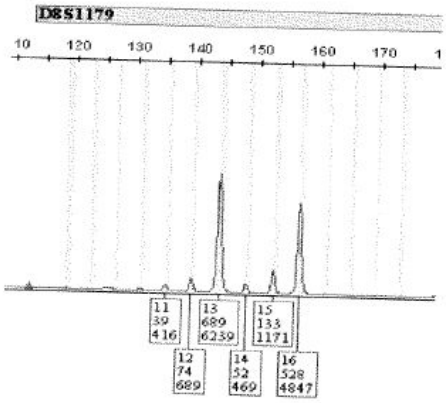
We report the graph sent to us on May 10, 2011 below:



Campione ID 681 200048896 (Rep.165B – gancetti reggiseno)
Legenda: Il riquadro posto sotto l'allele indica, in sequenza, i seguenti valori
-ALLELE- ALTEZZA PICCO IN RFU- AREA PICCO-

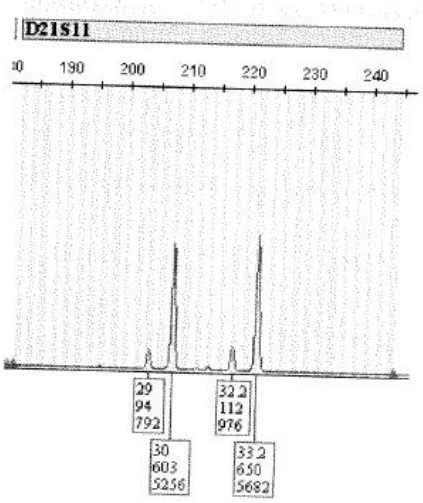
[121] In light of the numerical data relative to the heights of the peaks present in the electropherogram, and having explained the criteria for defining stutter, we can make the following observations concerning the interpretation of the peaks performed by the Technical Consultant.

Below, we analyze certain DNA markers, already subject to dispute, reporting for each of them the numerical values relative to the peak heights; the heights will be used to determine whether the peaks graphically present should be interpreted as alleles or stutter, according to the Recommendations of the International Society of Forensic Genetics (ISFG).



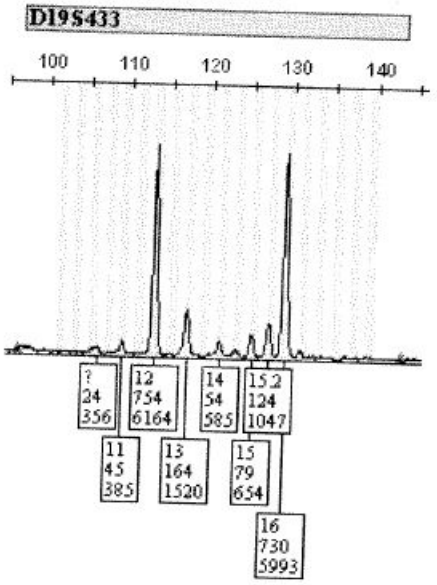
Picco 13 ↑ 689 = allele
Picco 14 ↑ 52 (39.09% dell'allele 15) = allele
Picco 15 ↑ 133 (25.18% dell'allele 16)= allele
Picco 16 ↑ 528= allele

D21S11



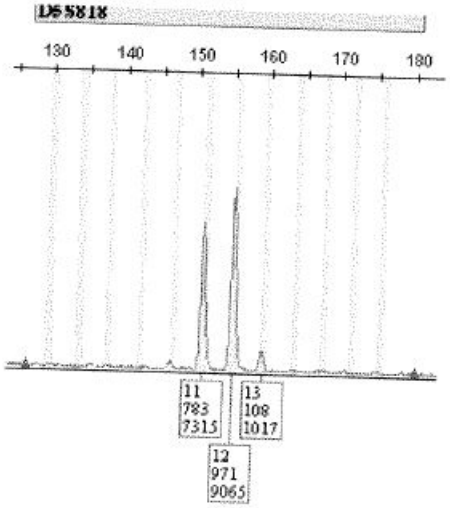
Picco 29 ↑ 94 (15.58% dell'allele 30) = allele
Picco 30 ↑ 603 = allele
Picco 32.2 ↑ 112 = allele
Picco 33.2 ↑ 650 = allele

D19S433



Picco 12 ↑ 754 = allele
Picco 13 ↑ 164 = allele
Picco 14 ↑ 54 = allele
Picco 15.2 ↑ 124 = allele
Picco 16 ↑ 730 = allele

D5S818



Picco 11 ↑ 783 = allele
Picco 12 ↑ 971 = allele
Picco 13 ↑ 108 = allele (non posizione stutter)

[123] We can assert that, relative to markers *D8S1179, D21S11, D19S433*, [and] *D5S818*, there was an ***erroneous interpretation*** of the peaks present in the electrophoretic plot, in that ***peaks with height over 50 RFU*** (***D19S433***: peak 14 ↑ 54) or which ***exceeded the threshold of 15%*** of the major allele (***D8S1179***: Peak 14 ↑ 53 (39.09% of allele 15); ***D21S11***: Peak 29 ↑ 94 (15.58% of allele 30) or were ***not in stutter position*** (***D5S818***) and which, therefore, ***should have been considered alleles***.

Regarding the evaluation of stutter we reiterate that the Technical Consultant, despite having affirmed that there are "***…recommendations for correct interpretation, so they're guidelines***" (GUP 10-04-2008, p.102), did not, in practice, correctly apply the recommendations made explicit in the ISFG guidelines.

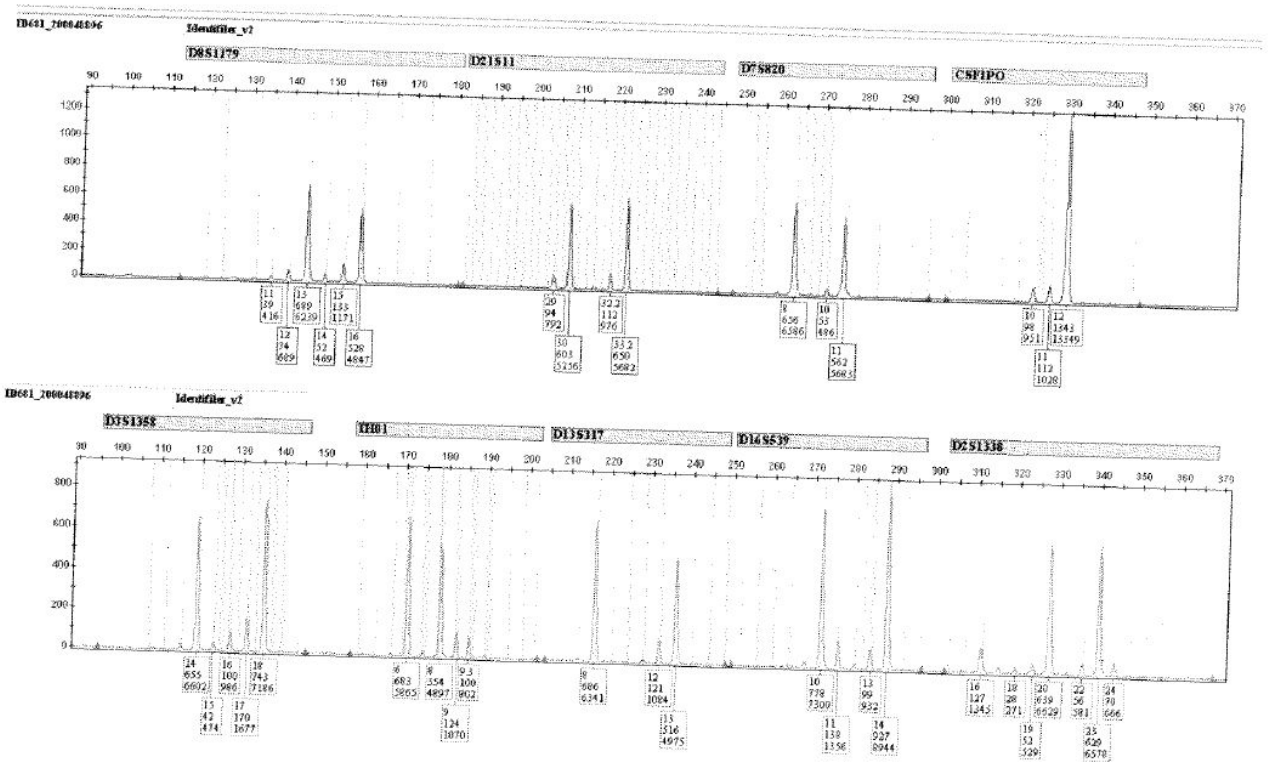
In fact, in paragraph 6 "***Treatment of stutter***" ("*DNA Commission of the International Society of Forensic Genetics: recommendations on the interpretation of mixtures". For Sci Int, 2006*) the following recommendation is reported:

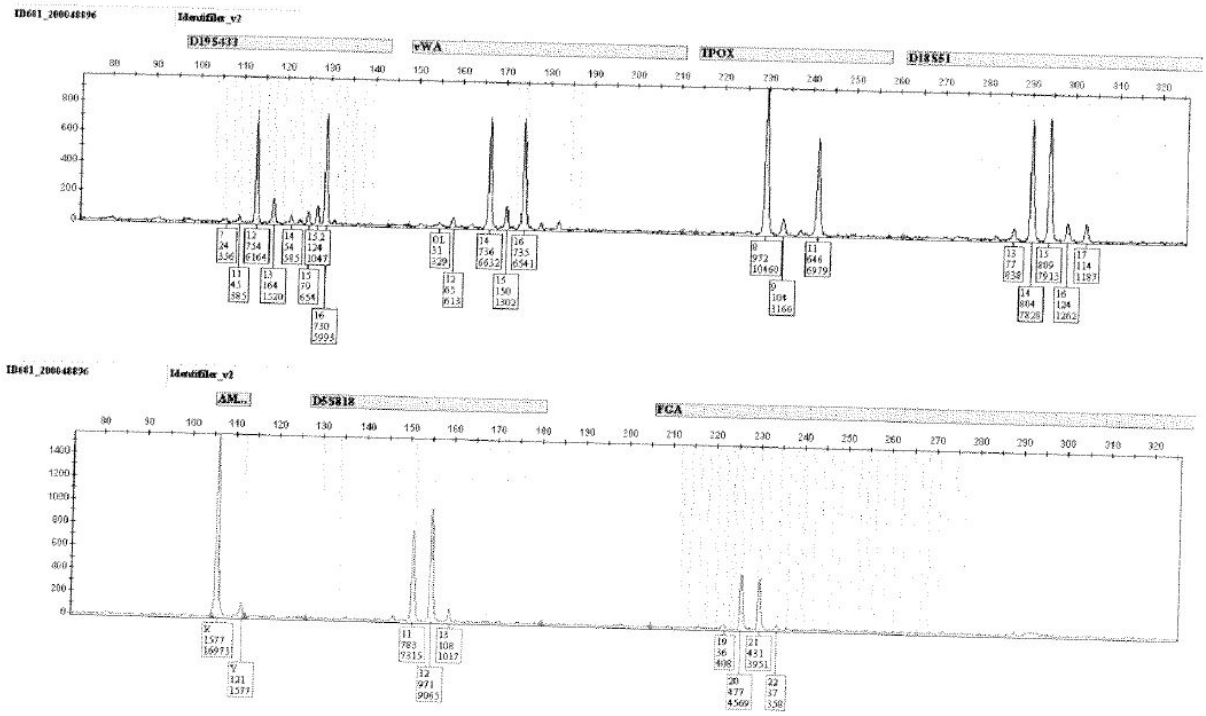
"***Recommendation 6***: *If the crime profile is a major/minor mixture, where minor alleles are the same size (height or area) as stutter of major alleles, then the stutters and minor alleles are indistinguishable. Under these circumstances, alleles in stutter position that do not support* H_p *[the prosecution's hypothesis] should be included in the assessment".*

This recommendation was entirely disregarded by the Technical Consultant, where she states "*So as you correctly say, the height, or rather the indication, to put it better, is, as you rightly point out, not recorded for certain peaks appearing in this electropherogram; it's obviously true that we can see that they aren't there, but this is for a simple reason, because since* ***I interpreted this mix, I obviously took it as my responsibility to regard these alleles, so to speak, or these peaks, as insignificant, because from my point of view they're stutter,*** **[124]** ***they're artifacts that are absolutely described and measured, quantified both in the literature and in the kit that I use***." (GUP 10-04-08, p. 100-101).

Applying *Recommendation No. 6 of the ISFG* (that is, *where in a mixture the alleles of the minor contributor are of the same height or area as the stutters and thus*

the alleles and stutters are not distinguishable, <u>the alleles in stutter position that do not support the prosecution theory should be included in the assessment</u>) to the present case, and taking into account the analogous consideration regarding the possible occurrence of allelic drop-out (*ISFG, point no.7*), it follows that all the peaks present in the individual DNA markers, as reported in the attached electropherogram, should be considered alleles.





[125] Thus, the interpretation of the electropherogram is rather more complex and involved [*articolata*] than what is reported in the RTIGF. Indeed, even if one were to arbitrarily limit the application of Recommendation 6 to peaks in stutter position above the threshold of 50 RFU, ***this still reveals a profile deriving from the mixture of several individuals consisting of a major contributor and several minor contributors***, as summarized in the table below:

DNA	RTIGF	Interpretazione elettroferogramma (ISFG: Racc. 6)	Interpretazione elettroferogramma (Racc. 6: solo picchi altezza superiore 50 RFU)
D8S1179	13 15 16	11-12-13-14-15-16	12-13-14-15-16
D21S11	30 32.2 33.2	29-30-32.2-33.2	29-30-32.2-33.2
D7S820	8 11	8-10-11	8-10-11
CSF1PO	10 12	10-11-12	10-11-12
D3S1358	14 16 17 18	14-15-16-17-18	14-16-17-18
TH01	6 8 9 9.3	6-8-9-9.3	6-8-9-9.3
D13S317	8 12 13	8-12-13	8-12-13
D16S539	10 11 14	10-11-13-14	10-11-13-14
D2S1338	16 20 23 24	16-18-19-20-22-23-24	16-19-20-22-23-24
D19S433	12 13 15.2 16	11-12-13-14-15-15.2-16	12-13-14-15-15.2-16
VWA	12 14 15 16	12-14-15-16	12-14-15-16
TPOX	8 9 11	8-9-11	8-9-11
D18S51	14 15 16 17	13-14-15-16-17	13-14-15-16-17
D5S818	11 12	11-12-13	11-12-13
FGA	20 21	19-20-21-22	20 21

AMPLIFICATION OF THE Y CHROMOSOME

[127] Regarding the subsequent amplifcation of the extract for the Y chromosome, on p.34 of the RTIGF we read:

"*Analogously to what has already been reported regarding the autosomic STRs (see p.31), in order to obtain the genetic profile (haplotype) of the Y chromosome [of the] DNA specimen of male origin, the following gene regions of interest were amplified via the use of pairs of specific oligonucleotide-primers for the regions supporting [*fiancheggianti*] the following polymorphisms:* ***DY3S391, DYS439, DYS38911, DYS438, DYS437, DYS19, DYS392, DYS393, DYS390, DYS385****, using the "Y-Filer" commercial kit of Applied Biosystems (Foster City, CA) according to the information contained in the relevant "User Manual". The amplification products were subjected to capillary electrophoresis by means of the ABIPRISM 3130 Genetic Analyzer instrument, using the "Gene Mapper" analysis software."*

We recall that, with respect to the *AmpFISTR Yfiler* kit, the instructions provided by the manufacturer for a correct interpretation are the following:

- *No. samples x 9.2 µL of AmpFISTR Yfiler PCR Reaction Mix.;*
- *No. samples x 0.8 µL of AmpliTaq Gold DNA Polymerase;*
- *No. samples x 5 µL of AmFISTR Yfiler Primer Set;*
- *10 µL DNA*
- *Final reaction volume 25 µL;*

The range of DNA concentration advised by the manufacturer is 0.5-1.00 ng/µL.

Given what is reported in the RTIGF (p.202) and the absence of specific annotations in the SAL, it must be concluded that no modification was made to the instructions provided by the kit manufacturer regarding the volume to be used for the amplification reactions; that is, that a total volume of 25 µL was employed, consisting respectively of:

15 µL of amplification Mix + 10 µL of extracted DNA for the Yfiler.

Therefore, the amount of DNA used for the PCR reaction was 1.15ng (0.115 ng/µL x 10 µL = 1.15 ng).

[128] This amount of DNA lies in the range suggested by the kits (0.5-1.00 ng/µL of template DNA).

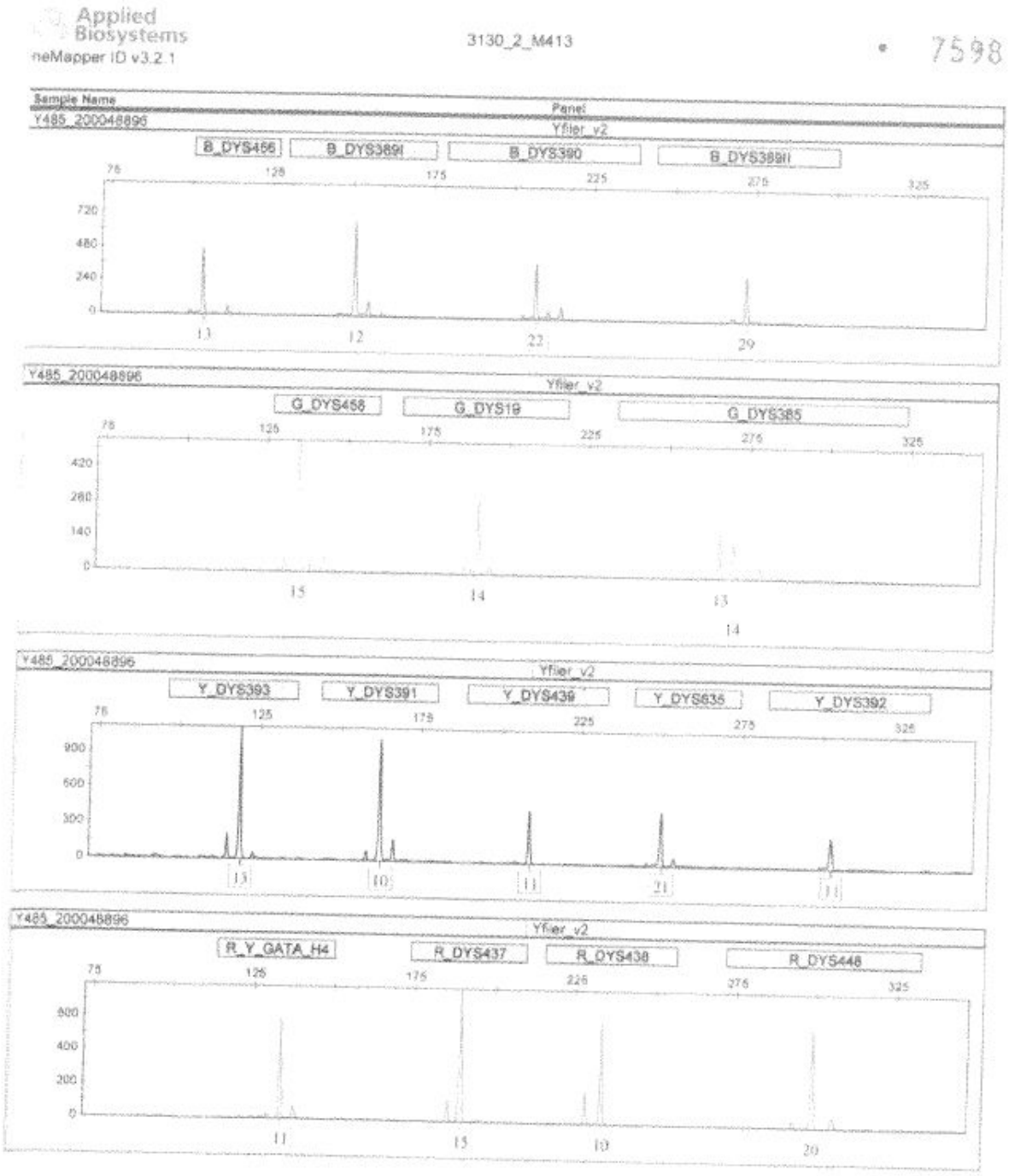
CAPILLARY ELECTROPHORESIS

The amplification product of the Y chromosome obtained from Exhibit 165B was subjected to capillary electrophoresis by means of the *ABIPRISM 3130 Genetic Analyzer* using the "*Gene Mapper*" analysis software.

Since the record does not show that any modification was made to the electrophoresis protocol, we assume that the running conditions were the standard ones indicated by the kit.

We now report the graph relating to the electrophoretic run of the DNA amplified from sample 165B dated "*Jun 10,2008 01:06 PM*" appended to the RTIGF:

[129]



Jun 10,2008 01:06PM, CEST

Printed by: gmid

Page 1 of 1

[130] The Technical Consultant concludes that "*The analysis of the Y chromosome has permitted the determination of the **Y haplotype** shown in **Table 165-II** relative to the DNA extracted from trace B. This result also confirms the presence of DNA belonging to Raffaele SOLLECITO in the analyzed trace, since the **Y haplotype** obtained is equal to that **belonging to Raffaele SOLLECITO** (comparison made with the Y haplotype already reported in Table 30-II of p.63, extrapolated from the genetic analysis of the salivary swab taken from the same [person]*."

We report below the table summarizing the results obtained by the Technical Consultant for the Y chromosome:

	SOLLECITO R.
locus genetico	aplotipo Y traccia B
DYS456	13
DYS389I	12
DYS390	22
DYS389II	29
DYS458	15
DYS19	14
DYS385	13, 14
	13
	10
	11
	21
	11
YGATAH4	11
DYS437	15
DYS438	10
DYS448	20

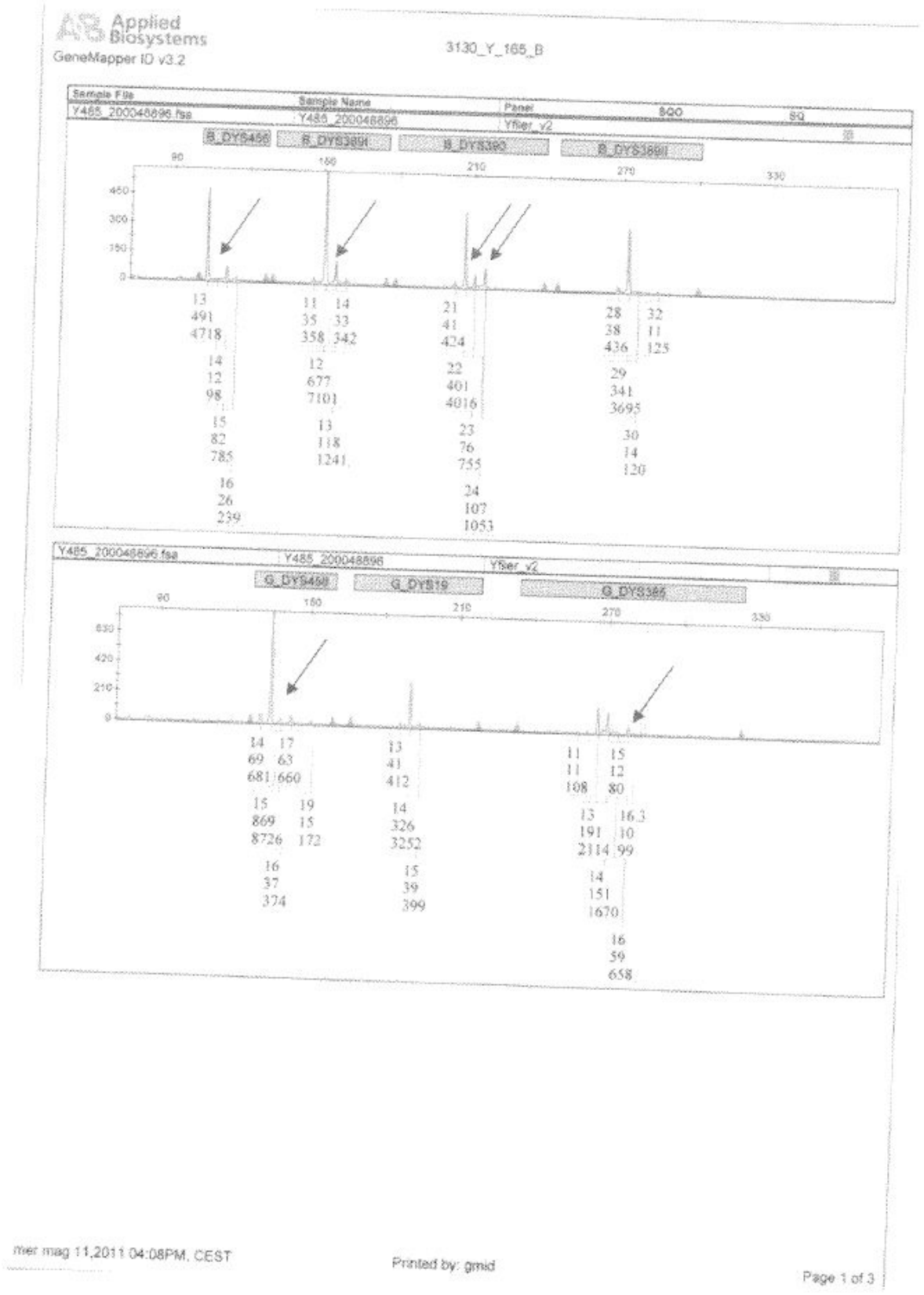
[131] Subsequently, Dr. Stefanoni provided us with the electrophoretic graph (*May 11, 2011 04:08 PM*) of the same run but with the indications relative to the height and the areas of all the peaks present in the aforementioned graph.

In light of the numerical values relative to the height of the peaks present in the electropherogram, we can make the following observations regarding the interpretation of the alleles performed by the Technical Consultant.

Observation of the electropherogram shows that, besides the peaks indicated in the RTIGF as alleles, additional peaks exceeding the threshold of 50 RFU are present, which despite not being in stutter position were not taken into consideration by the Technical Consultant.

Below, we report the electropherograms with an arrow marking the peaks not reported in the RTIGF:

[132]


Applied Biosystems
GeneMapper ID v3.2
3130_Y_165_B
Sample File
Y485_200048696.fsa
Sample Name
Y485_200048696
Panel
Yfiler_v2
SQO
SQ
B_DYS456
B_DYS389I
B_DYS389II
G_DYS458
G_DYS19
G_DYS385
Printed by: gmid
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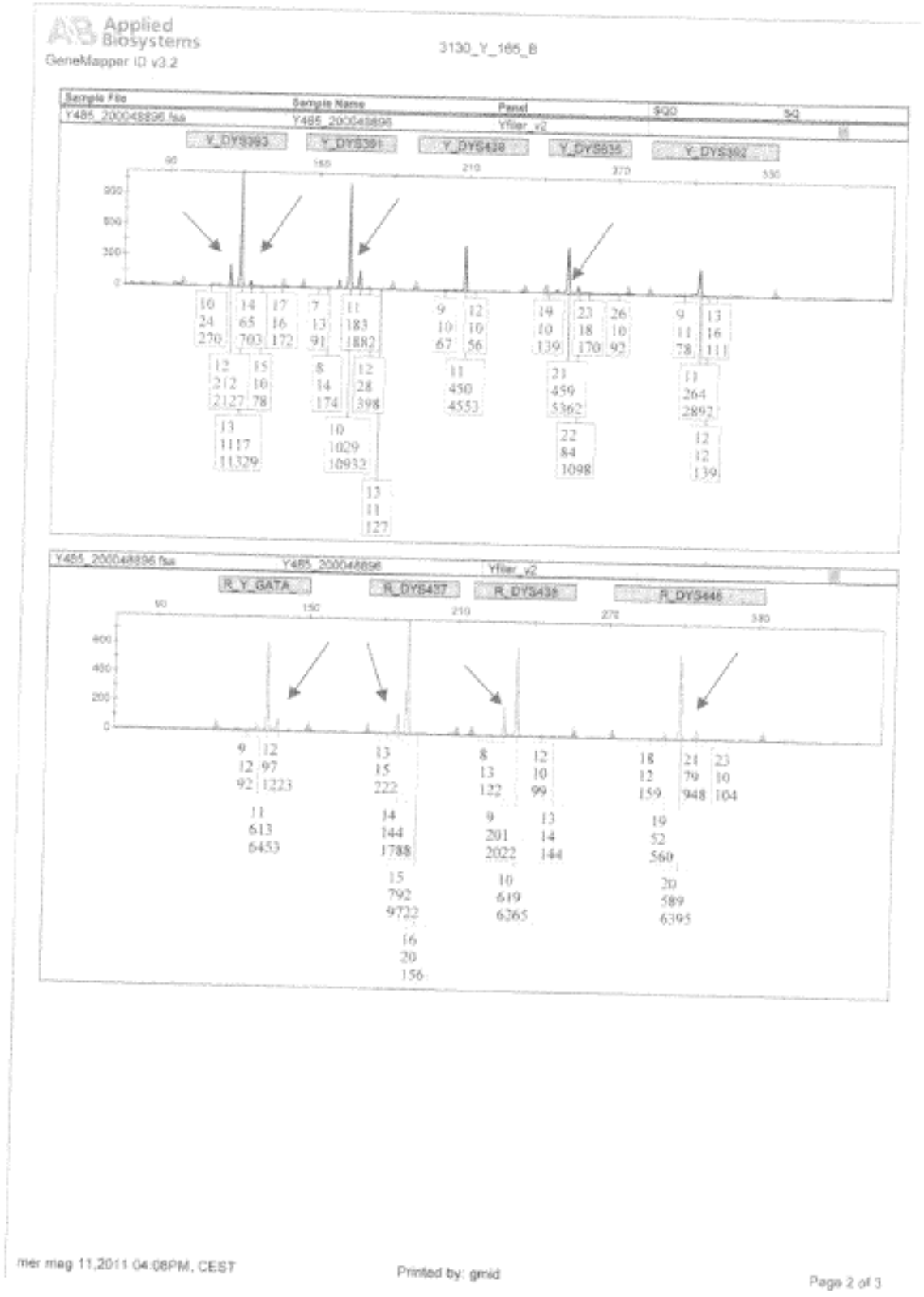
[133]


Applied Biosystems
GeneMapper ID v3.2
3130_Y_165_B
Sample File
Sample Name
Panel
SQ
Y485_20004896.fsa
Y485_20004896
Yfiler_v2
Y_DYS393
Y_DYS391
Y_DYS439
Y_DYS635
Y_DYS392
Y485_20004896.fsa
Y485_20004896
Yfiler_v2
R_Y_GATA_
R_DYS437
R_DYS438
R_DYS446
mer mag 11,2011 04:08PM, CEST
Printed by: gmid
Page 2 of 3

[134] What has been illustrated above shows that more alleles are present in the electropherogram relative to the Y chromosome than are reported in the RTIGF.

In the following table, the alleles revealed by a careful reading of the electropherogram are summarized:

LOCUS	RTIGF	NS LETTURA	ALLELI NON LETTI
DYS456	13	13, 15	15 (↑ 82)
DYS389I	12	12, 13	13 (↑ 118)
DYS390	22	22, 23, 24	23 (↑ 76), 24 (↑ 107)
DYS389II	29	29	-----
DYS458	15	15, 17	17 (↑ 63)
DYS19	14	14	-----
DYS385	13,14	13, 14, 16	16 (↑ 59)
DYS393	13	12, 13, 14	12 (↑212=18.97% allele 13) 14 (↑ 65)
DYS391	10	10, 11	11 (↑ 183)
DYS439	11	11	-----
DYS635	21	21, 22	22 (↑ 84)
DYS392	11	11	-----
YGATA	11	11, 12	12 (↑ 97)
DYS437	15	14, 15	14 (↑144=18.18% allele 15)
DYS438	10	9, 10	9 (↑201=32.47% allele 10)
DYS448	20	20, 21	21 (↑ 79)

It follows from this that several minor contributors of male sex are present in the DNA extracted from Exhibit 165B, confirming what was already observed in the electropherogram of the autosomic STRs and which was not revealed by the Technical Consultant.

Thus we agree with Dr. Stefanoni's assertion regarding "*the extrapolation of a genetic profile deriving from a mixture of biological substances belonging to at least two individuals, at least one of male sex*" but we cannot accept the conclusion stating

that "*the genetic profile is compatible with the hypothesis of a mixture of biological substances (presumably flaking cells) belonging*" **only** "*to Raffaele Sollecito and Meredith Susanna Cara Kercher"*.

[135] We find that the Technical Consultant arrived at this conclusion restricted to two individuals (Meredith Kercher and Raffaele Sollecito) following an incorrect interpretation of the electropherograms of the autosomic STRs, as a result of disregarding the recommendations of the ISFG concerning the correct interpretation of mixtures ("***Recommendation 6***: *If the crime profile is a major/minor mixture, where minor alleles are the same size (height or area) as stutter of major alleles, then the stutters and minor alleles are indistinguishable. Under these circumstances, alleles in stutter position that do not support* H_p *[the prosecution's hypothesis] should be included in the assessment"*) and point 7 of the aforementioned ISFG recommendations (***Point no. 7***: Drop-out: "*The consideration on drop-out is analogous to that on stutter"*). Had these recommendations been followed, they would have allowed one to reach the conclusion that several minor contributors were present in the trace besides the victim (major contributor).

The latter assertion is supported by the electropherogram relative to the Y chromosome, where several alleles are present that, despite being particularly evident, were not taken into consideration by the Technical Consultant.

The genetic profile thus derives from a mixture of unidentified biological substances (it will be recalled that no test was performed with a view toward revealing the presence of flaking cells, and so the claim is without scientific basis), whose larger component is represented by the DNA of the victim and whose smaller component is represented by DNA from several individuals (cf. autosomic STRs) of male sex (cf. Y chromosome), of which one of the Y haplotypes corresponds to the Y haplotype of Raffaele Sollecito

[136] Regarding the reliability of the item with specific reference to "*possible contamination*", we find it appropriate to examine the **means by which and circumstances under which Exhibit 165B was acquired**.

The item was recovered 46 days after the crime, in a context highly suggestive of environmental contamination.

The DNA obtained, though sufficient in quantity to permit analysis, does not satisfy the minimum quality requirements, due to clear environmental contamination.

Various peaks (cf. table of autosomic STRs and Y-chromosome haplotype) which should have been considered alleles until proven otherwise, were not taken into consideration in the analyses; yet their presence was indicative of the fact that, besides Kercher and Sollecito, other unidentified individuals were represented in the genetic traces found at the crime scene. In this regard, it was necessary to proceed to further amplifications of the extracted DNA in order to confirm the presence of the various haplotypes present at the crime scene — something which was not done, even though a sufficient amount of extracted material was available (cf. SAL: 50 µL of extracted material).

Furthermore, **the documentation regarding possible contamination of the item, both before and after recovery, is inadequate**. The mere fact that the amplification control — which was not provided — was negative is not enough to rule out environmental contamination of the item previous to the extraction and amplification of the DNA. **It would have been necessary to obtain the allele profiles present in the surrounding environment**.

The item was recovered on the floor, where it predictably had contact with ambient dust, which, in closed environments frequented by humans, is composed to a large extent of elements of human origin (cells, hairs, etc.).

It has been demonstrated that dust from closed environments can contain tens of micrograms of DNA per gram, with the amount of DNA depending on the level of frequentation by individuals and on the amount of dust that accumulates in the relevant environment.

It has been thoroughly demonstrated that the presence of ambient dust constitutes a significant source of contamination in forensic investigations, since the DNA deriving from such dust can present itself in the form of alleles in analyses of polymorphisms.

[137] The risk of incorrectly interpreting such environmental contaminants can be minimized only by taking the care [*accortezza*] to institute **extremely strict control protocols** including **the analysis of extracts from sterile cotton swabs soaked with sterile buffer that have passed on ambient surfaces to take dust samples** (*Toothman MH et al., 2008*).

In any case, the allele profiles obtained from ambient dust, or from samples contaminated with ambient dust, can be indicative of the individuals who have frequented that environment.

Nevertheless, the aforementioned authors explain that it is difficult to generalize to a direct correlation between human frequentation and levels of dust with the quantity and quality of human DNA therein present, because of the potential effects of other uncontrolled factors. Indeed, environmental variables, including light, heat, and humidity can degrade DNA, and residues of detergents (such as bleach) can destroy the DNA. Moreover, the ventilation system can act as a vehicle for transferring dust among different rooms, introducing DNA from individuals that have not necessarily frequented a specific environment (*Toothman MH. et al., 2008*).

In order to advance interpretive hypotheses, it would have been necessary, in our opinion, ***to proceed to multiple amplifications on Exhibit 165B, whose alleles should have been compared with the alleles obtained from multiple amplifications performed on extracts from multiple samples of ambient dust***.

Only alleles found on Exhibit 165B, and not in ambient dust, could be considered of possible evidentiary value — independently of the height and area of the corresponding peaks. This [comparison] not having been done, the allele profiles of Exhibit 165B should not, in our opinion, be considered probative.

Moreover, given what has been explained above relative to the inspection methods, having seen the documentation in the record, and in particular the DVD of the filmed investigation of the scene [*indagini di sopraluogo*], the official photos of the Scientific Police, and the statements made in court, **we find that the universally noted inspection procedures and correct protocols of collection and sampling of items were not performed on Via della Pergola, even [those designed] to prevent [*scongiurare*] the risk of environmental contamination**.

[138] And in particular, conspicuous lackings [*palesi carenze*] were revealed on the following points:

- **Specific areas for the containment of contamination were not delimited**, or rather this was done only at the outermost level, without worrying about more internal levels of security including the "*crime* scene"; this however carries with it the possibility of contamination arising from possible transfers between internal environments of the residence, including the room in which the body of Meredith Kercher was found (*crime scene*).

- Related to the above, **no security corridor was created for internal access with anticontamination criteria between the various environments**; and indeed on

these first two points, Dr. Stefanoni herself has stated, in the deposition of 10-4-2008 on pp.38-39: "…*since we had our foot-covers [*calzari*], and had been entering many times obviously for the inspection activities in that room, and since the blood was abundant…the floor was certainly dirtier the second time, quote unquote, that is the blood had actually been spread around compared to the beginning of the inspection… this is the point, that is contamination is to be understood as something foreign [*esogeno*] brought in from the outside onto the crime scene… the only thing that could have happened in general in any inspection is that something could have been transferred which however is already there, if this something was moved, this can't be ruled out but certainly nothing was brought in from the outside….Q:..So the most you can assume is a movement of DNA already present at the site?…A: Yes…*" And again on pp. 83-84: "*…Q: However the foot-covers aren't being changed while you move around the house? A: It was possible to go back in…Q:…you went in and out of the rooms without changing the foot-covers…? A: Yes…*"

- **There was no appropriate location set up for the storage of disposable material** used during the inspections (the wastebasket present in Sollecito's house was even used); in this sense we emphasize the possible increase in the risk of contamination due to the stock [*giacenza*] of material used and thus contaminated.

- **There were deficiencies in the records of access to the area of the crime**, that is a record was not correctly kept of who entered the environment. Not only that; in the immediacy of the event, there was access by personnel of the 118 without any protective clothing, personnel who among other things touched and removed **[139]** even the duvet which covered the body of Meredith (deposition of Monica Napoleoni, hearing of 2/27/2009, p. 228-229): "… *A. I stepped into the room while the lady [*dottoressa*] from the 118 uncovered the corpse. PM:…when you went in, did you have your suit on? A: …no, I wore foot-covers and sterile gloves… PM: so everyone who went in had these? A: Yes, yes, of course. Yes now except, um, the 118 personnel …*" And again; access records were held to be unnecessary [*superflua*] (pp 262-263): "*…Attorney: do you know whether your presence was recorded? A: What do you mean recorded? …Attorney: Was your entry noted? Because I can't find it anywhere? A: Our entry wasn't noted because we went with the Public Minister. What am I supposed to do, make notes if there's a Public Minister there?…But when there's a Public Minister I don't make notes and neither do my colleagues…*".

- **Specific anticontamination clothing was not continually and correctly used**, and this includes gloves, foot-covers, masks, and head covers. Even at the crime scene, in fact, there were persons present who wore only foot-covers and a pair of gloves, that is, they were in Meredith's room normally dressed; and we emphasize again how 118 personnel entered without any precaution and even disturbed [*movimentato*] the crime scene (see the preceding transcription); there were also investigative personnel who were not correctly wearing masks and head protection.

- **No indication was given of the correct use of disposable gloves**, and in particular on whether they were changed at the moment of collection of each sample object.

- **Foot-covers were not changed inside the residence** (see transcripts above), which gives rise to the possibility of a potential contamination. In this connection we emphasize what was explained above relative to the ***floor which: "…is largely a depository of evidence but at the same has the greatest contamination potential..". We recall that the clasp was regularly observed and filmed at the very first inspection and remained on the floor for some 46 days, and how there were additional inspections and displacements of objects during this temporal hiatus".***

- **The protocol during the collection of samples was not correct**, i.e. failure to use disposable tweezers [*pinzette*], no preventive drying of biological traces, extraction on areas of low concentration, failure to take **[140]** control samples, failure to wrap certain samples in paper, rushed sampling in open air [*agitato campionamento nell'aria*].

- **The items were handled by more than one person without changing gloves**, extracted without using appropriate tweezers (Deposition of Dr. Stefanoni, 10/4/2008, pp. 39-40); "…*Q:...so the most you can imagine would be a movement of DNA already present on site? A: Yes…Q:…as regards then the reasoning you just gave, this also holds as a description of the change of hand which is of course always photographed or rather filmed…Why does this piece of fabric with the two clasps pass from the hand of one operator to the hand of another operator? A: But if the gloves are clean it absolutely doesn't matter".*

- **The initial position of discovery on the floor of the clasp was not the same after 46 days**; and moreover it was touched several times in succession by several operators, **after having been collected from the floor, and repositioned again on**

the floor; in disregard therefore of what is envisioned in Techniques of Crime Scene Investigation (*Techniques of Crime Scene Investigation*, *Barry Fischer – CRC ed. 2003*), that is: ***Accurately record the position of objects before removing them (caution: do not try to put the objects back in their original position).***

ASSESSMENT OF THE FORENSIC GENETIC TESTS CONDUCTED BY THE SCIENTIFIC POLICE ON ITEM 165B (BRA CLASPS)

[141] From what has been explained above, we can draw the following conclusions about the analytical procedures performed on Exhibit 165B.

- Regarding the nature of the material taken from the item, **there does not exist evidence which scientifically confirms the presence of presumed flaking cells**. Hence the hypothesis formulated by the Technical Consultant about the nature of the material taken from Exhibit 165B is wholly arbitrary in that it is not supported by objective findings;

- From the electrophoretic graph relative to the **autosomic STRs**, we can assert that relative to the markers *D8S1179, D21S11, D19S433, D5S818*, there was an **erroneous interpretation of the peaks present in the electrophoretic graph** in that peaks were considered stutter whose height was above 50 RFU (D19S433 peak 14↑54), exceeded the threshold of 15% of the major allele (D8S1179, D21S11, D5S818), or were not in stutter position (D5S818), and thus should have been considered alleles. It follows from this that in the DNA extracted from Exhibit 165B are present several minor contributors that were not revealed by the Technical Consultant;

- The electrophoretic graph relative to the **Y chromosome** markers shows, besides the peaks indicated in the RTIGF as alleles, the **presence of additional peaks** with heights that exceed the threshold of 50 RFU which, despite not being in stutter position, were not taken into consideration by the Technical Consultant. It follows from this that in the DNA extracted from Exhibit 165B are present several minor contributors which were not revealed by the Technical Consultant, confirming what was already observed in the electropherograms of the autosomic STRs.

- We find that the Technical Consultant arrived at this restrictive conclusion (presence of only two individuals: victim and Raffaele Sollecito) following an incorrect interpretation of the autosomic STRs as a result of having **disregarded the recommendations of the ISFG** concerning the correct interpretation of mixtures, recommendations which, had they been followed, would have allowed one to conclude that several minor contributors were present in the trace besides the victim's

profile (major contributor). Hence we agree with Dr. Stefanoni's statement regarding **[142]** "*the extrapolation of a genetic profile deriving from a mixture of biological substances belonging to at least two individuals, at least one of male sex*" but ***we cannot accept the conclusion*** stating that "*the genetic profile is compatible with the hypothesis of a mixture of biological substances (presumably flaking cells) belonging"* **only** "*to Raffaele Sollecito and to Meredith Susanna Cara Kercher*" insofar as, from what has been explained above, a mixture is present in which several contributors of male sex are present (a circumstance supported by the electropherogram relative to the Y chromosome, where several alleles are clearly present which, despite being particularly evident, were not taken into consideration by the Technical Consultant);

- The item was recovered 46 days after the crime, in a context highly suggestive of environmental contamination. The risk of incorrectly interpreting such environmental contamination from dust could have been minimized only by taking the care [*avendo l'accortezza*] to institute **extremely stringent control procedures**, including **the analysis of extracts from sterile cotton swabs soaked with sterile buffer passed on ambient surfaces to take samples of dust, a procedure which was not carried out;**

- Taking into account what has been explained relative to the inspection methods, having seen the documentation in the record, and in particular the DVD of the filmed investigation of the scene [*indagini di sopraluogo*], the official photos of the Scientific Police, and the statements made in court, we find that the universally noted **inspection procedures and correct protocols of collection and sampling of items were not applied** on Via della Pergola, even [those designed] to minimize environmental contamination and contamination from handling. From this it follows that it cannot be ruled out that the results obtained from Exhibit 165B derive from contamination phenomena in any phase of the collection and/or handling of the item.

CONCLUSIONS

[143] Based on the considerations explained above, we are able to respond as follows to the inquiries posed at the assignment hearing:

"*Having examined the record and conducted such technical investigations as shall be necessary, the Expert Panel shall ascertain:*

1. whether it is possible, by means of a new technical analysis, to identify the DNA present on items 165b (bra clasp) and 36 (knife), and to determine the reliability of any such identification"

- The tests that we conducted to determine the **presence of blood** on item 36 (knife) and item 165B (bra clasps) yielded a **negative result**.

- The cytomorphological tests on the items **did not reveal the presence of cellular material**. Some samples of item 36 (knife), in particular sample "H", present granules with a circular/hexagonal characteristic morphology with a central radial structure. A more detailed microscopic study, together with the consultation of data in the literature, allowed us to ascertain that the structures in question are attributable to **granules of starch**, thus matter of a vegetable nature.

- The quantification of the extracts obtained from the samples obtained from item 36 (knife) and item 165B (bra clasps), conducted via Real Time PCR, **did not reveal the presence of DNA**.

- In view of the absence of DNA in the extracts that we obtained, with the agreement of the consultants for the parties, we did not proceed to the subsequent amplification step.

[144] 2. "*if it is not possible to carry out a new technical analysis, shall evaluate, on the basis of the record, the degree of reliability of the genetic analysis performed by the Scientific Police on the aforementioned items, including with respect to possible contamination.*"

Having examined the record and the relevant documents, we are able to report the following conclusions regarding the laboratory analyses performed on Item 36 (knife) and Item 165B (bra clasps):

ITEM 36 (KNIFE)

[145] Relative to the genetic analysis performed on **trace A** (handle of the knife), **we agree with the conclusion reached by the Technical Consultant regarding the attribution of the genetic profile obtained from these samples to Amanda Marie Knox.**

Relative to **trace B** (blade of the knife) we find that **the technical analyses performed are not reliable** for the following reasons:

1. **There does not exist evidence which scientifically confirms that trace B (blade of knife) is the product of blood.**

2. **The electrophoretic profiles exhibited reveal that the sample indicated by the letter B (blade of knife) was a Low Copy Number (LCN) sample, and, as such, all of the precautions indicated by the international scientific community should have been applied.**

3. Taking into account that none of the recommendations of the international scientific community relative to the treatment of Low Copy Number (LCN) samples were followed, **we do not accept the conclusions regarding the certain attribution of the profile found on trace B (blade of knife) to the victim Meredith Susanna Cara Kercher, since the genetic profile, as obtained, appears unreliable insofar as it is not supported by scientifically validated analysis**;

4. **International protocols of inspection, collection, and sampling were not followed;**

5. **It cannot be ruled out that the result obtained from sample B (blade of knife) derives from contamination in some phase of the collection and/or handling and/or analyses performed.**

ITEM 165B (BRA CLASPS)

Relative to Item 165B (bra clasps), we find that **the technical analysis is not reliable** for the following reasons:

1. **There does not exist evidence which scientifically confirms the presence of supposed flaking cells on the item;**

2. **There was an erroneous interpretation of the electrophoretic profile of the autosomic STRs;**

3. **There was an erroneous interpretation of the electrophoretic profile relative to the Y chromosome;**

4. **The international protocols for inspection, collection, and sampling of the item were not followed;**

5. **It cannot be ruled out that the results obtained derive from environmental contamination and/or contamination in some phase of the collection and/or handling of the item.**

THE EXPERTS

Prof. Carla Vecchiotti

Prof. Stefano Conti

CORTE DI APPELLO DI PERUGIA
DEPOSITATO IN CANCELLERIA
da prof. Stefano Conti
Perugia, 29-6-2011
RESPONSABILE DELLA SEZIONE PENALE
CANCELLIERE
(Dr.ssa Francesca Romana Batsella)