BLOOD PREANALYTICAL STUDIES/TRIALS

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WHY TRIALS?

- USEFUL TOOL TO STIMULATE THE ATTENTION ON A SPECIFIC TOPIC
- THE RESULTS DISCUSSION FACILITATES THE COMPREHENSION OF THE CRITICAL POINTS
- IF CHANGES ARE NEEDED, THEY ARE WELL ACCEPTED

FROM THE EU SPIDIA PROJECT

- **WP1.2: Evidence-based Quality Guidelines for the pre-analytical phase of Blood Samples (M1-M48)**
- **Partners: UNIFI, QIAGEN, TATAA, PRENALYTIX, DIAGENIC, ImmunID**
- **AIMS: Identification of critical steps in the pre-analytical phase of blood samples**
- **Analytical Targets: Genomic DNA from whole blood, Cell-free (plasma) DNA, RNA from whole blood**

SPIDIA WP 1.2. The Design of the SPIDIA trials. 1

- *SPIDIA invites Laboratories performing Molecular Diagnostics to participate "for free" to one or more of the planned SPIDIA trials*
- *SPIDIA sends the (same) sample/s (whole blood, plasma) to the participants and asks them to perform the extraction procedure using their own protocol and reagents,*
- *Participants then send back the extracted DNA/RNA to SPIDIA for further analysis, plus details about reagents and protocols used for the extraction phase*

SPIDIA WP 1.2. The Design of the SPIDIA trials. 2.

- *At SPIDIA facilities, it will be performed:*

- *1. the **analysis** of the extracted DNA/RNA samples*
- *2. the "**report**" and the "**score**" for each participant, which includes the comparison of the performance of the single Laboratory with that of the other participants.*

SPIDIA WP 1.2. The Design of the SPIDIA trials. 3.

- *Participants with "a lower score" will be invited to join in further SPIDIA activities which include:*

- *1. Participation to SPIDIA training courses, in order: A. to discuss the performance of the laboratory and then B. to revise the critical aspects that can affect the pre-analytical phase.*
- *2. Participants are also invited to perform the trials for the second time taking into consideration alternative procedures/reagents that should be able to improve the "score"*
- *3 Results of the first and second trial will be used to monitor the expected improvement of the performance of the pre-analytical phase*

Expected impact of the SPIDIA trials for the Scientific Community and for the European Commission

- On the basis of the information and the data produced during the trials, SPIDIA will propose to the Scientific Community “evidence-based” guidelines for the pre-analytical phase in blood specimens.
- Availability of these data can serve also as a basis for officially recognized standardisation activities by CEN (European Committee for Standardization) partner of the Consortium.

THE SPIDIA BLOOD TRIALS

- Three trials have been planned:
- 1. **SPIDIA-DNA** to monitor the performance of the pre-analytical phase for genomic DNA analysis in whole blood
- 2. **SPIDIA-DNAPlas** to monitor the performance of the pre-analytical phase for both genomic DNA analysis in whole blood and *for cell-free DNA analysis in plasma*
- 3. **SPIDIA-RNA** to monitor the performance of the pre-analytical phase for RNA analysis in whole blood

Why “plasma” DNA Analysis?

- 1. Plasma DNA analysis has been increasingly explored for different clinical diagnostic purposes.
- Previous studies have shown that the concentration of plasma DNA is increased in patients with neoplastic diseases, pregnancy-related complications, traumas and certain autoimmune diseases, suggesting its use as a “surrogate” tissue/entity and thus as a target for new diagnostics.

Why the SPIDIA-DNAPlas Trial?

- *It has been reported a critical role of the pre-analytical phase in "plasma" DNA analysis due to:*
- Lower amount in comparison to the DNA extracted from whole blood (1/100 or less)
- Critical UV analysis (may need fluorescent dyes)
- Need to avoid the presence of DNA released from blood cells (high speed centrifugations..)
- DNA Integrity evaluation seems to play a role in Cancer patients

Where we are now for the activities planned in the SPIDIA WP1.2?

- **DISSEMINATION OF THE INFOS ON SPIDIA AIMS IN ORDER TO REACH THE EXPECTED NUMBER OF APPLICATIONS**
- Active role of the EFCC with invitation letters sent to the Presidents and National representatives of the EU community of Clinical laboratories. Additional Mailing lists from Partners and other EU projects (about 3000 e mail contacts).

- **Development of Web Pages for collection of Applications at the www.efcclm.org web site.**

- **CLOSING DATE FOR APPLICATIONS has been Febr.5, 2010**

APPLICATIONS:

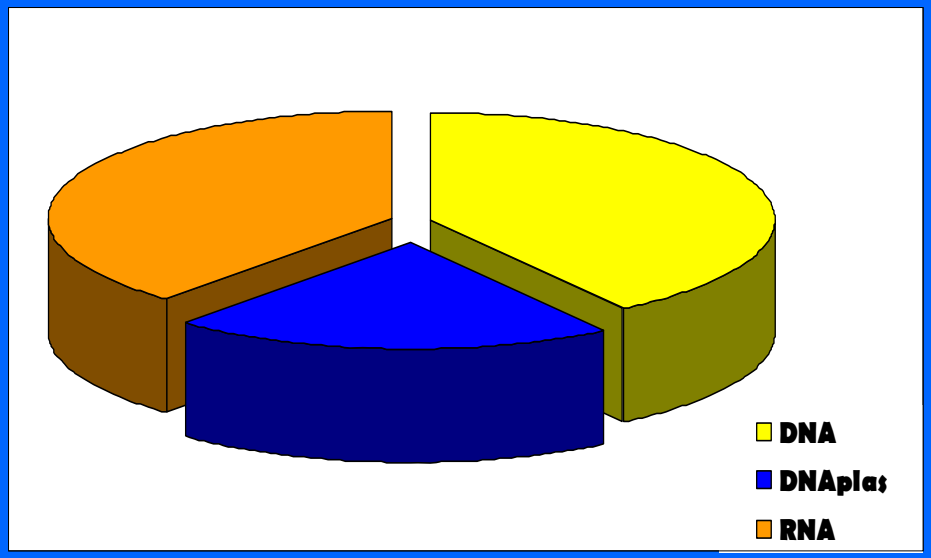
Number of Labs per Country that have applied to the DNA, DNAplas and RNA trials for a total of 322 applications

	TOT LABS	DNA	PLAS	RNA
Austria	8	6	2	3
Belgium	10	6	4	9
Bulgaria	1	0	1	1
Croatia	7	4	2	4
Czech Republic	7	6	1	4
Denmark	2	1	1	1
Estonia	4	2	2	1
Finland	3	3	0	3
France	14	10	2	9
Germany	24	14	5	13
Greece	9	3	3	3
Hungary	4	4	0	2
Ireland	3	3	0	0
Israel	4	3	0	4
Italy	45	22	19	27
Latvia	1	0	1	0
Lithuania	1	0	1	0
Norway	2	0	1	2
other countries	2	2	0	2
Poland	2	0	2	0
Portugal	9	6	3	3
Romania	4	2	2	4
Slovenia	1	1	0	1
Spain	5	4	2	2
Sweden	13	6	5	6
Switzerland	2	1	1	1
The Netherlands	6	5	1	1
Turkey	7	2	5	6
United Kingdom	19	15	1	12

SEVENTH FRAMEWORK PROGRAMME

SPIDIA

SPIDIA Programmes	TOTAL Applications
DNA	131
DNAplas	67
RNA	124
Total applications	322
Total Labs	219



SEVENTH FRAMEWORK PROGRAMME

SPIDIA

SHIPMENT OF SPIDIA-DNA AND SPIDIA-DNAPlas KITS
March 17, 2010

* Contains 1.2 mL of a pool human whole blood, cytrate anticoagulated (HIV-; HBV-;HCV-)

SPIDIA-DNA Kit. SUMMARY OF THE INSTRUCTIONS

BLOOD SAMPLE: 1.2 ml/participant

Actions for the participants:

1. ***Extract genomic DNA using your own protocol and reagents,***
2. ***perform spectrophotometric measurements and calculations;***
3. ***report results and information of the protocol and reagents used for the extraction on the www.efcclm web site;***
4. ***send back the extracted DNA to SPIDIA for further analysis***

THREE PRE-EXTRACTED DNAs (in water, 1.0 ml)

(These solutions are used to verify the accuracy of the UV instrument of the participant)

1) ***One sample containing High Quantity and High Quality of DNA***
2) ***One sample containing Low Quantity and High Quality of DNA (about 1/10)***
2) ***One sample containing High Quantity and Low Quality of DNA (contamined with BSA)***

Actions for the participants:

1. ***Perform spectrophotometric measurements;***
2. ***report results.***

* Contains 1.2 mL of a pool human whole blood, cytrate anticoagulated (HIV-; HBV-;HCV-)

SPIDIA PILOT STUDIES

- During the first year of the SPIDIA project, Pilot studies have been planned in order to revise all the phases of the trials (shipment, instructions, web site pages, SPIDIA analysis, etc.).
- The results of these pilot studies are not statistically significant and they should be considered only as indications.
- For the SPIDIA-DNAplas kit, 11 laboratories of 5 countries have participated to the pilot study.
- For the SPIDIA-RNA kit, 10 laboratories of 5 countries have participated to the pilot study.

SPIDIA-DNA

WHAT SPIDIA HAS PLANNED TO TEST IN THE EXTRACTED DNA FROM THE PARTICIPANTS? *(in order to contribute to monitor the performance of the pre-analytical phase for genomic DNA analysis in whole blood)*

1. COMPARISON OF THE UV ANALYSIS PROCESS

2. EVALUATION OF DNA INTEGRITY/FRAGMENTATION AFTER EXTRACTION

3. PRESENCE OF POSSIBLE INTERFERENCES AFFECTING DNA AMPLIFICATION ASSAYS

PROPOSED ACTIVITIES BY SPIDIA

GENOMIC DNA EVALUATION FROM SPIDIA-DNA

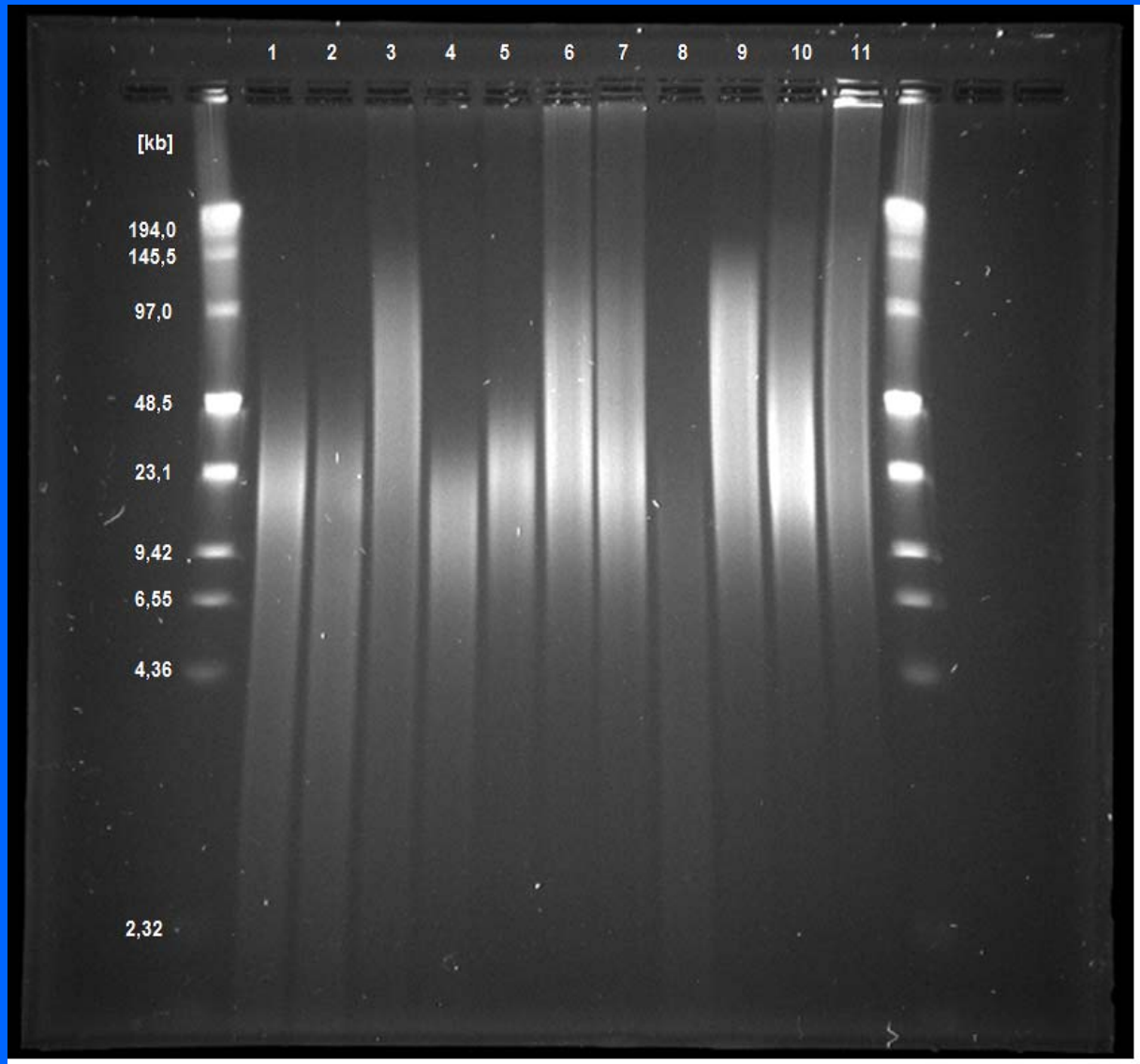
- 1. DNA UV analysis of Quality and Quantity (by Nanodrop or similar)(UNIFI) and comparison to the data produced by the participants
- 2. DNA Integrity (high MW) (by Pulsed field gel electrophoresis)(QIAGEN)
- 3. DNA Quantification by a Real Time PCR Assay (Target Gene: the single copy gene *RNAase P*)**,(UNIFI)**
- 4. Evaluation of the Presence of interferences on the Real Time PCR amplification kinetics (by Kineret analysis developed by TATAA),
- 5. Evaluation of the performance of the extracted DNA in the IMMUNID Diagnostic test (gene rearrangement analysis for leukemia applications)

Pulsed field gel electrophoresis

- Quantity of the extracted genomic DNA: **800 ng**
  DNA Gel: 1% agarose gel (Ultra Pure Agarose, Invitrogen),
- **DNA Size Marker: Low Range PFG Marker (2,03-194 kb; New England Biolabs)**
- Assay Conditions: Electrophoresis performed for **20 hours** at 10 to 12°C with 6 V/cm and a switch time of 1-12 seconds. The gel was **stained** for 1,5 hours using 0,5 ‰ **ethidium bromide** solution and de-stained for 1 hour in distilled water. Documentation was performed using a EASY Win32 system (Herolab).

Pulsed field gel electrophoresis.

11 Labs participated in the SPIDIA-DNA pilot study, performing the genomic DNA extraction



GENOMIC DNA EVALUATION AT SPIDIA FACILITIES

- 1. DNA UV analysis of Quality and Quantity (by Nanodrop or similar)(UNIFI) and comparison to the data produced by the participants
- 2. DNA Integrity (high MW) (by Pulsed field gel electrophoresis)(QIAGEN)
- 3. DNA Quantification by a Real Time PCR Assay (Target Gene: the single copy gene **RNAase P),(UNIFI)**
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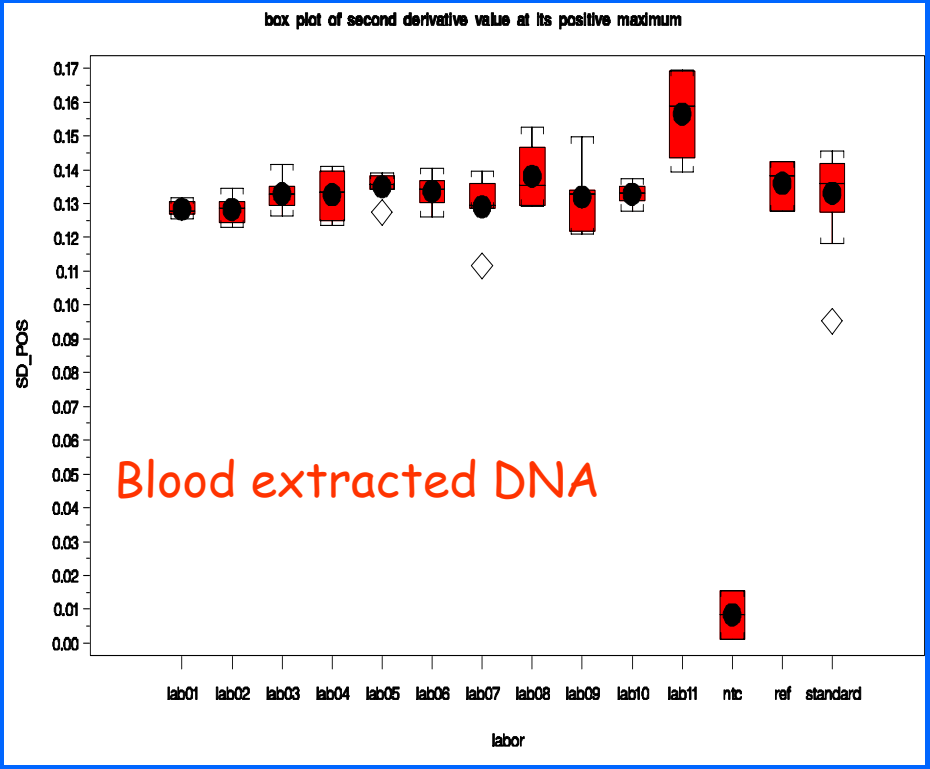
- 5. Evaluation of the performance of the extracted DNA in the IMMUNID Diagnostic test (gene rearrangement analysis for leukemia applications)

Presence of interferences by Kinetics Outlier Detection (Kineret Software)

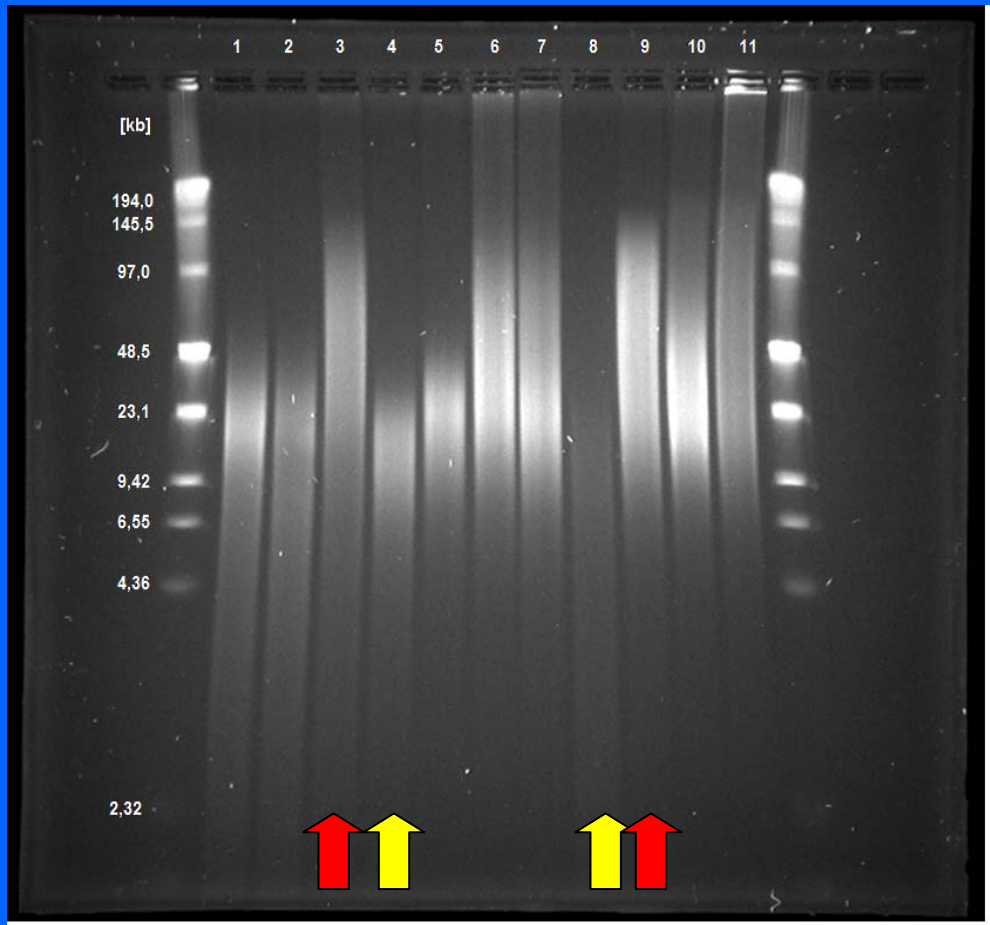
- The software requires reference samples (assumed without interferences) and unknowns (from the participants) performed in triplicate and at two dilutions in the same run.
- Fluorescence data (absolute quantification by real time PCR using a seven-point standard curve) are processed by Kineret software (www.kineretsoftware.com).
- *Values of the second derivative in its positive maximum (SD_pos) of the amplification curve are used to evaluate presence of Interferences in a Real Time PCR assay*

PCR interferences by KINERET



PCR_id	PCR_name	SD_pos	status
22	REFERENCE	0.14251	
27	TEST lab1 1:10	0.13183	
28	TEST lab1 1:100	0.12549	
31	TEST lab2 1:10	0.12308	
34	TEST lab2 1:100	0.12770	
37	TEST lab3 1:10	0.13503	
42	TEST lab3 1:100	0.13127	
43	TEST lab4 1:10	0.12938	
48	TEST lab4 1:100	0.12498	
49	TEST lab5 1:10	0.13913	
54	TEST lab5 1:100	0.13464	
55	TEST lab6 1:10	0.13347	
60	TEST lab6 1:100	0.12601	
61	TEST lab7 1:10	0.11158	
66	TEST lab7 1:100	0.13588	
67	TEST lab8 1:10	0.14675	
72	TEST lab8 1:100	0.12924	
73	TEST lab9 1:10	0.13220	
78	TEST lab9 1:100	0.12096	
79	TEST lab10 1:10	0.12782	
84	TEST lab10 1:100	0.13497	
85	TEST lab11 1:10	0.15394	
90	TEST lab11 1:100	0.14365	
91	NTC	.	

A preliminary comment on the Pulsed field gel electrophoresis analysis.



High MW DNA Integrity Profile:

Good in Labs 3 and 9 (RED arrows)

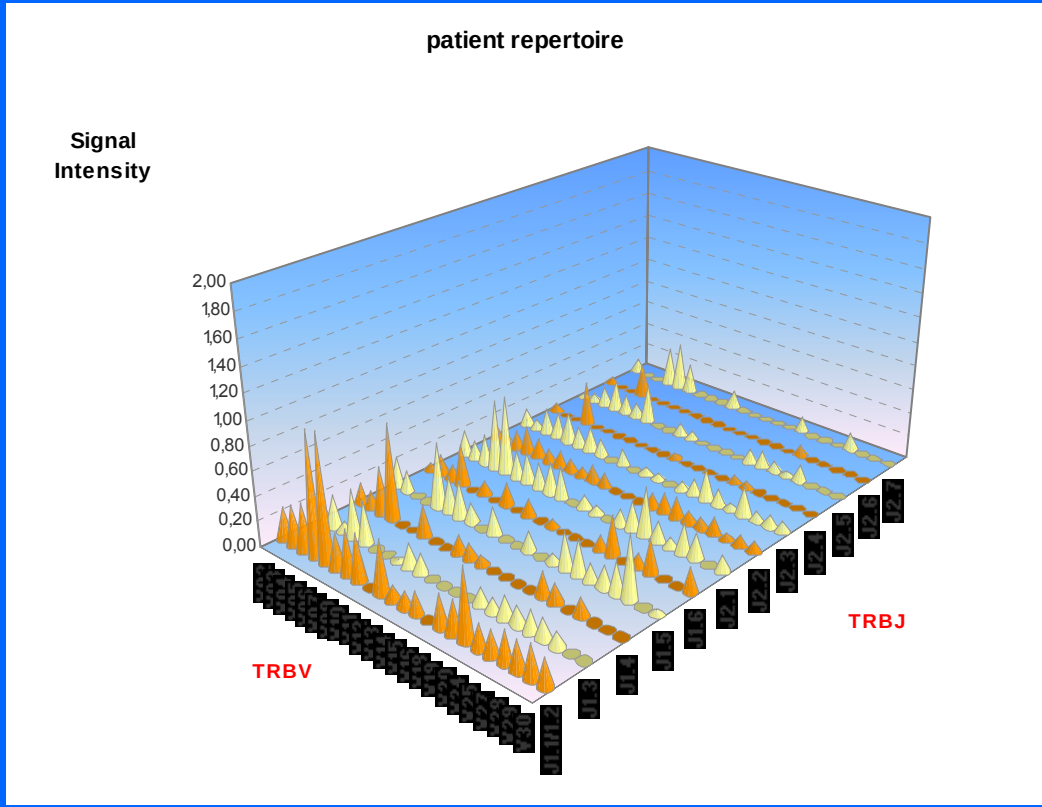
Low in Labs 4 and 8 (YELLOW Arrows)

High MW DNA Integrity Profile: Effect on Real Time PCR analysis

- All Lab 3, 9, 4, 8 had “Good and comparable performance” on DNA Quantification, PCR Efficiency and Interferences

High MW DNA Integrity Profile:
Effect on a Diagnostic tests that requires High quality not fragmented genomic DNA

ImmunID has developed molecular diagnostic tests for routine use, based on gDNA analysis requiring high quality DNA (gene rearrangement for leukemia applications)

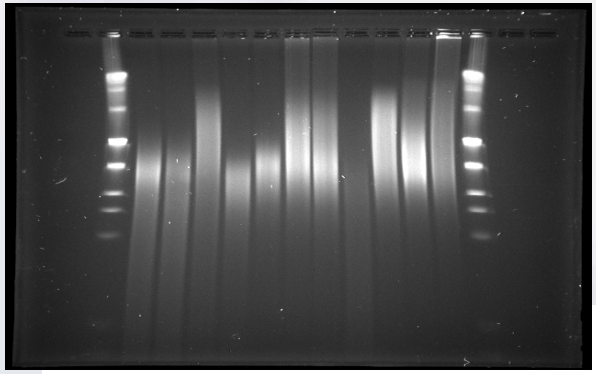


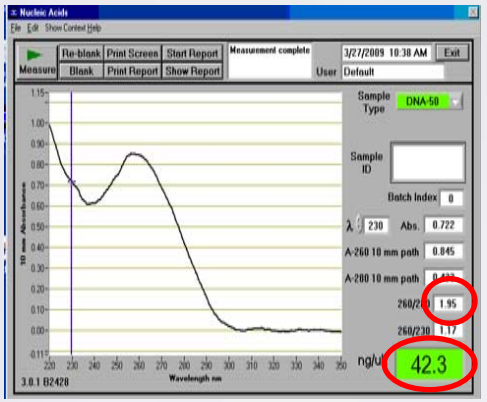
Lab 9 (GOOD INTEGRITY PROFILE) gave a reliable result with this diagnostic test

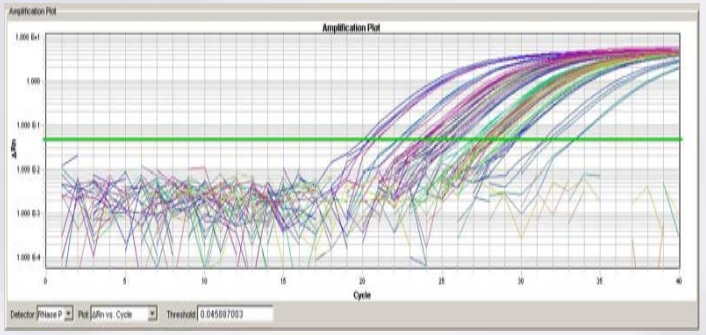
Lab 8 (LOW INTEGRITY PROFILE) gave unreliable result with this diagnostic test

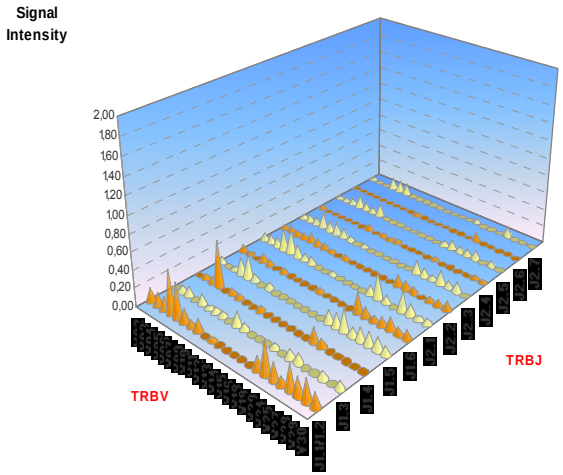
SPIDIA Analysis of genomic DNA extracted from blood by the participants:

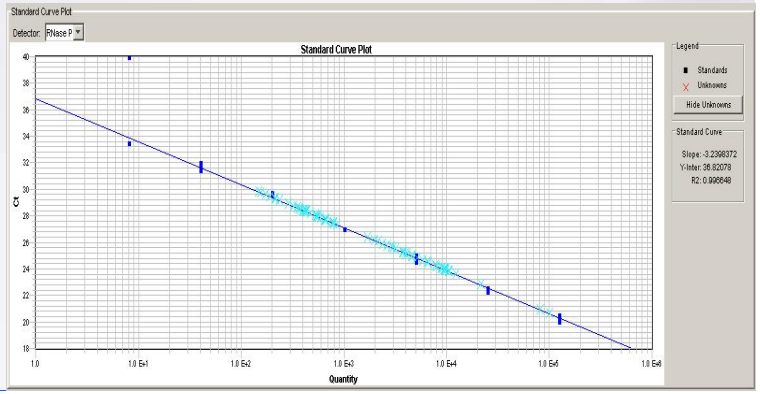
UNIFI Nanodrop quality and quantity of the extracted DNA
real-time PCR quality and quantity of a target gene
TATAA KINERET ® PCR interference
QIAGEN pulse elettrophoresis DNA integrity
IMMUNID Tested in a genetic assay for diagnostic purpose







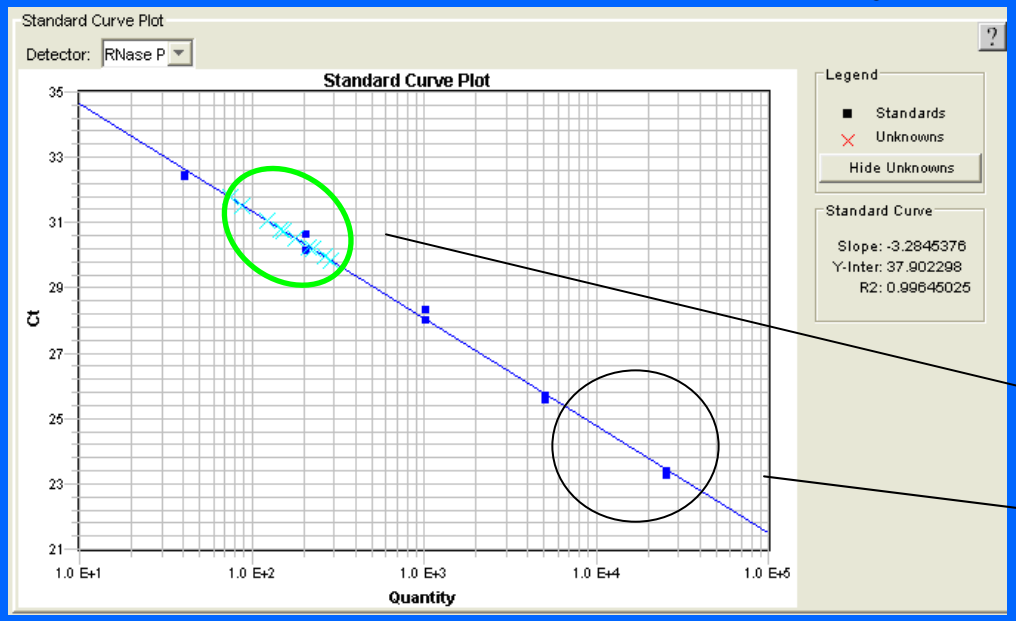




SPIDIA-DNAPlas

TAQMAN assay: DNA quantity evaluation

by RNase P assay with standard curve



Standard curve (DNA standard)

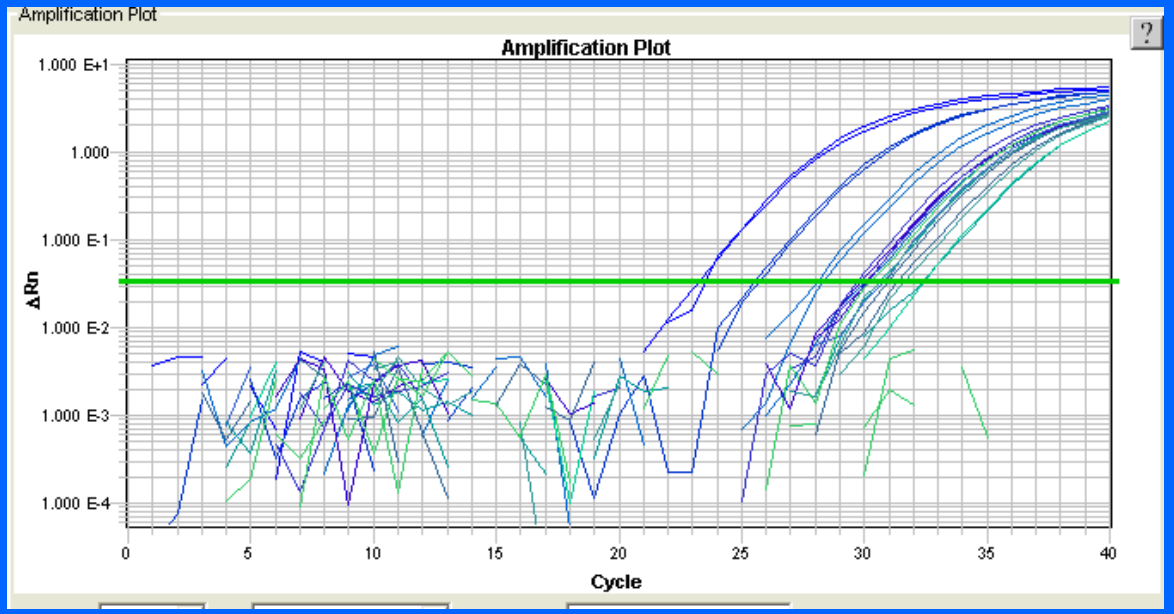
Plasma DNA LESS THAN 100 FOLD

Genomic DNA

Amplification Plot (DNA standard)

Quantity of a DNA target (Real Time for a single copy gene)

Interferences (efficiency of Real Time data evaluated by TATAA)

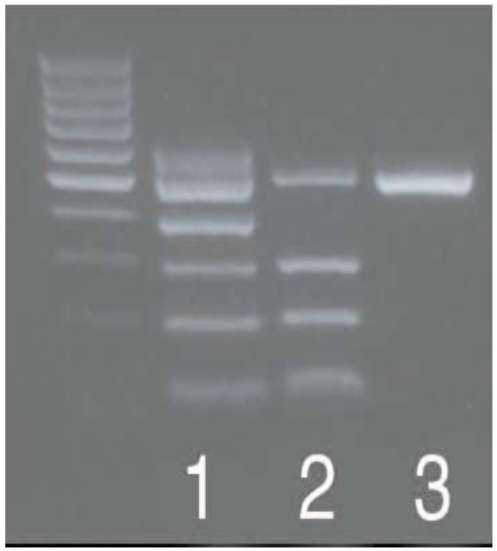


DNA INTEGRITY
(low MW; range 100-600 bp)

DNA Quality Ready Kit

The Isohelix DQC kit is a PCR reaction designed to check the quality and presence of the Human DNA before you start your full research or testing program. The DQC kit can also be used to provide comparative data against different types of DNA Isolation methods and the presence of PCR inhibitors.

- **Checks Human DNA Quality prior your Research.**
- **Use it to select the most efficient Isolation protocol.**

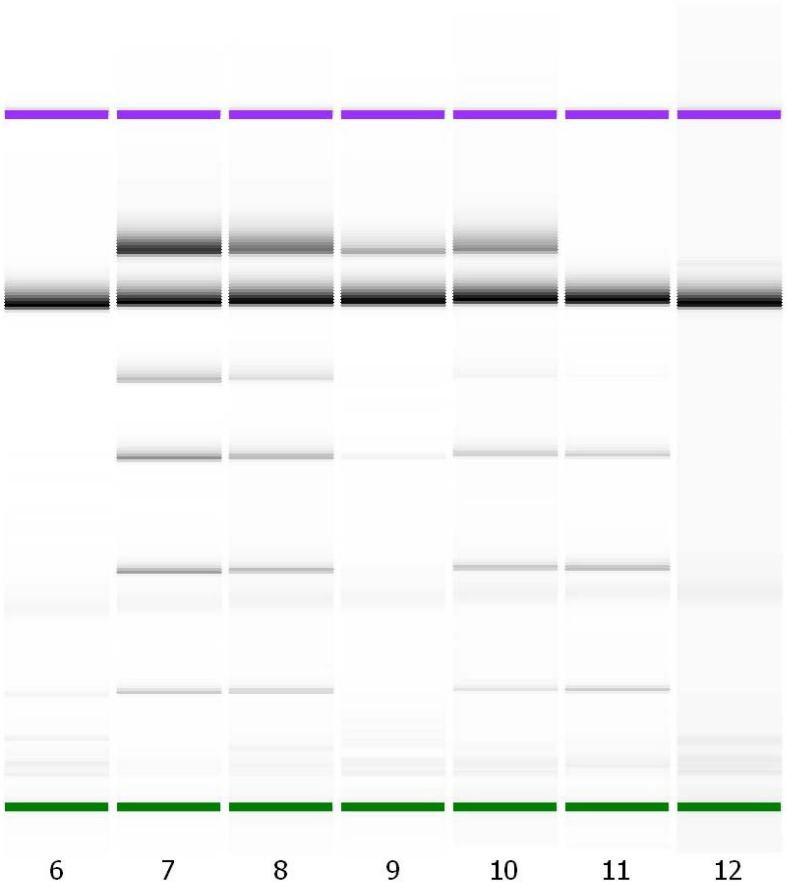


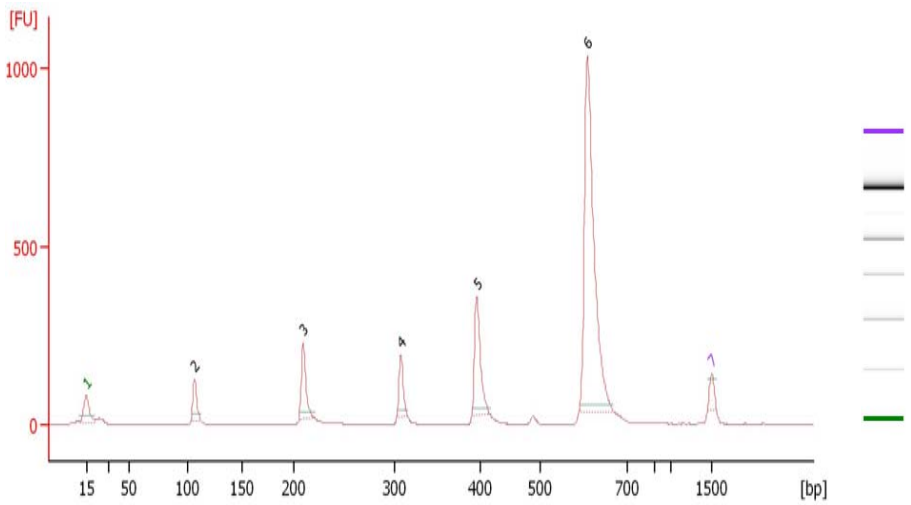
Isohelix

Lane 1	Acceptable results containing good quality DNA
Lane 2	Unacceptable the genomic DNA is visibly starting to degrade
Lane 3	Completely unacceptable only the positive control is present

integrity

Isohelix plus Agilent analysis





Whole blood DNA – sample 6

Peak [nmol/l]	Size [bp] Observations	Conc. [ng/µl]	Molarity
1	15	4.20 Lower Marker	424.2
2	107	4.05	57.4
3	210	6.47	46.7
4	308	3.97	19.5
5	396	9.65	36.9
6	609	41.57	103.4
7	1,500	2.10 Upper Marker	2.1

C. Analysis of DNA5

C.1. Evaluation of DNA integrity (ISOHELIX plus Agilent Analysis)

Analysis of bands concentration

LAB	vol_extrac_plas_ ul	vol_elu_plas_ ul	B1	B2	B3	B4	B5	B6	SUM*	N. of bands
1	1000	50	4.1	6.57	6.5	4.69	22.57	21.86	3.3145	6
2	200	50	3.13	3.96	3.47	1.79	19.1	13.13	11.1450	6
3	200	150	2.62	0.53	0.46		22.12	5.89	23.7150	5
5	400	60	1.7	3.31	2.38	0.51	20.7	10.36	5.8440	6
7	200	20	3.9	5.33	2.74	0.23	25.27		3.7470	5
8	100	20	1.75	0.4			18.84	0.31	4.2600	4
9	500	20	3.23	5.76	5.98	5.46	13.74	20.27	2.1776	6
10	350	50	3.99	5.69	5.56	2.45	16.22	14.43	6.9057	6
11	500	20	4.55	7.79	7.53	6.16	15.7	18.61	2.4136	6

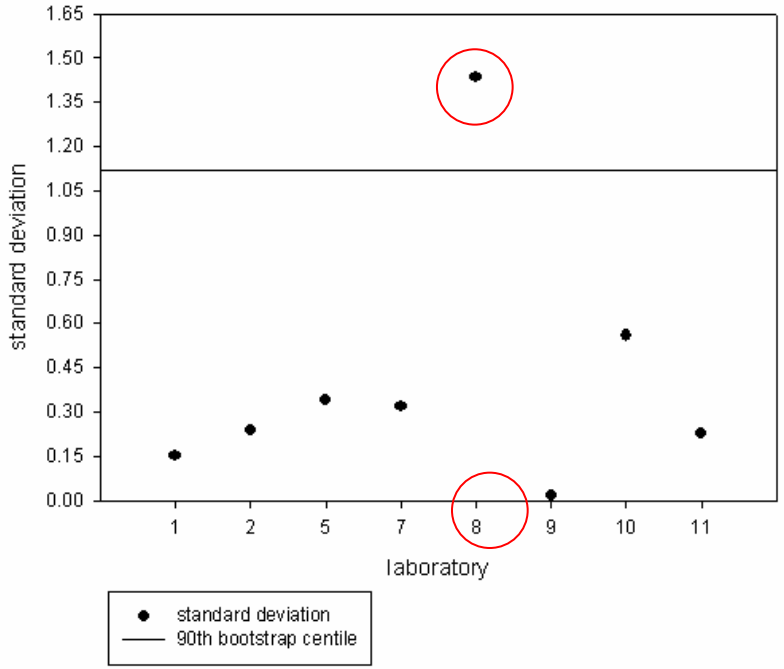
*sum of Bi = (Bi *vol_elu_plas_ul)/vol_extrac_plas_ul

C. Analysis of DNA5

C.2 Real Time PCR Analysis of DNA5

On DNA5 was performed an absolute quantification by real time PCR using a seven-point standard curve in triplicate.

Figure 13: Control chart of the standard deviation of the ct replicates for unknown samples



Notes: The horizontal continuous lines in Figure 13 correspond to the 90th bootstrap centile of the distribution of standard deviations of the ct replicates for the unknown sample.

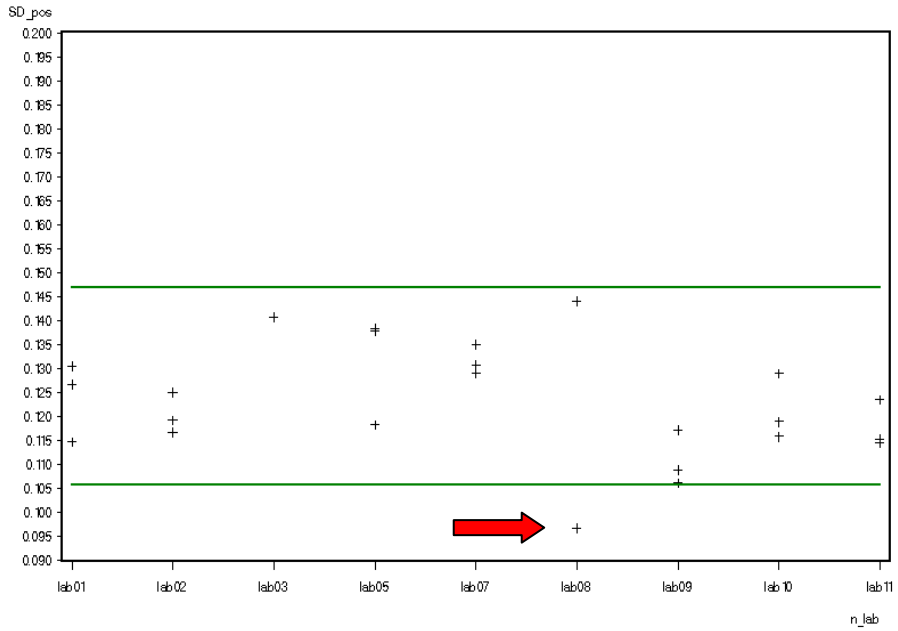
6 Lab 4 and 6 did not extract DNA from plasma sample and for lab 3 only 1 ct replicate is available.

C. Analysis of DNA5

C.3 PCR Efficiency and Interferences

Fluorescence data (absolute quantification by real time PCR using a seven-point standard curve) corresponding to three replicates of the DNA5 were obtained for each lab. These data were processed by Kineret software (www.kineretsoftware.com)

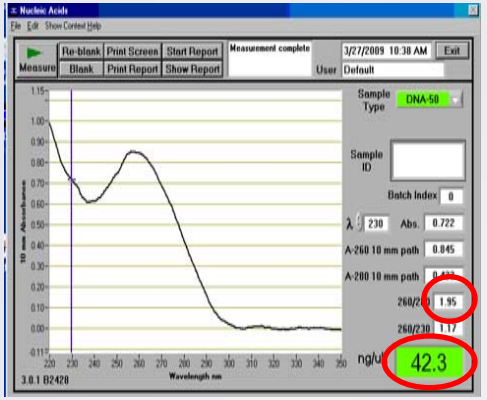
Figure 15: Efficiency (each participating lab versus standards).



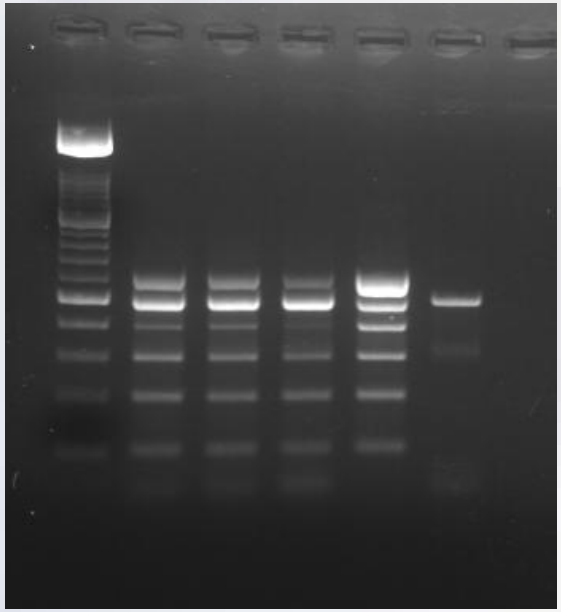
*Notes: Figure 15 reports the values of the second derivative in its positive maximum (SD_pos) of the fluorescence curve for all the available replicates. The two limit, represented by green lines, were obtained as: LQ/UQ±1.5*IQR where, LQ and UQ are respectively, the 25th centile and the 75th centile of the SD_pos distribution of the standards used to generate the standard curve for DNA5 quantification; IQR is the Interquartile range (75th centile – 25th centile) of the same distribution*.

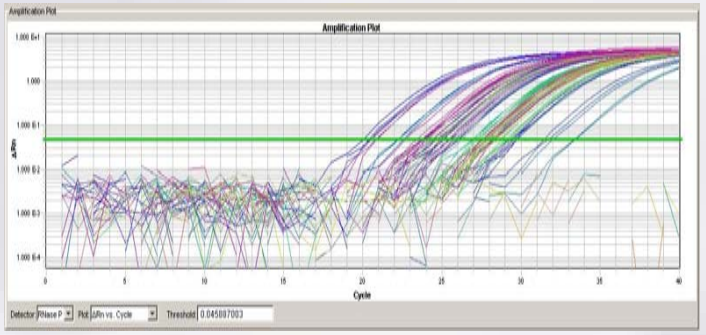
Analysis of "Plasma" DNA extracted by the participants:

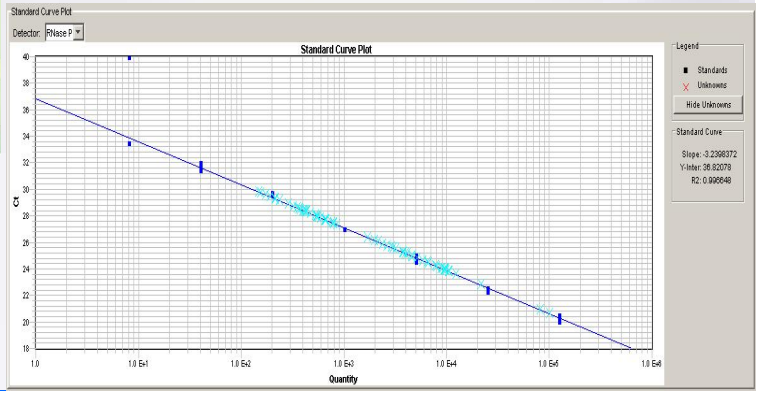
UNIFI Nanodrop quality and quantity of the extracted DNA with fluorescent dyes
real-time PCR quality and quantity of a target gene
TATAA KINERET ® PCR interference
UNIFI. ISOHELIX assay to test Low MW DNA integrity on DNA extracted from plasma samples



Isohelix DNA plasma







RT-PCR at 45 cycles

SPIDIA-RNA

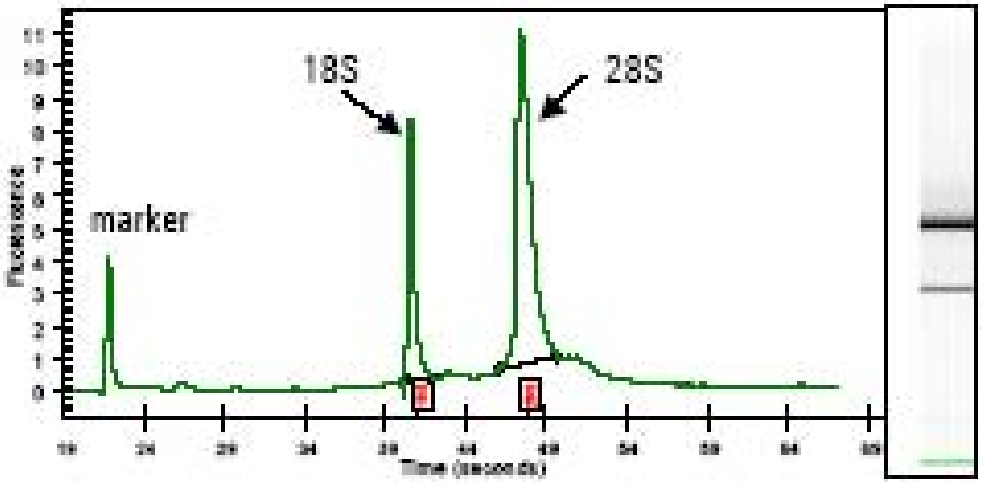
WHAT SPIDIA HAS PLANNED TO TEST IN THE EXTRACTED RNA FROM THE PARTICIPANTS?
(in order to contribute to monitor the performance of the pre-analytical phase for RNA analysis in whole blood)

1. REVISE THE UV ANALYSIS
2. EVALUATE RNA INTEGRITY
3. EVALUATE RNA STABILITY
3. REVEAL PRESENCE OF INTERFERENCES AFFECTING AMPLIFICATION ASSAYS

RNA degradation vs time can be monitored by electrophoresis

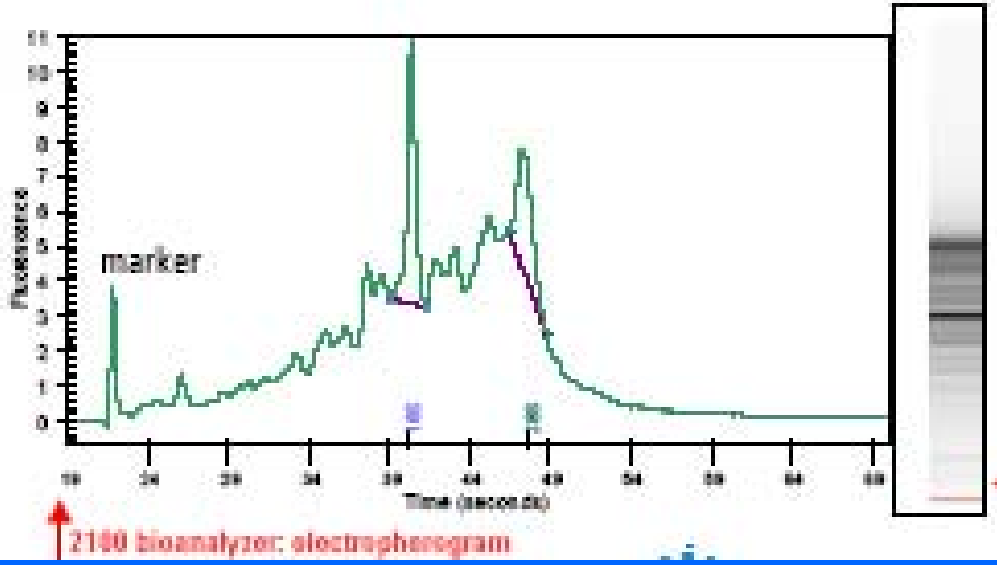
RNA LabChip kits

Analysis of Total RNA Integrity



Typical first QC step after RNA sample prep prior to microarrays or real-time PCR

High quality total RNA

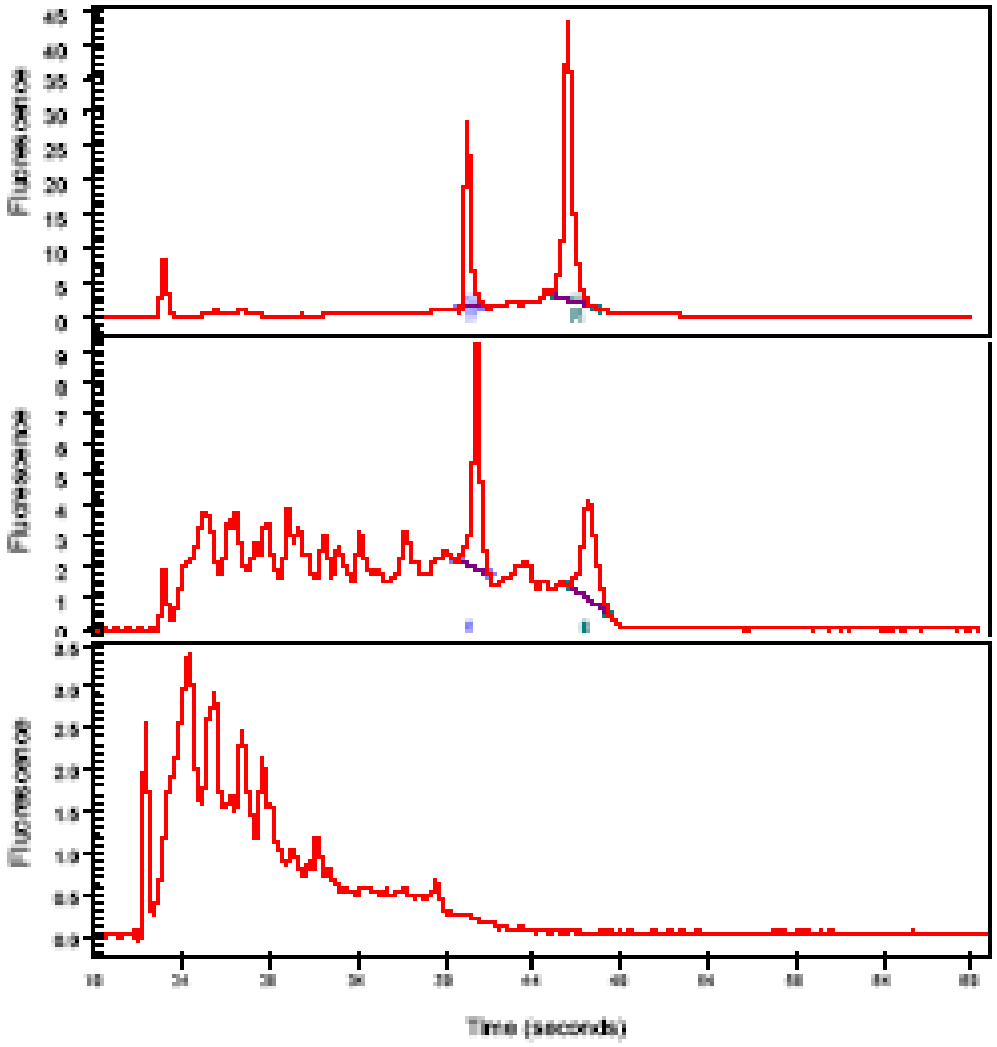


Partially degraded total RNA

2100 bioanalyzer: single lane gel-like image

RIN analysis (Agilent) monitoring of the RNA degradation

RIN Application – Assessment of RNA Integrity



Intact RNA: RIN 10

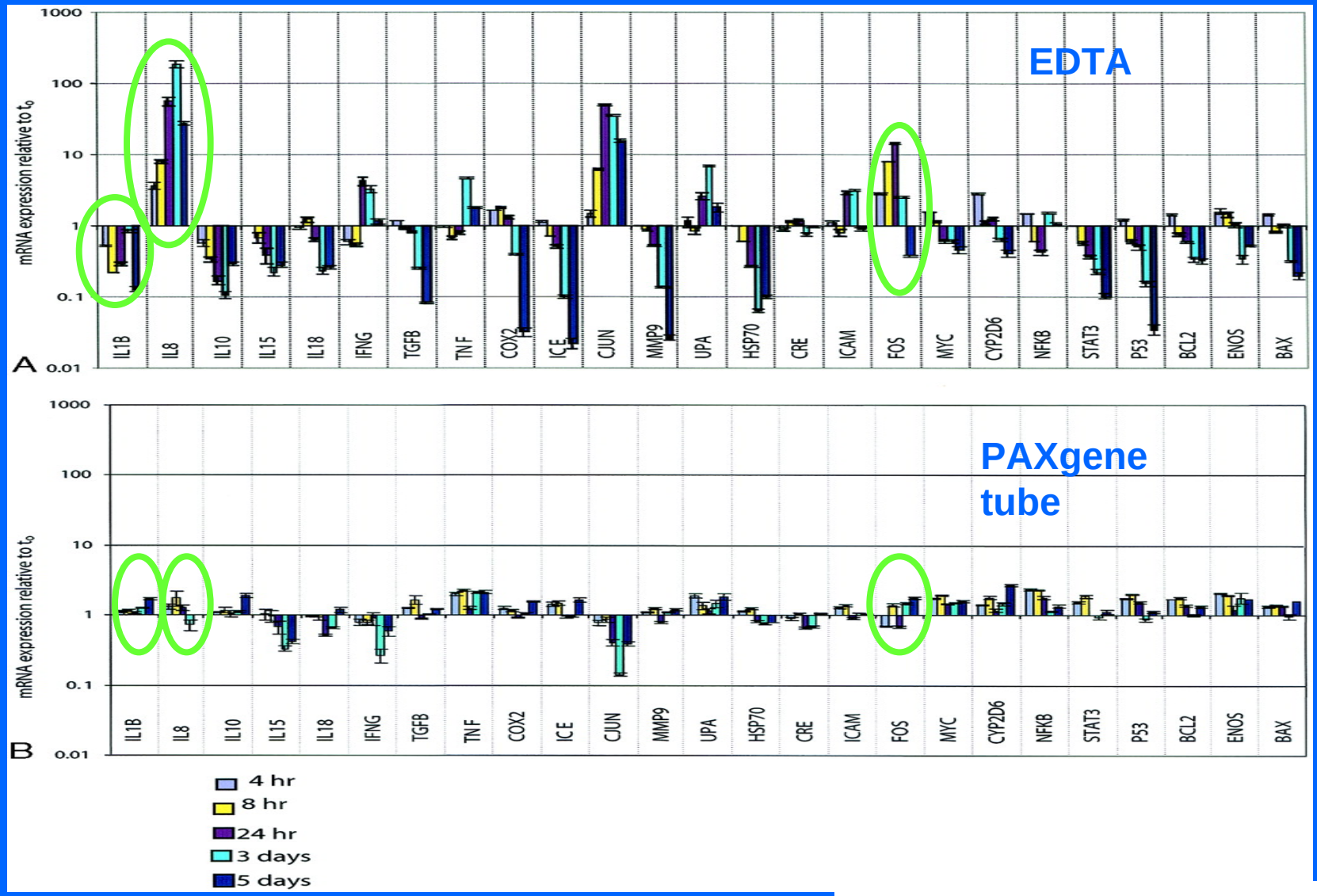
Partially degraded RNA: RIN 5

Strongly Degraded RNA: RIN 3

HOWEVER **OTHER SPEFICIC FACTORS** CAN ALSO AFFECT mRNA EXPRESSION IN BLOOD VS. TIME EVENTUALLY INDUCING OVEREXPRESSION OR DOWN REGULATION ONLY ON SPECIFIC TARGETS

RNA expression

Effect of storage conditions on specific mRNA expression



Rainen, L. et al. Clin Chem 2002;48:1883-1890

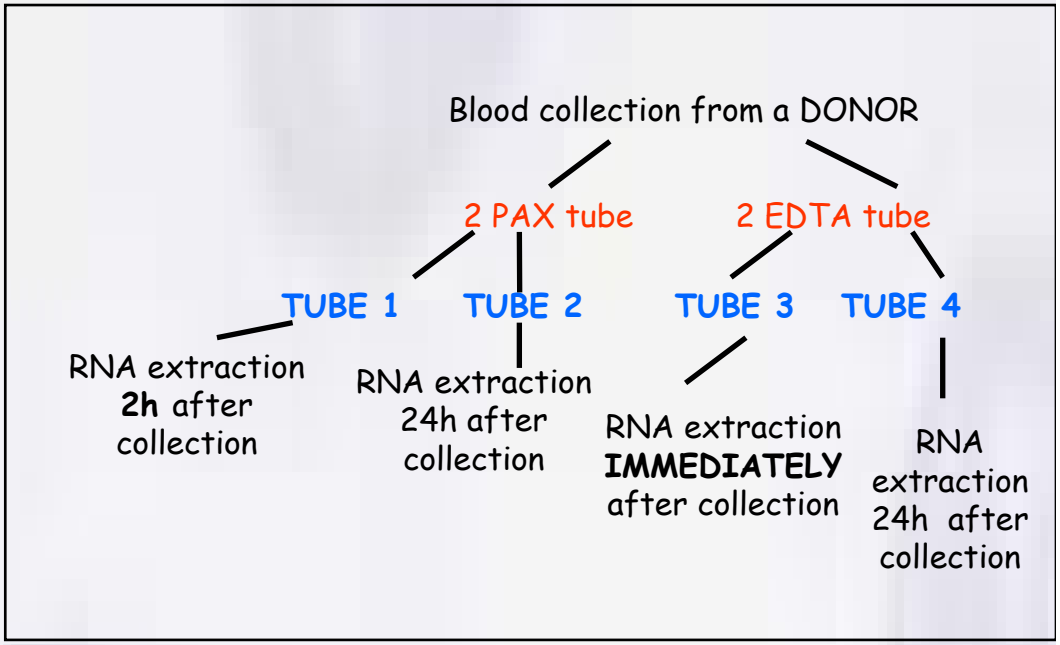
Clinical Chemistry

2)

SPIDIA- RNA pilot study

The labs participating in the pilot study had to supply blood samples obtained from a DONOR, two in a PAXgene tube (tube 1 and tube 2) and two in EDTA tube (tube 3 and tube 4).

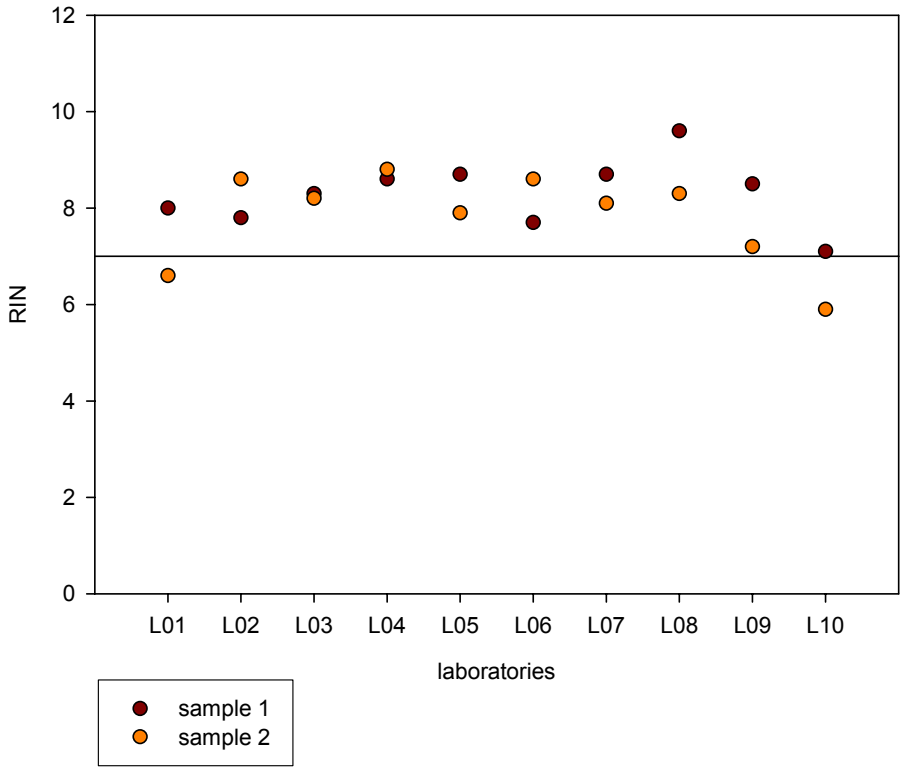
Tube 1 PAXGene tube extraction 2 hrs after the collection
Tube 2 PAXGene tube extraction 24h after collection
Tube 3 EDTA tube extraction immediately after the collection
Tube 4 EDTA Tube extraction after 24h after collection



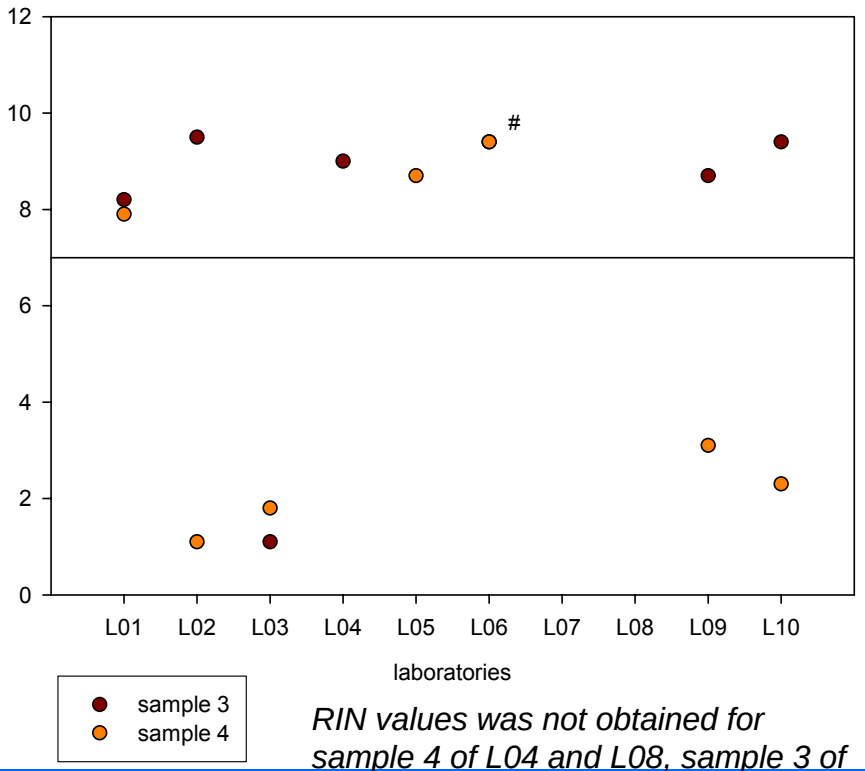
Storage/extraction intervals (after 24 h) - lab-specific donor

Evaluation RNA integrity (RIN) – Pilot study: 10 labs

Evaluation of RNA integrity (RIN) -PAXgene time comparison



Evaluation of RNA integrity (RIN) - EDTA time comparison

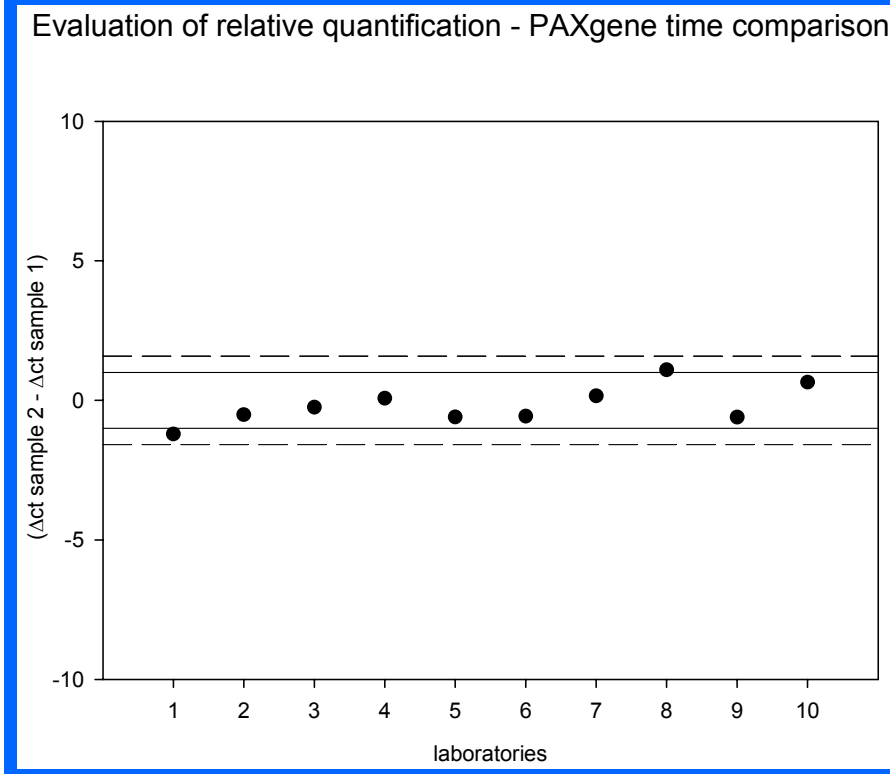


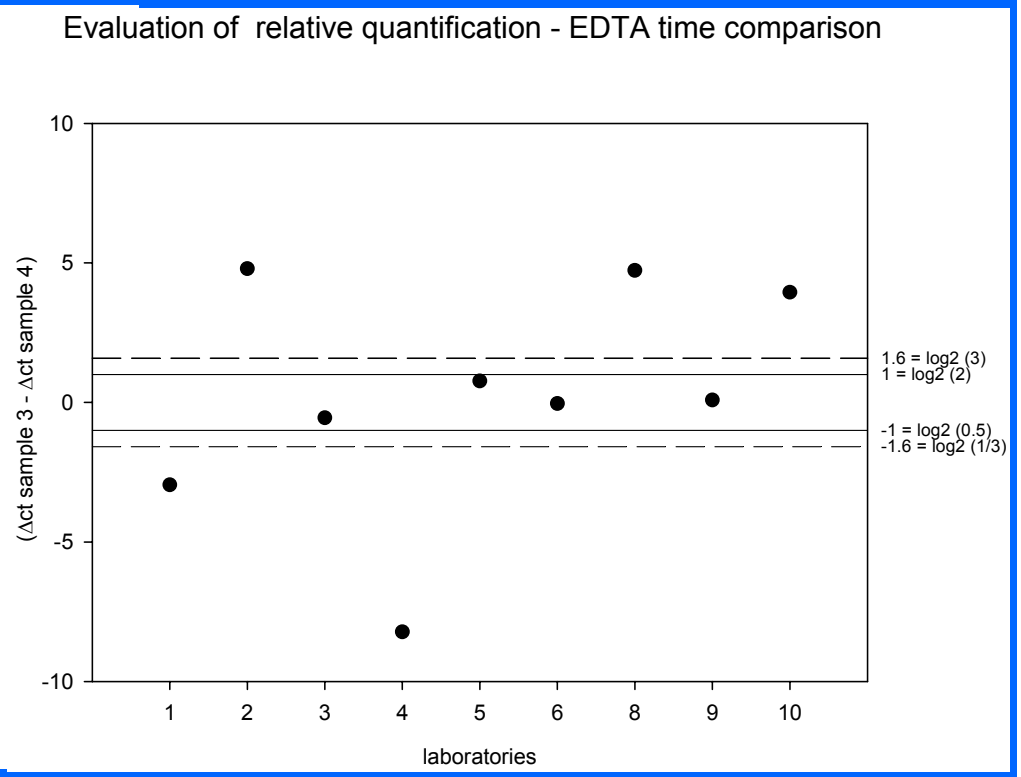
RIN values was not obtained for sample 4 of L04 and L08, sample 3 of L05 and L08

L07 did not sent RNA extracted from Tube B, Tube 3 and Tube 4

Storage/extraction intervals (after 24 h) - lab-specific donor

Evaluation of the relative quantification – real-time PCR data



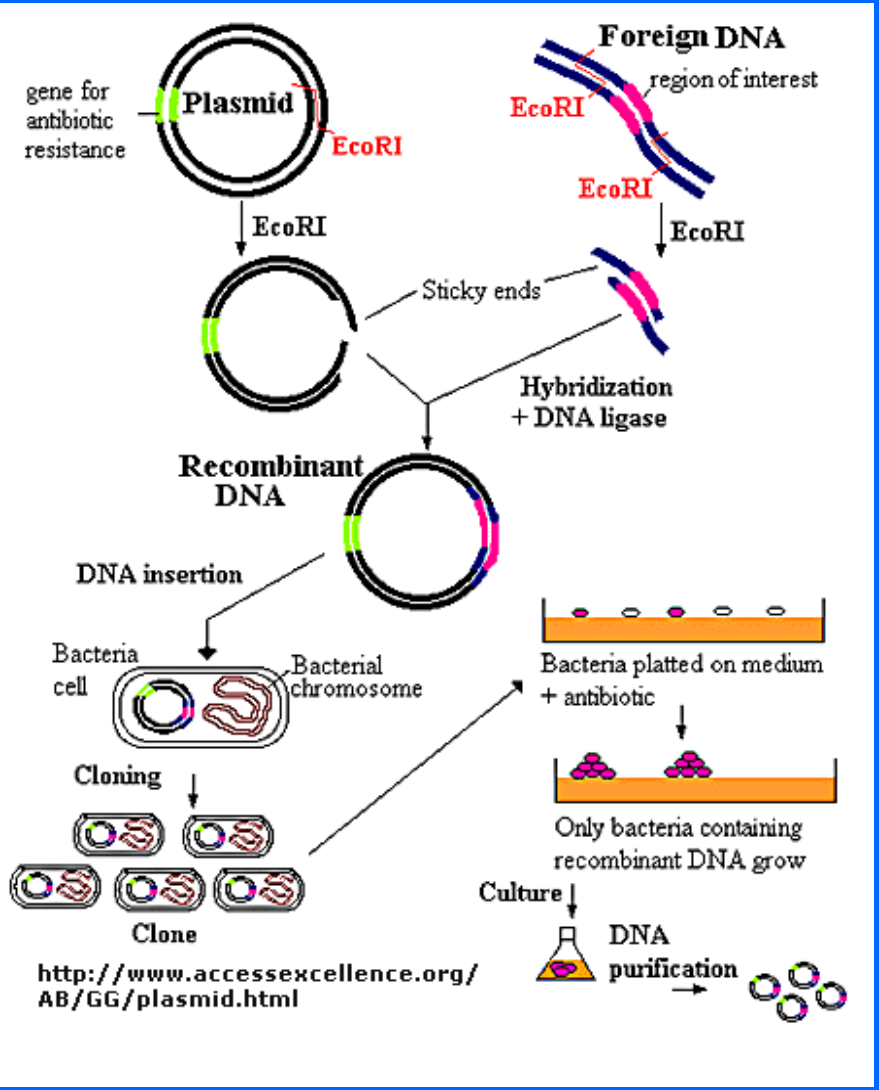


PLANNED SPIDIA-RNA TRIAL

Quantitative evaluation by Real Time RT-PCR of the following genes:

- ✓ **GAPDH** – housekeeping gene for relative measurements
- ✓ **IL1β** - relatively stable or down regulated in blood vs. time
- ✓ **IL8** – unstable and overexpressed vs. time – high variability
- ✓**C-fos** unstable and overexpressed vs. time – medium variability

TO MONITOR DIFFERENCES IN GENE EXPRESSION IS IMPORTANT TO IMPROVE THE ASSAY STANDARDIZATION

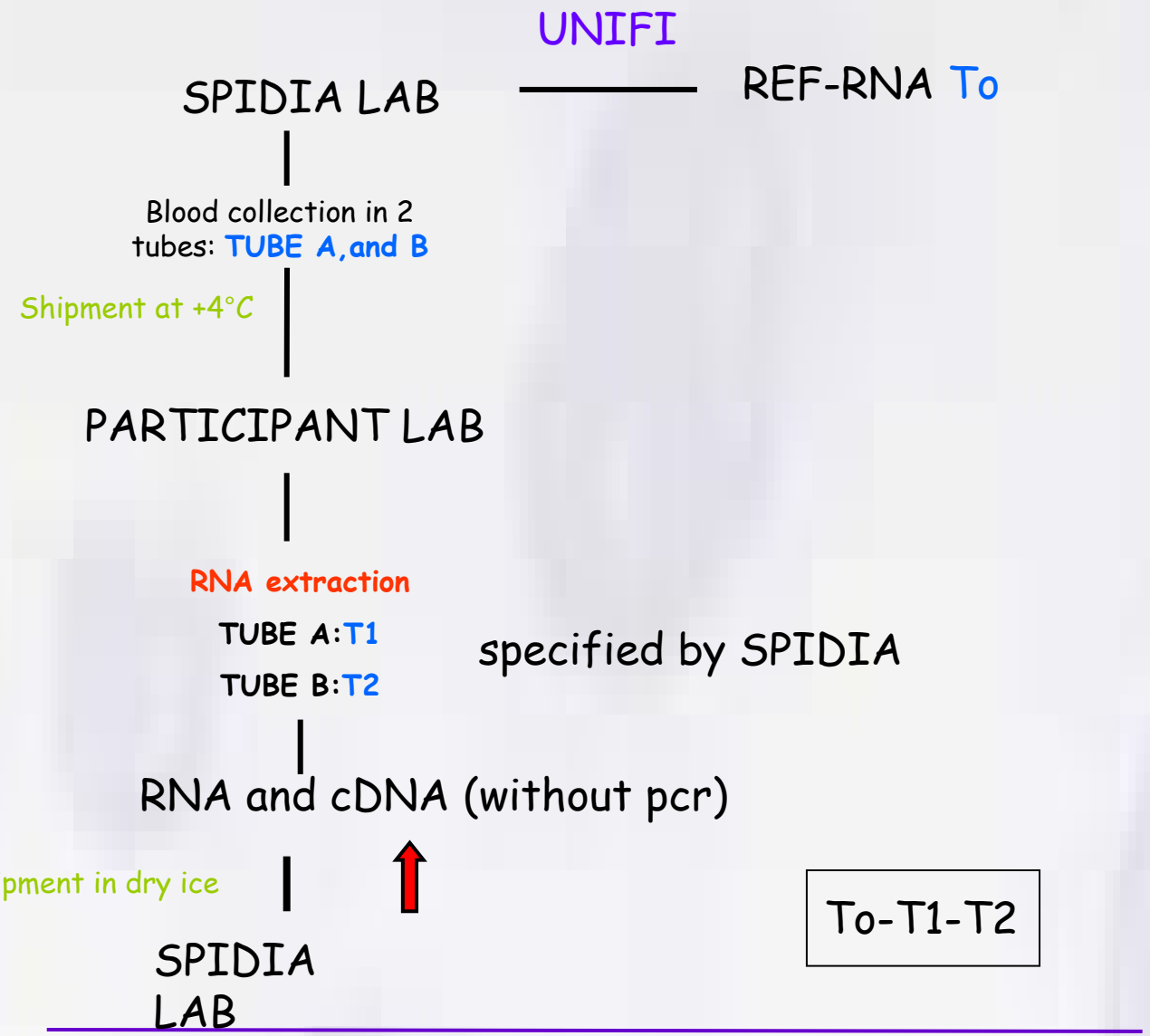


Target sequences for GAPDH, **IL1**β, IL8 and C-FOS genes have been cloned in a specific plasmid, in order to to have a reproducible and unique standard and thus to perform absolute/relative and reproducible measurements of these mRNAs by real-time PCR technique during the study

SPIDIA- RNA ring trial

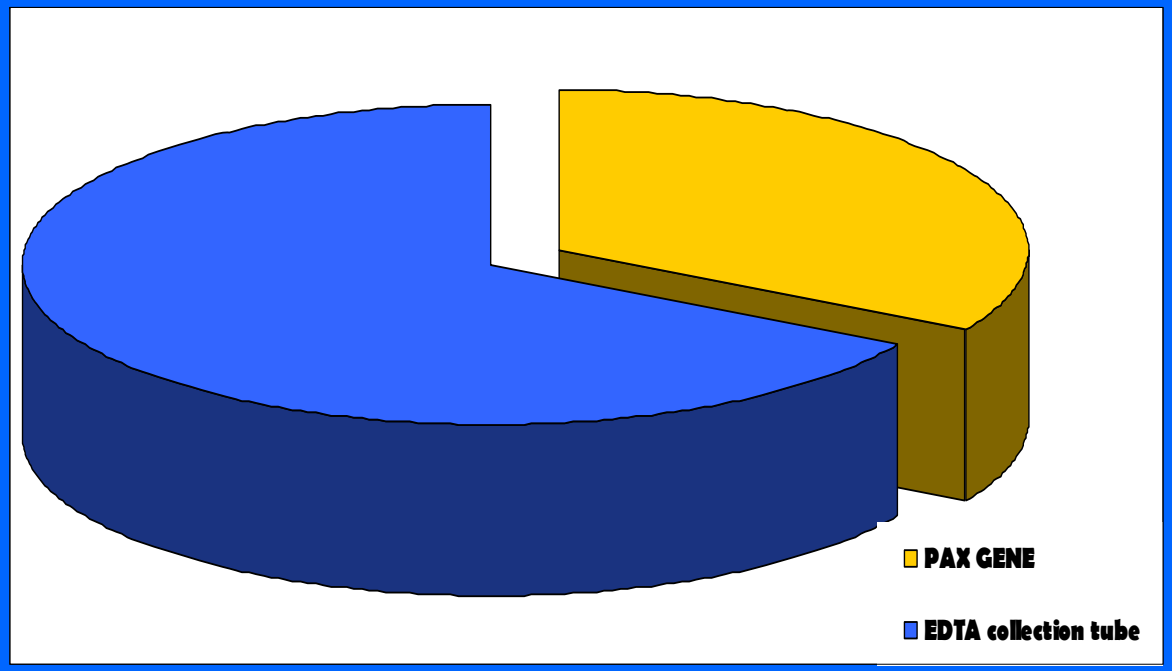
JANUARY 2009

Questionnaire:
Which kind of tube do you use for RNA Assay in Blood?



	RNA	PAX	EDTA
Austria	3	1	2
Belgium	9		9
Bulgaria	1	1	
Croatia	4		4
Czech Republic	4		4
Denmark	1		1
Estonia	1		1
Finland	3	1	2
France	9	1	7
Germany	13	10	2
Greece	3	1	2
Hungary	2		2
Ireland	0		
Israel	4	1	3
Italy	27	10	15
Latvia	0		
Lithuania	0		
Norway	2	1	1
other countries	2		2
Poland	0		
Portugal	3	2	1
Romania	4		4
Slovenia	1		1
Spain	2		2
Sweden	6	4	2
Switzerland	1	1	
The Netherlands	1	1	
Turkey	6	2	4
United Kingdom	12	1	10
TOT	124	38	82

Distribution of RNA applications on the basis of the selected protocol relative to blood collection



SEVENTH FRAMEWORK PROGRAMME

SPIDIA

Preliminary considerations from the SPIDIA-DNAs pilot studies

- Most of the Clinical labs do not have the possibility to monitor “in deep” the performance of the pre-analytical phase (Importance of SPIDIA)
- Additional analysis, in the extracted DNA, can reveal “hidden” unsatisfactory performance of the pre-analytical phase.
- The impact of these “unsatisfactory performances” of the pre-analytical phase remains to be clearly elucidated but they seem to be relevant depending on the kind of Molecular methods in which the extracted DNA will be used.

Preliminary considerations from the SPIDIA-RNA pilot studies

- The “Instability” of the RNA in Blood is challenging when planning a ring trial.
- The RIN value is not sufficient for the evaluation of the performance of the pre-analytical phase.
- Additional specific markers should be tested in order to test RNA stability.
- These additional markers should be identified (spidia wp1.3) and “accepted” by the scientific community.
- The efficiency of the RNA Retro transcription needs to be investigated and it should be included as part of the monitoring of the pre-analytical phase.

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