ANNEXE 6 f

Résultats test éducationnel pour le test EPO WADA

Sandra Ferary

De: Analyses [analyses@afld.fr]
Envoyé: lundi 17 décembre 2007 16:21
À: Adeline Molina; Sandra Ferary; Francoise Lasne; Nathalie Crépin; Esther Cerpolini; Nathalie Mechin
Objet: [Fwd: Reports on the WADA educational PT tests (urinary EPO and finasteride)]

Pièces jointes: analyses.vcf

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-------- Message original --------
Sujet: Reports on the WADA educational PT tests (urinary EPO and finasteride)
Date: Mon, 17 Dec 2007 10:25:11 -0500
De: Maziar, Violet <Violet.Maziar@wada-ama.org>
Pour :: <analyses@afld.fr>

Dear Laboratory Director,

This message is to inform you that the WADA final reports on finasteride and urinary EPO educational tests were sent to your laboratory on December 17-2007 by courier. We would appreciate if you handle these reports with the utmost confidentiality*.*

Please note that in this educational PT test your laboratory is coded as laboratory* 28. *

Should you have any comments or questions regarding this educational test, please do not hesitate to contact me.

On behalf of the WADA Science Department, we would like to wish you a Happy Holiday Season and all the best for 2008.

Kind regards,

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Final Report

WADA Educational EPO test

The WADA Educational Program is designed to assist the WADA-accredited laboratories and laboratories in probationary phase of WADA accreditation to harmonize the identification and reporting of substances newly added to the WADA Prohibited List. The program also seeks to improve the analytical techniques of the laboratories in order to maintain state-of-the-art standards and thus remain at the forefront of the global fight against doping in sport.

The WADA Educational Program is considered to be of particular help to laboratories that have newly implemented method(s) and want to compare their own data with other participants. The purpose of this program is purely educational; therefore the results of the WADA educational proficiency tests are not taken into account in evaluation of laboratory performance in the WADA PT program.

As part of the WADA educational test, during the week of December 11, 2006 three (3) urine EPO samples were distributed to the twenty four (24) WADA-accredited laboratories which indicated their capability to perform the urinary EPO tests.

The laboratories were asked to submit the results of this educational test by January 31, 2007. There were some late submissions with the last results being received on March 30, 2007. WADA made several follow-up enquiries with some laboratories in order to collect the missing data or clarify the provided information.

Urinary EPO (WADA EDUC 007, 008, 009)

Sample preparation

Three urine EPO samples were prepared by the WADA PT sample provider and stored at -20°C ± 4°C. The samples were analyzed for the presence of EPO prior to their release to the laboratories.

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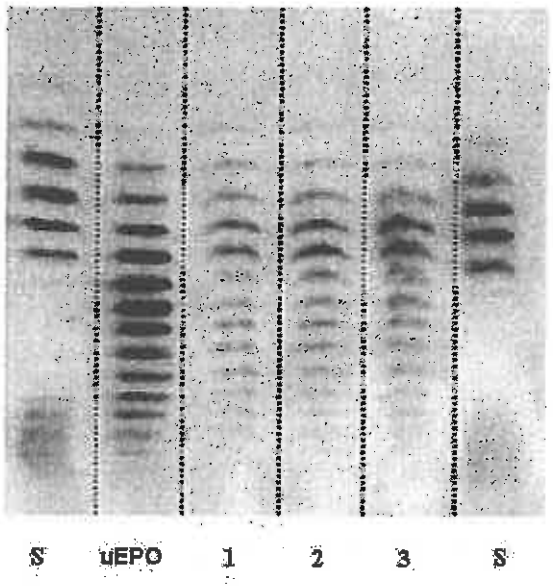
WADA EDUC 007 ("Borderline sample").

WADA EDUC 007 sample was prepared following subcutaneous injection of three consecutive doses (50 IU/kg) of Epoetin beta (commercial name: EPO Beta NeoRecormon®) to two volunteers every 48 hours.

The electropherograms of the standard solution (**S**, containing both rEPO 1800 IU/L and NESP 6ng/mL), urinary EPO (solution of **uEPO** 3 IU/mL) and three replicates of WADA EDUC 007 are shown below:



S - Standard solution (rEPO 1800mIU + NESP 6 ng/mL)
uEPO - Solution of uEPO (3 IU/mL)

The sample was evaluated by the WADA PT sample provider in accordance with the WADA Technical document TD2004EPO. The results of all three replicates fulfilled the acceptance, identification and stability criteria described in the TD2004EPO and therefore were reported as an adverse analytical finding for recombinant EPO.

1. Adverse analytical finding for rEPO;
2. Adverse analytical finding for rEPO;
3. Adverse analytical finding for rEPO.

It should be emphasized that for educational purposes, the WADA EDUC 007 sample was deliberately prepared as a "borderline sample", therefore, in some cases the results of this sample may have been evaluated as negative.

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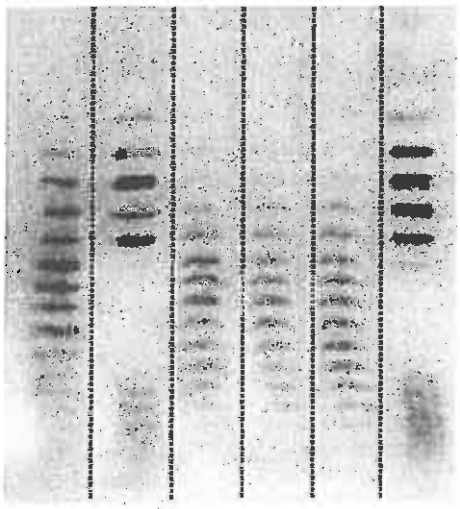
WADA EDUC 008 (endogenous EPO)

WADA EDUC 008 was a blank urine containing endogenous EPO. The electropherograms of the urinary EPO (solution of **uEPO** 3 IU/mL), standard solution (**S** containing both rEPO 1800 IU/L and NESP 6ng/mL) and three replicates of WADA EDUC 008 are shown below:



S - Standard solution (rEPO 1800mIU + NESP 6 ng/mL)
uEPO - Solution of uEPO (3 IU/mL)

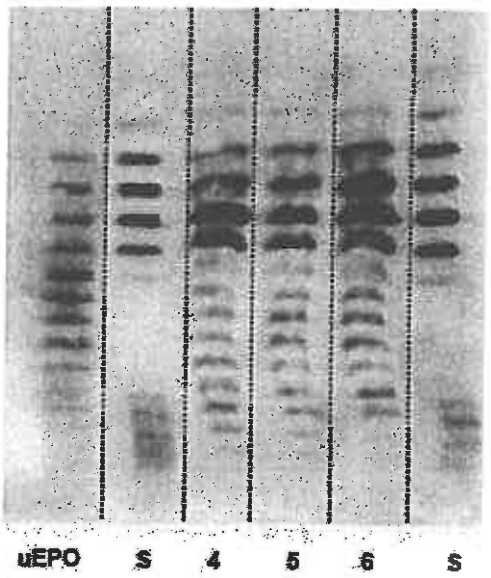
The sample was evaluated in accordance with WADA TD2004EPO. All three replicates were found to be negative for the presence of rEPO by the WADA PT sample provider:

1. Negative for the presence of rEPO;
2. Negative for the presence of rEPO;
3. Negative for the presence of rEPO.

WADA EDUC 009 (recombinant EPO)

WADA EDUC 009 sample was obtained following administration of rEPO to two (2) healthy volunteers. Three consecutive doses (50 IU/kg) of Epoetin alfa (commercial name: Epogen®) were injected subcutaneously every 48 hours.

The electropherograms of the standard solution (**S** containing both rEPO 1800 IU/L and NESP 6ng/mL), urinary EPO (solution of **uEPO** 3 IU/mL) and three replicates of WADA EDUC 009 are shown below:



S - Standard solution (rEPO 1800mIU + NESP 6 ng/mL)
uEPO - Solution of uEPO (3 IU/mL)

The sample was evaluated in accordance with WADA TD2004EPO, based on which all three replicates were interpreted as meeting all criteria to report as an adverse analytical finding for recombinant EPO :

4. Adverse analytical finding for rEPO;
5. Adverse analytical finding for rEPO;
6. Adverse analytical finding for rEPO.

Receipt of Samples

A strong indication of degradation was reported for samples WADA EDUC 007, 008 and 009 by laboratory 34.

Laboratory results

All the participating laboratories analysed and evaluated WADA EDUC 007, 008 and 009 samples by applying the acceptance, identification and stability criteria described in the WADA technical document "Harmonization of the Method for the Identification of Epoetin Alfa and Beta (EPO) and Darbepoetin Alfa (NESP) by IEF-Double Blotting and Chemiluminescent Detection" (WADA TD2004EPO).

Quality of the electropherograms

The quality of the electropherograms varied widely among the laboratories. The gels provided by laboratories 4, 5, 12, 23, 25 and 28 are considered to be of good quality (showing clear band separation, good band intensity and linearity). Gels judged of average quality were produced by laboratories 1, 7, 8, 9, 13, 17, 18, 24, 30, 31, 32 and 34 (the quality of gels needs improvement in order to achieve acceptable band separation). The gels submitted by laboratories 2, 3, 11, 15, 26 and 33 are considered of poor quality and justify a strong recommendation for thorough review of experimental procedure (see Appendix A: Electropherograms).

Due to gel overload, laboratories 4, 12, 23 and 33 had to repeat the assay by applying a lower volume (or lower concentration) of retentate thus achieving well defined bands, and consequently, acceptable gels. In this connection, it is recommended that the protein content in the sample is measured prior to analysis (before applying the sample to the gel) in order to minimize the possibility of protein overload on the gels.

The majority of the laboratories assessed band intensity by performing a densitometric analysis. Twelve (12) laboratories (2, 3, 4, 5, 11, 12, 13, 17, 23, 24, 30 and 32) utilized the GASepo software to analyse the gel images. Laboratories 15 and 28 evaluated the gel images by using the AIDA Image Analyzer software.

Some laboratories used two different software packages for the analysis of the images. Laboratory 11, for example, assessed the gel images by using both the standard laboratory software package, Gauge (Fuji), and utilized the GASepo software for cross checking the results. Laboratory 13 also used the MultiGauge (v3.0) software in addition to the GASepo.

Two different types of software, GASepo and AIDA, were used by laboratories 5 and 23. Laboratory 3 performed a cytometric analysis and assessed the gel images by using the GASepo software.

Several laboratories did not indicate the type of software used in the assessment of the gel images.

Acceptance criteria

WADA TD2004EPO defines the requisite acceptance criteria that the image shall fulfil prior to applying the identification criteria necessary to determine the presence of rEPO or NESP.

1. Spots, smears, areas of excessive background or absent signal in a lane that significantly interferes with the application of the identification criteria shall invalidate the lane.
2. Comparison to reference samples shall allow assignment of band numbers in the athlete's sample.

The acceptance criteria for the educational urine EPO samples (EDUC 007, 008 and 009) were fulfilled by a majority of the participating laboratories.

Some laboratories, however, commented on the quality of their gel images. Laboratory 4, for example, noted the presence of an excessive spot in the area of rEPO which interfered with the ability to meet the acceptance criteria.

Laboratory 11 reported that the poor quality of the gels caused issues in assessing the results. The laboratory emphasized, however, that the poor quality of the gels is a recent issue which is currently being investigated by the laboratory.

For the confirmation analysis of sample EDUC 007, laboratory 13 was not able to evaluate the profile properly due to the absence of signal in the lane.

As a general comment, laboratory 23 indicated that the sample profiles had broad bands thus spreading the signal over to the neighbouring lanes and consequently causing difficulties in the densitometric calculations. This laboratory had to repeat the screening procedure of sample EDUC 009 as well as utilize an additional clean-up of the sample in order to meet the acceptance criteria of WADA TD2004EPO (see Laboratory Comments for more details).

In the case of laboratory 24, the identification criteria were not met for 2 samples: EDUC 007 and EDUC 009. It should be noted, however, that laboratory 24 reported that the samples were stored refrigerated rather than frozen (see Laboratory Comments). According to the laboratory, this may explain the difference between screening and confirmation results, as well as the quality of the EPO bands, due to the possible degradation of EPO in the samples.

A number of reasons were noted by laboratory 26 (samples were not frozen on arrival, variability of the reagent suppliers (especially antibodies) and camera resolution) as possible explanations for the laboratory's poor gel quality.

Some laboratories, though identifying the presence of spots or smears on the gel, considered that the gel images were valid for evaluation.

Identification criteria

WADA EDUC 007:
Sixteen (16) participating laboratories confirmed and reported the presence of recombinant EPO in the sample (see Table 1).

Based on the screening procedure, laboratory 2 reported a suspicious result, however, did not proceed to the confirmation claiming that "the sample was not meeting the WADA criteria". Laboratory 4 also reported the sample as suspicious (ID criteria were not met) and recommended further target testing.

Six laboratories (8, 9, 11, 13, 24 and 31) reported the WADA EDUC 007 sample as negative. According to laboratories 11 and 13, the gel image acceptance criteria had not been fulfilled. Further, the sample was reported as negative by laboratories 8, 9, 24 and 31 since the identification criteria were not met (see Table 1 and Laboratory comments for more details). Laboratory 24 commented that the sample volume was not sufficient to repeat the confirmation analysis.

Laboratories 5 and 23, using two different types of software for assessing the gel image, noticed that the results of the densitometric analysis varied with the software application. Laboratory 5 specified that the sample had to be reported negative with GASepo software, but would have been considered positive with AIDA software. Laboratory 23 mentioned that sample evaluation by the AIDA-algorithm gave a ratio (the second most intense band in basic area to the most intense band in endogenous area) slightly higher compared to the GASepo software (versions 1.7 and 2.0 respectively)

WADA EDUC 008:
Twenty one (21) out of twenty four (24) laboratories reported the WADA EDUC 008 sample as negative for recombinant EPO. Laboratory 1 reported an inconclusive result for the EDUC 008. Laboratory 15 considered its result for sample EDUC 008 as not valid due to the fact that the identification criteria were not met.
Laboratory 26 interpreted sample EDUC 008 as containing a positive profile.

WADA EDUC 009:
Twenty (20) participating laboratories detected and reported the presence of rEPO in WADA EDUC 009.

Even though the sample was found to contain rEPO, laboratory 11 did not perform the confirmation analysis due to insufficient sample volume and some technical difficulties encountered by the laboratory.

Laboratory 24 evaluated the sample as negative since the acceptance criteria were not met.

Laboratories 32 and 34, although suspecting the presence of rEPO based on the screening, reported the sample as negative since the identification criteria were not fulfilled in the confirmation procedure (see Table 1 and Laboratory Comments).

Stability criteria

The stability test was performed by all participating laboratories. All WADA educational urine EPO samples (EDUC 007, 008 and 009) were found to have met the stability criteria as described in the WADA TD2004EPO.

Conclusion

All laboratories correctly applied the acceptance, identification and stability criteria as described in the WADA TD2004EPO, but a few laboratories were unable to identify the presence of rEPO. In particular, this was the case for the borderline sample. In addition one false positive as well as several false negatives were reported in the negative and positive samples, respectively (see Table 2). In some cases, the misidentifications were a consequence of technical problems, e.g. insufficient sample volume, protein overload, storage conditions, poor gels, all of which are considered issues that can be resolved. Some laboratories correctly pointed out that a second opinion would have been sought for true anti-doping control samples or that they would recommend that the testing authority perform follow-up tests.

Based on these results, it is evident that some laboratories need to review their procedures in order to improve the quality of their gels and enable the proper interpretation of results.

Table 1

Results of the WADA urinary educational EPO test (as reported by laboratories)

Lab code	WADA EDUC 007		WADA EDUC 008		WADA EDUC 009	
	Screening	Confirmation	Screening	Confirmation	Screening	Confirmation
1	rEPO	rEPO	Inconclusive results (opinion for testing authority provided)	Inconclusive results (opinion for testing authority provided)	rEPO (opinion for testing authority provided)	rEPO (opinion for testing authority provided)
2	Some suspicius results, but not meet WADA criteria so this sample was analysed neither for active test nor for confirmation.	Not performed	Negative		rEPO	rEPO
3	Positive --> Stability Test	rEPO -α or -β	Negative--> No further testing.		Positive --> Stability Test	rEPO -α or -β
4	Suspicious for the presence of rEPO, confirmation analysis and stability test to be performed	Due to the fact that the differences between the highest peak in the basic area and the peak in the endogenous area are not higher than twice, the sample would be considered as suspicious for the presence of rEPO, but not as an adverse analytical finding. Therefore, further target testing would be recommended	Pattern similar to endogenous EPO	Pattern similar to endogenous EPO	Failure due to inhomogeneity of the retentate, test to be repeated	rEPO
5	Synthetic erythropoietin cannot be excluded	rEPO	No synthetic erythropoietin found, no further action		Synthetic erythropoietin cannot be excluded	rEPO
7	Recombinant EPO was found to be present in this sample.	rEPO	Recombinant EPO is absent in this sample.		Recombinant EPO was found to be present in this sample.	rEPO
8	Sample 007 showed 3 acceptabel consecutive bands in the recombinant area. Two of these bands had higher intensity that the most intense bands in the endogenous area. It was decided to perform a second analysis using the confirmation protocol.	Following the WADA criteria, sample did not comply the identification criteria number 3. The profile observed could be compatible with a borderline case. The sample would be reported as negative, with additional comment recommending a follow up of the athlete	The sample would be reported as negative.		The profile obtained showed a higher intensity of the recombinant bands and fulfilled all identification criteria described in WADA document. Following our SOP, the sample was analysed using the confirmation protocol.	rEPO

Lab code	WADA EDUC 007		WADA EDUC 008		WADA EDUC 009	
	Screening	Confirmation	Screening	Confirmation	Screening	Confirmation
9	Presence of exogenous EPO. The results need to be confirmed.	EDUC 007 is turned out to be negative because it does not meet the criteria (the 2 most intense bands exist not only in basic area but also in endogenous area)	Negative		Presence of exogenous EPO. The results need to be confirmed.	rEPO
11	Most likely contains endogenous EPO, with a high basic distribution . Analysis was repeated (findings are consistent with initial analysis).	Not performed	Endogenous EPO. Analysis was repeated (findings are consistent with initial analysis).		rEPO	Required, but not performed. Insufficient sample left due to technical difficulty encountered
12	Sample 007 (both aliquots) were matching the criteria for positivity; however, the intensity of the band signal was very low.	1. Second gel (confirmation) The bands did not appear clearly distinguishable, due to sample overload. However, the stability test did not show signs of activity (Lane #9). 2. The analysis was therefore repeated once again, this time processing a lower amount of retentate. The stability test was also performed again. The third gel results sample EDUC 007 matches the criteria of positivity	Negative		Sample 009 (both aliquots) were matching the criteria for positivity	rEPO
13	Negative	The lane for the repetition was considered as not valid, because there is an absence of signal in the lane that do not allow to evaluate the profile properly.	Sample EDUC 008 was considered stable and negative based on the screening and repetition results.	Negative	Only conclusion is provided	rEPO
15		Positive		Not valid		rEPO
17		rEPO	Does not contain rhEPO			rEPO
18	Suspicious	Confirmed BRP	Negative		Suspicious	rEPO

Lab code	WADA EDUC 007		WADA EDUC 008		WADA EDUC 009	
	Screening	Confirmation	Screening	Confirmation	Screening	Confirmation
23	AAF with rEPO is suspected. Sample EDUC 007 was forwarded to a confirmation procedure.	AAF containing rEPO.	The sample appears negative. Due to the band broadening, densitometric calculations were difficult, and the sample was re-run.	Negative	The sample is forwarded to a confiirmation analysis with additional sample clean-up prior ro the IEF-procedure.	rEPO
24	The sample is suspicious for EPO and will be transferred to confirmation analysis	All the identification criteria were NOT met and the sample should be reported as negative *Remark*: The sample volume was not sufficient for repetition of confirmation analysis.	The sample is negative. Due to the nature of the educational test, we decided to reanalyse it with the confirmation batch.	Repeat analysis: the sample is negative	The sample is suspicious for EPO and will be transferred to confirmation analysis	The acceptance criteria were NOT met and the sample should be reported as negative
25	Presumptive AAF requiring confirmation	AAF. Presence of rhEPO	Negative for the presence of rEPO or NESP		Presumptive AAF requiring confirmation	rEPO
26		Positive profile		Positive profile		rEPO
28	The sample is positive amd needs to be confirmed	According to the TD2004 EPO criteria, this sample is positive	The sample is negative.		The sample is positive and needs to be confirmed	rEPO
30	EDUC 007 was found to have isoform bands consistent with the presence of both endogenous and recombinant EPO.	EDUC 007 confirms the presumptive finding from the screen data, that is the presence of recombinant EPO	EDUC 008 was found to have a relatively weak signal but shows a profile typical of endogenous human EPO, as seen in th enegative control, ant therefore this sample considered to be negative.	EDUC 008 was re-run as sufficient urine remained EDUC 008 shows a weak signal, but a typical endogenous EPO isoform pattern confirming the negative result	EDUC 009 showed strong evidence of the presence of recombinant EPO.	rEPO
31	Only conclusion provided	Negative (Because the two most intense bands in the basic area are not *twice* intense than any other bands in the endogenous area).	Only conclusion provided	Negative	Only conclusion provided	rEPO

Lab code	WADA EDUC 007		WADA EDUC 008		WADA EDUC 009	
	Screening	Confirmation	Screening	Confirmation	Screening	Confirmation
32	EPO alpha/beta positive; require confirmation	Positive	Sample 008 looks like negative; repeated for better image.	Sample 008 has been negative in the both screening an dconfirmation tests, and the image was repeatedly weak. Conclusion: No prohibited substances found	EPO alpha/beta positive and suspicious for NESP also; require confirmation.	In contrast with screening conclusions, the confirmation showed that sample 009 does not meet WADA identification criteria since TWO of the most intensive bands turned 3 and 4 while being 2 and 3 before. Moreover, NESP lanes disappeared as well. Therefore, sample 009 was found negative. Conclusion: No prohibited substances found.
33	It was suspected that the sample contains rEPO and the sample underwent confirmatory analysis	WADA EDUC 007 is reportable as AAF for rEPO	Negative for both rEPO and Nesp. No further analysis required.		The sample was believed to contain rEPO and a confirmatory analysis was carried out.	rEPO
34	The screening of this urince sample shows some strong indications of degradation, but nevertheless presents a suspect profile, indicating the presence of rEPO. For that reason, a confirmation was performed.	Presence of rEPO. All WADA crotería are fulfilled. Result: positive.	The screening of this urine sample presents a typical negative profile, but as this sample was received with other urine samples showing indications of degradation, we decided to perform a confirmation.	Result: negative	The screening of this urine sample shows some strong indications of degradation but the presence of rEPO seems obvious.	The Confirmation of this sample highlights once again the degradation, and therefore all criteria are not fulfilled as it is not possible to identify 3 well defined bands corresponding to the standard. Consequently, we return the result as suspect. Result: negative Remark: suspect. The result for recombinant erythropoietins, NESP included, is considered as suspicious for one of the followinfg reasons: - The isoelectric pattern of the sample does not meet all hte criteria of positivity. - All the acceptability criteria of the technique are not entirely fulfilled.

Table 2
Final laboratory results (EDUC 007, EDUC 008 and EDUC 009)

Lab code	Sample EDUC 007 "Borderline sample"	Sample EDUC 008 Negative for rEPO and NESP	Sample EDUC 009 Adverse Analytical Finding for rEPO
1	rEPO	Inconclusive	rEPO
2	Suspicious*	Negative	rEPO
3	rEPO	Negative	rEPO
4	Suspicious**	Negative	rEPO
5	rEPO	Negative	rEPO
7	rEPO	Negative	rEPO
8	Negative**	Negative	rEPO
9	Negative	Negative	rEPO
11	Negative*	Negative*	rEPO*
12	rEPO	Negative	rEPO
13	Not valid	Negative	rEPO
15	rEPO	Not valid	rEPO
17	rEPO	Negative	rEPO
18	rEPO	Negative	rEPO
23	rEPO	Negative	rEPO
24	Negative	Negative	Negative
25	rEPO	Negative	rEPO
26	rEPO	Positive profile	rEPO
28	rEPO	Negative	rEPO
30	rEPO	Negative	rEPO
31	Negative	Negative	rEPO
32	rEPO	Negative	Negative
33	rEPO	Negative	rEPO
34	rEPO	Negative	Negative

* Results obtained during the screening analysis, confirmation not performed

** Further testing is recommended

Laboratory comments

Laboratory code	Comments
1	As the overall comments, I would like to inform you that we did not apply second opinion policy for reporting results of the educational PT for urine EPO. If the EPO samples were real, we would ask other WG member laboratories the second opinion as required by WADA. EDUC 008: Inconclusive results were obtained. (Opinion on EDU008 for testing authority only: Testing authority is advised to check previous urine EPO and blood EPO screen tests history of the athlete coded EDU008 if available or proposed to follow the athlete by subsequent urine EPO test, when the previous test history is not available for this athlete. The test result suggested weak sign of basic EPO bands, however, no conclusive test result could be obtained due to low urinary EPO concentration and limited availability of the specimen). EDUC 009: Urine sample coded was found to contain recombinant EPO. (Opinion on EDU009 for testing authority only: This sample is also suspected to contain Darbepoietin (NESP), however the bands were not identified as NESP. The test result related to recombinant EPO only was notified in the results).
2	EDUC007: The bands in screening showed some suspicious results but not meet WADA criteria so this sample was not analysed neither for active test nor for confirmation.
4	EDUC 007: Due to the fact that the differences between the highest peak in the basic area and the peak in the endogenous area are not higher than twice the sample would be considered as suspicious for the presence of rEPO, but not as an adverse analytical finding. Therefore, further target testing would be recommended. EDUC 008: In sample EDUC 008 only the bands (isoforms) of a normal urine sample could be found. Therefore the sample would be reported negative regarding rEPO and NESP. EDUC 009: Sample EDUC 009 showed significant differences depending on the used aliquot. The screening of the first sample aliquot yielded only very weak signals in the area of recombinant EPO which invalidated this test. In the second IEF run from a second urine aliquot strong signals for the recombinant isoforms in the basic area were detected. However, both lanes showed some smears which is typically for protein overload. The third IEF run with remaining aliquots of the first and second sample preparation confirmed this finding. Weak signals for the first preparation, recombinant isoforms for the second preparation with additional weak isoforms in the endogenous and acidic area. In summary, this sample would be reported as an adverse analytical finding for rEPO.
5	EDUC 007 Remarks: Considering the agreements at the Paris Meeting in 2005 for the ratio between basic and endogenous bands (the two most intense bands in the basic area must be twice or more intense than any band in the endogenous area), this sample has to be reported negative with GASEpo software evaluation, and positive with AIDA – software evaluation. The main reason to this divergence is the different style of background correction: while with AIDA-valley-to-valley integration of the 1D-blot was employed (as described by F. Lasne, Paris 2005), GASepo uses subtraction of a background plane of the 2D-data. As has been shown by an oral contribution

Laboratory comments

	to the Manfred Donike Workshop in Cologne 2006, valley-to-valley integration leads to a statistical overestimation of lower abundant bands and shows a time dependence of the results as a function of the acquisition time of the camera. Sample EDUC 009 Considering the agreements at the Paris Meeting in 2005 for the ratio between basic and endogenous bands (the two most intense bands in the basic area must be twice or more intense than any band in the endogenous area), this sample stays positive.
11	Gel images were assessed using both Image Gauge (Fuji) and GASepo software. Image Gauge is the standard software package in this laboratory. GASepo is being used for checking, and is being cross checked for routine use. Screening procedure: Blot quality – the blot suffers from distortion and from uneven signal development, especially in the lower part of the blot and in patches in the mid part of the blot. The negative control could not be visualised, which is unusual. The standard rhEPO, BRP-2 from the EDQM, was useable. Summary (screening): The gel is not of good quality and this has caused problems in assessing the results. EDUC007 – Probable endogenous EPO with a relatively basic isoform distribution, though uneven signal development in the endogenous area makes the exact isoform distribution unclear. EDUC008 – Endogenous EPO. The uneven quality of the signal development makes the exact isoform distribution unclear, but if this were corrected, the distribution would still be endogenous in appearance. EDUC009 – There is a strong set of basic bands in this sample, with a probable appearance of recombinant EPO in the presence of a small amount of endogenous EPO (consistent with a naive user or a spiked sample). However, the uneven signal development in this lane makes it impossible to make a finding of recombinant EPO, especially as the position of the weak signal may mask stronger endogenous bands. The analysis was repeated: Blot quality – the blot suffers from uneven signal development, with blotching especially in the right half of the blot. Summary (repeated analysis): The gel is not of good quality having several blotches and this has caused problems in assessing the results. EDUC007 – Probable endogenous EPO with a relatively basic isoform distribution, though blotching in the basic area makes the exact isoform distribution unclear. It supports the first analysis. EDUC008 – Endogenous EPO. Despite some blotching at the cutoff. The distribution of isoforms is consistent with endogenous EPO as in the initial analysis. EDUC009 – There is a strong set of basic bands in this sample, with the appearance of recombinant EPO in the presence of a small amount of endogenous EPO (consistent with a naive user or a spiked sample). This lane is straight and clean, and supports a finding of recombinant EPO Conclusion: EDUC007 – The sample most likely contains endogenous EPO, with a high basic distribution. The isoforms appear to have a single smooth distribution according to their pI, as opposed to EDUC009 below. The band distribution is lower

Laboratory comments

	than the rHEPO by one band making it different to rHEPO. No further action would be taken on this sample. EDUC008 – The sample is assessed as containing endogenous EPO. No further action would be taken on this sample. EDUC009 – The sample is assessed as containing recombinant EPO upon screening. The ratio of the appropriate bands is >2. Although there is some signal in the endogenous area there is a marked discontinuity in the distribution of the intensity of the bands at the cutoff, suggesting two populations of isoforms. This is consistent with the sample being either a spiked sample, or with the sample being collected after an initial rhEPO injection. This sample would require a confirmation analysis and stability check before an adverse analytical finding could be made. There was insufficient sample to do this after the technical difficulty encountered. Given the relatively small sample, and had this been an A or B sample, the overall poor gel quality even though this sample band was clear, would have made an adverse analytical finding difficult. We are currently investigating the reasons for this problem as it has only happened recently. We need to ensure the gels run properly before we undertake more analysis.
12	Following the results of this first assay, a new gel was processed (Jan 8-10 2007), to carry out the confirmation analysis and the stability test for samples ED009 and to repeat the analysis of sample ED007 (one aliquot only, but this time loading a higher amount of retentate on the gel), including the stability test for this sample also. The image analysis of this second gel confirmed that sample ED009 (Lane #12) matched the criteria for positivity, and, also, that the results of the stabiity test (Lane #13) were negative. Sample ED009 would therefore have been reported as positive by our laboratory, provided that – according to the WADA requirements and also to our internal procedures – this result would have been confirmed by at least two other WADA accredited laboratories, including at least one of the Authors of the WADA technical document "TD2004EPO". With respect to sample ED007 (Lane #8), the bands did not appear clearly distinguishable, due to sample overload. However, the stability test did not show signs of activity (Lane #9). The analysis was therefore repeated once again (Jan 10-12 2007), this time processing a lower amount of retentate. The stability test was also performed again. This third gel image is here reported. As it can be seen, sample ED007 matches the criteria of positivity and therefore it would have undergone the same process as for sample ED009 (transmission of the gel images to other WADA accredited laboratories performing the EPO/NESP analysis, including the Authors of the WADA technical document "TD2004EPO"). As a final, additional comments on the study, we would like to stress out the importance of collecting a volume of urine as high as possible whenever the analsyis for EPO/NESP is required.
13	Conclusion: 1. The three gels used for the analysis of the samples were considered as valid according to our Internal Standard Procedure.

Laboratory comments

	2. The sample with WADA code EDUC007 internal code 06142408, was considered stable and negative based on the screening result, because the lane for the repetition was considered as not valid, because there is an absence of signal in the lane that do not allow to evaluate the profile properly. 3. The sample with WADA code EDUC008 internal code 06142409, was considered stable and negative based on the screening and repetition result. For the repetition 30 ml were taken, the double of our standard volume. 4. The sample with WADA code EDUC009 internal code 06142410, was considered stable and Adverse Analytical Finding of recombinant EPO.
15	The areas under the profiles in each lane has been calculated using the AIDA image analysis software connected to the Chemiluminescent Imager.
17	It should be noted that for WADA EDUC 007, 008, 009, the regular procedure for the evaluation for the results was not applied, i.e. exceptionally no second opinion from a "reference" laboratory was requested. The results were specifically and strictly interpreted according to the WADA TD2004EPO, as requested.
18	For the screening analysis, a gel image is provided while for the confirmation analysis gel images together with their densitometry profile analysis are submitted.
23	General comments: The blots were analysed densitometrically using two different softwares: GASepo and AIDA. The samples profiles ran with very broad bands, with bleeding of signal over to neighbouring lanes as a result. For sample 009 we therefore found it necessary to reduce the total protein content by removing all non-glycosylated proteins using lectin affinity chromatography on whet germ agglutinin WGA). Sample EDUC 007. Conclusion: Using the criteria of TD2004EPO, we found the ratio (the second most intense band in basic area/the most intense band in endogenous area) to be 1.7 (screening) and 1.6(confirmation) respectively and the sample 007 to fulfil the stability criteria. This sample will be reported to be an adverse analytical finding, containing recombinant EPO. If the new criteria for EPO evaluation will be changed to "a ratio of approximately 2", this sample would be reported negative. We also notice that the densitometric analysis results will vary with the software applied. An evaluation by the AIDA-algorithm give a ratio slightly above 2. Sample EDUC 008 Screening 1: A ladder of weak bands stretching from the lower endogenous area into the basic area, very much like as the control urine. Visually no bands in the basic area stand out as stronger than any band in the endogenous area and the sample appears negative. Due to the band broadening discussed above, densitometric calculations were difficult, and the sample was re-run. Screening 2b: the most intense bands measured densitometrically are in the endogenous area. Conclusion: Sample EDUC 008 is negative.

Laboratory comments

	Sample EDUC 009 Screening 1: Strong labelling of bands 2-4 in the basic area and labelling of bands B, C, and D in the acidic area. Die to white spot covering most of the endogenous area and also band 1 in the basic area, the lane was regarded invalid. Screening 2a: There is labelling of 4 consecutive bands in the basic area, of which bands 2 and 3 are most intense. All of these bands are most intense than any band in the endogenous area. There is faint labelling of band corresponding to B in acidic area. The weaker labelling of screening II compared to I is due to the use of paper for sample –loading on gel in order to reduce band-width. We still notice that non-EPO urine proteins are disturbing the endogenous area. The sample is forwarded to a confirmation analysis with additional sample clean-up prior to the IEF-procedure. Confirmation EDUC 009: Strong labelling of bands 1-4 in the basic area, of which bands 2 and 3 are the most intense. The consecutive bands running through the entire endogenous area into the acidic area were all weaker that bands 1-3 in the basic area. The sample was this time partially purified on WGA (Wheat Germ Agglutinin) to remove bulk urine protein. Conclusion: Using the criteria of TD2004 EPO, we found the sample to fulfil the criteria for an adverse analytical finding, containing recombinant EPO. The ratio (the second most intense band in basic area/the most intense band in endogenous area) was calculated to be 5.7. If new criteria for EPO-evaluation will be changed to "a ration of approximately 2", this sample would still be reported positive.
24	Confirmation of EPO WADA EDUC 007 Acceptance criteria: The lane is smeared and the bands are cut, which complicates the band identification. Identification criteria: 1) there are only two consecutive bands with appropriate intensity in the basic area; 2) the two most intensive bands are consecutive and the most intense is 1; 3) the two most intense bands in the basic are more intense than any other band in the endogenous area (although the band is again more than 50% of the intensity of the band 1). Stability criteria: The activity test is negative Confirmation result: All the identification criteria were not met and the sample should be reported as negative. Remarks: The sample volume was not sufficient for repetition of confirmation analysis. WADA EDUC008 Acceptance criteria: The quality of exogenous area of the lane is improved and it may be concluded that there are no bands in the basic area. Reanalysed screening result:the sample is negative WADA EDUC009: Acceptance criteria: The exogenous EPO area of the lane is heavily dispersed and the split bands interfere the identification criteria. Identification criteria: 1) three consecutive bands can be seen in the basic area, but the split between invalidates the identification 2) the two most intensive bands are consecutive and the most intense is 2; 3) the two most intense bands in the basic are more intense than any other band in the endogenous area Stability criteria: The activity test is negative. Confirmation result: The acceptance criteria were not met and the sample should be reported as negative.

Laboratory comments

	Remarks:The sample volume was not sufficient for repetition of confirmation analysis. General remarks: Methyl red is applied to all the analyses. The laboratory made a mistake in the sample storage between screening and confirmation, and the samples were stored in cold room instead of freezer. The difference between the screening and confirmation results, as well as between the quality of the EPO bands may be due to degradation of EPO in the samples. Therefore, if there were samples still available, it would be of great interest for us to analyse the samples EDUC007 and EDUC009 again.
26	Comments: After analysis of the four samples sent by WADA, we have noted that we have some difficulties to obtain the same result in screening and confirmation. We think that the probable reasons of the poor quality of the picture may be: - The samples were not frozen at the arrival - The variability of the reagents suppliers (specially the antibodies) - The resolution of the camera is not performed - The negative urine present a profile of urine taken after strenuous exercise - The concentration of the Nesp reference (too high) didn't give a good separation of bands For these reasons the EPO test results are not always evidently interpreted. Conclusion: Our results according to the official WADA identification criteria for rhEPO are the following: - Sample EDUC007 : Positive profile - Sample EDUC008 : Positive profile - Sample EDUC009 : Positive profile For the three positive samples, we have not been able to distinguish between the endogenous profile and the exogenous one.
30	Screening: EDUC 007 was found to have isoform bands consistent with the presence of both endogenous and recombinant EPO. Although there is a small air bubble in the lane, it does not significantly interfere with the application of the identification criteria laid out in TD2004EPO for the prescence of recombinant EPO. The measured concentration of total EPO in the retentate using immunoassay (Immulite) was 425 IU/L. EDUC 008 was found to have a relatively weak signal but shows a profile typical of endogenous human EPO, as seen in the negative control, and therefore this sample is considerd to be negative. Immulite analysis of EPO determined a retentate concentration of 238 IU/L. EDUC 009 showed strong evidence of the presence of recombinant EPO, since the most intense bands for this lane are clearly in the recombinant region. However, there appears to be an area of 'absent signal' in the endogenous region that may hinder the application of the identification criteria. Immulite analysis of EPO determined a retentate concentration of 1500 IU/L, hence this retentate was diluted approximately three-fold before applying to the gel for IEF.

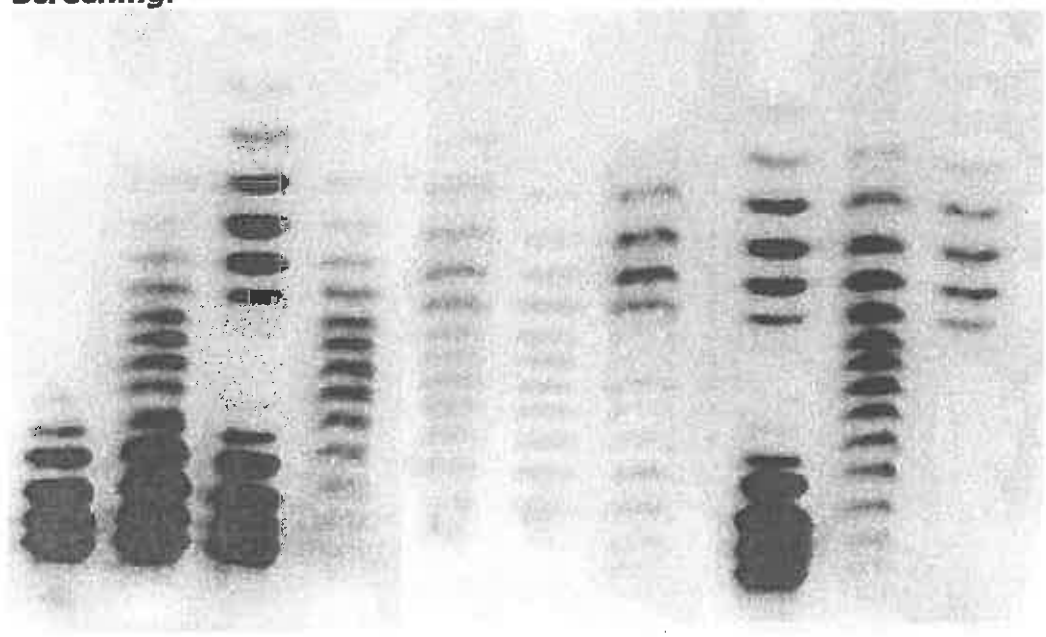
Laboratory comments

	Confirmation: Quantitative interpretation of the data using densitometry and Gasepo software confirms the visual assessment of EDUC 007 and EDUC 009 as adverse analytical findings for recombinant erythropoietin. The criteria complied with the specification in TD2004EPO for rEPO points 1, 2 and 3.
32	Screening: After the Screening procedure two samples namely 007 and 009 were tentatively identified as positive, the other sample code number 008 looks like negative but the signal was very weak. Each sample in the Screening procedure was made in two replicates, the replicates were quite identical. The raw data of the sample 009 showed that there are several visible bands in the NESP area, but the software does not recognize those bands, see screening report. The samples 007 and 009 has three most intensive bands in the basic area, and TWO of the most intensive are bands 2 and 3 for each sample in both tracks. It meets WADA criteria for positive results. Therefore after Screening it was concluded that - sample 007 is EPO α/β positive, - sample 009 is EPO α/β positive, and suspicious for NESP also, and require confirmation. Sample 008 was repeated for better image. Confirmation: Confirmation was done for all three samples with activity test. The samples were taken in the amount, given in the concentration column corresponding to each line, see Table at the last page of screening EPO report (page 21). The column shows the volume of the urine for the sample preparation and the volume of retentate used in the analysis afterwards. All three activity tests showed that there was NO any active urine. In contrast to screening conclusions, the confirmation showed that sample 009 does not meet WADA Identification criteria since TWO of the most intensive bands turned 3 and 4 while being 2 and 3 before. Moreover, NESP lanes disappeared as well. Therefore sample 009 was found negative. The picture of sample 007 was not clear enough, and raw data might be considered as not fulfilling WADA Acceptance criteria. Nevertheless GASepo software allowed us to define bands and conclude that the most intensive bands are in the basic area and TWO of the most intensive bands are 2 and 3. Therefore considering previous screening and confirmation results, we have concluded that the sample 007 is positive. The remaining sample 008 has been negative in the both screening and confirmation tests, and the image was repeatedly weak.
33	All samples, spikes and blanks were diluted to a lower level for the confirmatory analysis, as compared to the screen, with the intention of preventing overloading of the gel. Reference standards of lower concentration were also used

Appendix A: Electropherograms.

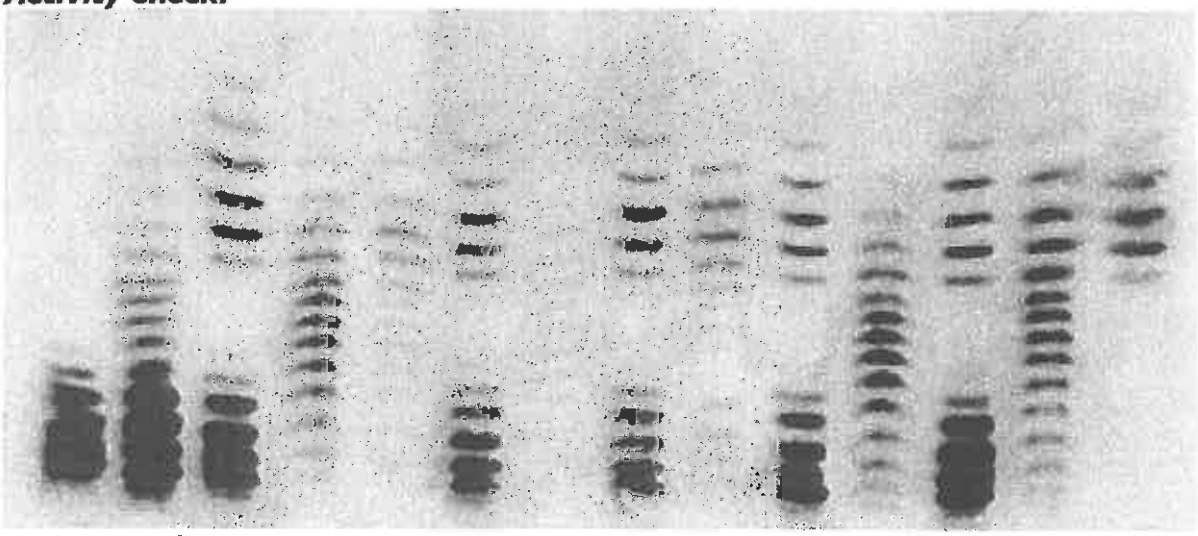
Laboratory 1

Screening:



Controls **007/ 008/ 009** WADA Educational Controls

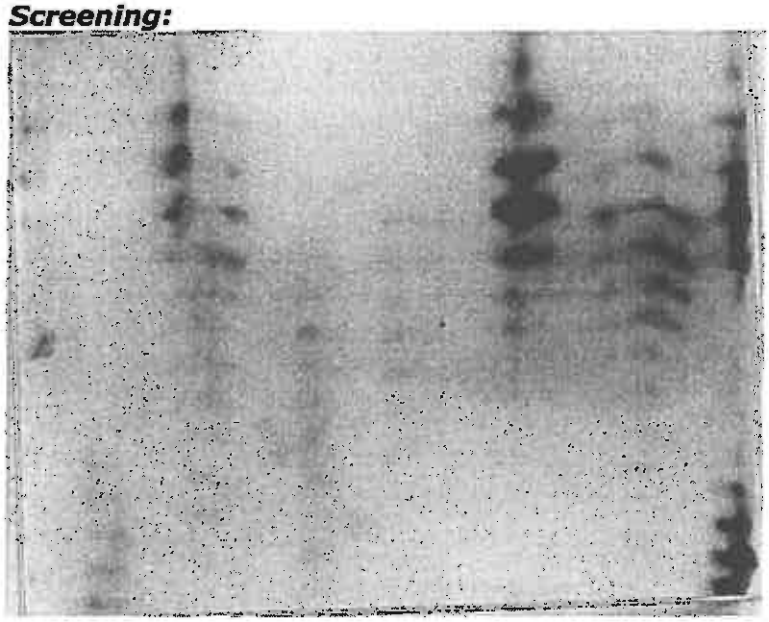
Activity check:

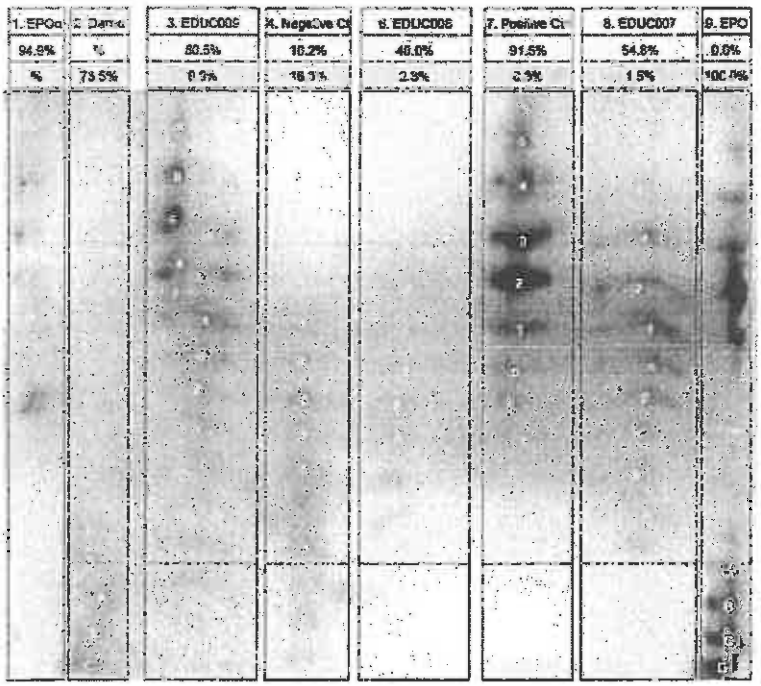


Controls ..... DFU/ **007** spiked/**008** spiked/**009** spiked DFU .....Controls .....

Laboratory 2

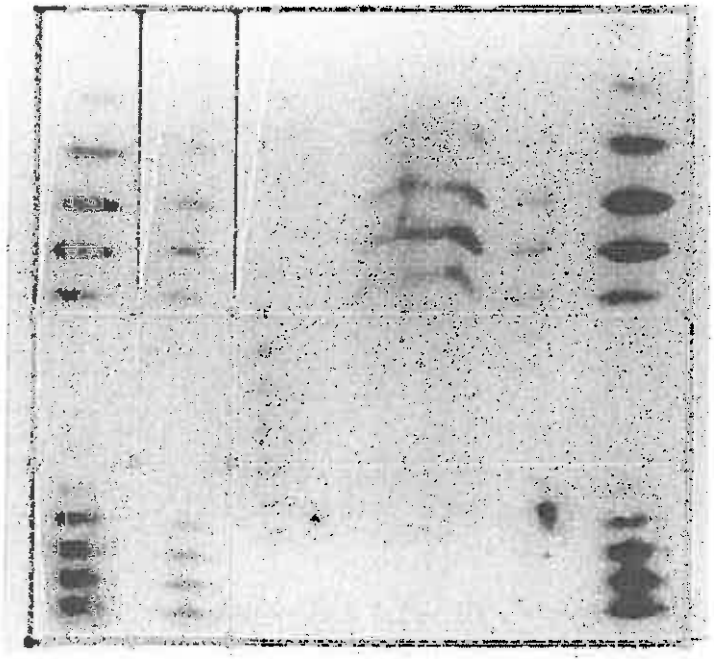
Screening:

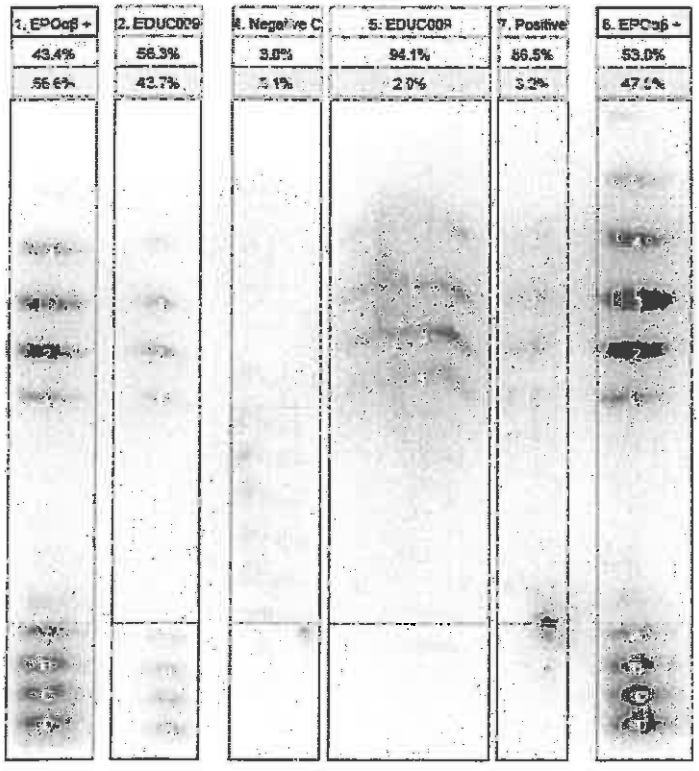




Laboratory 2

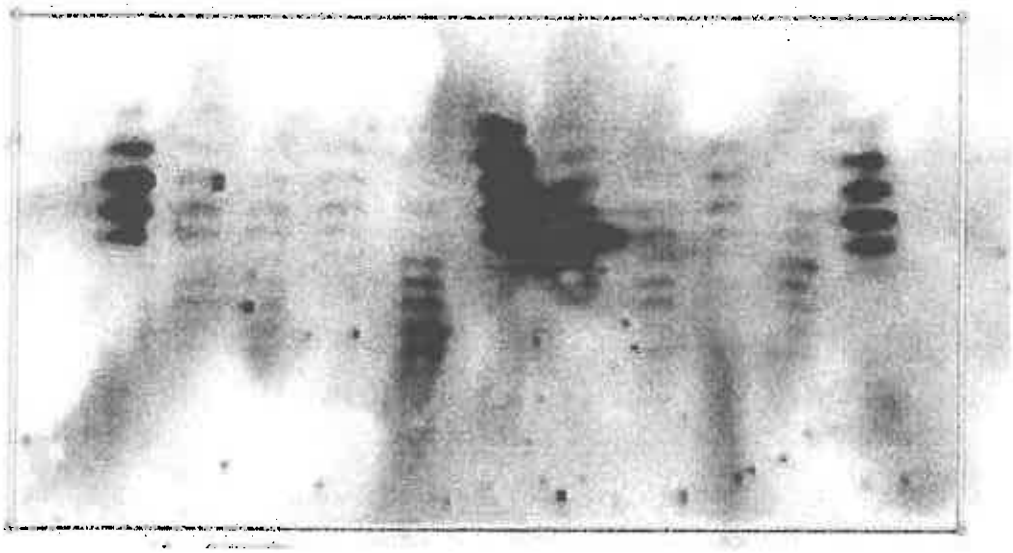
Confirmation:

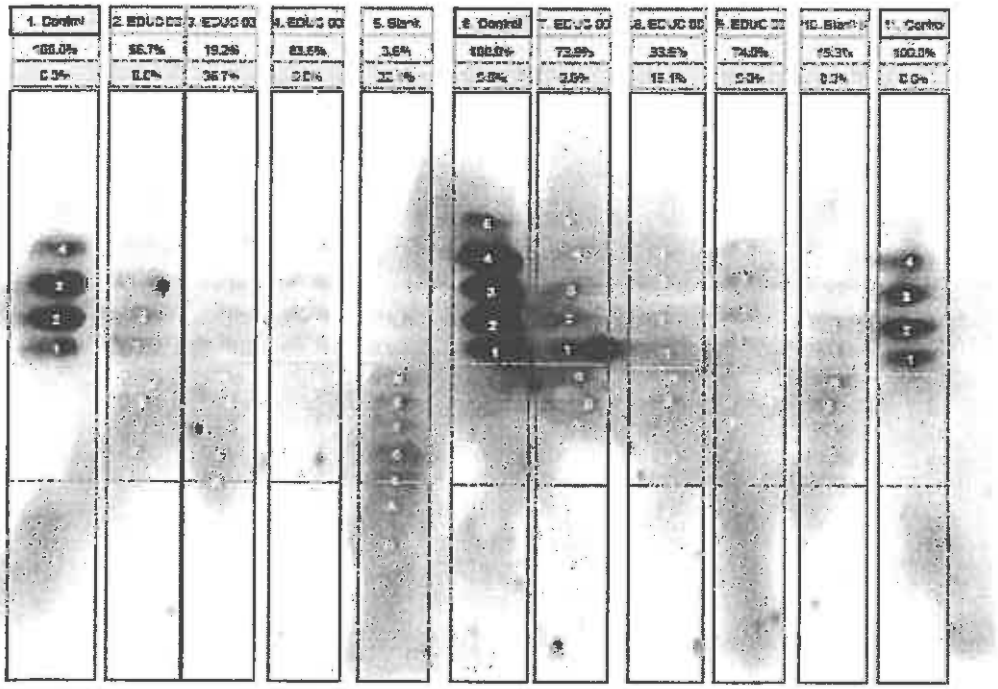




Laboratory 3

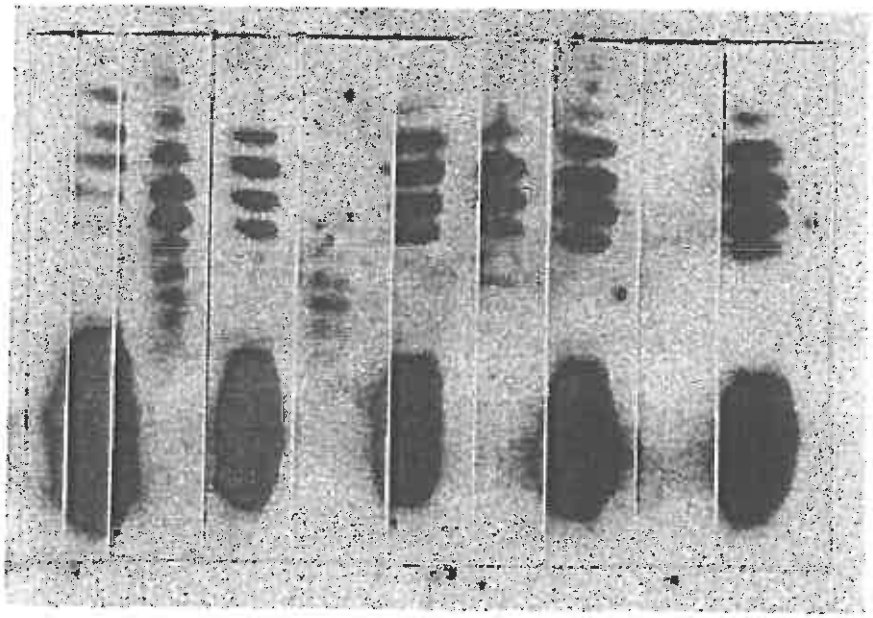
Screening:

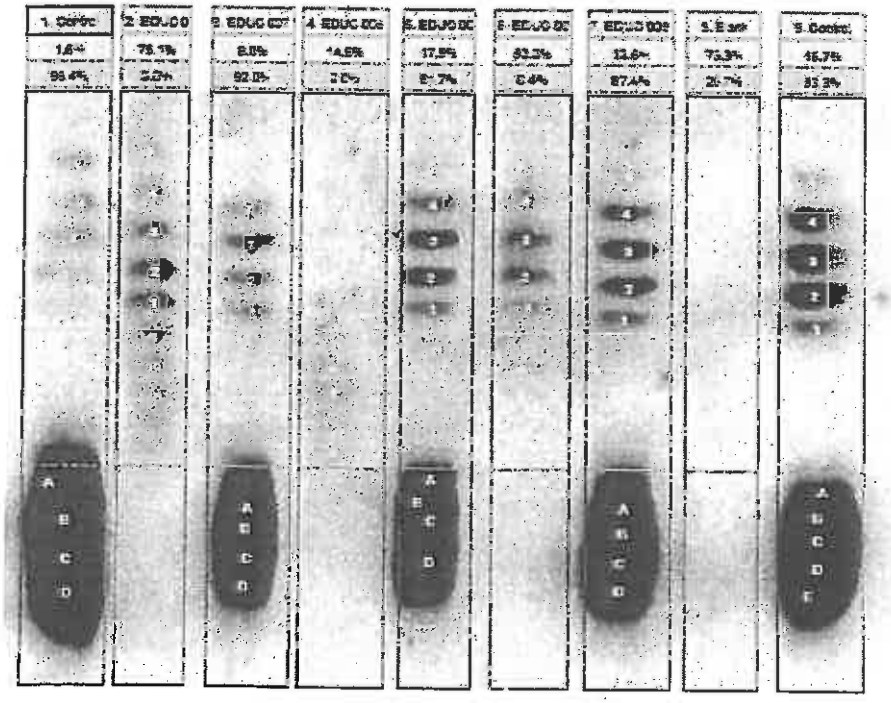




Laboratory 3

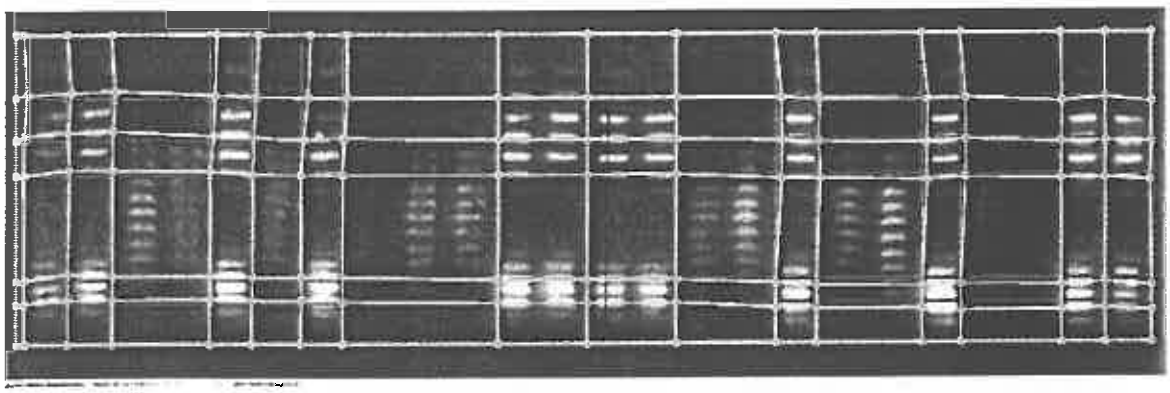
Confirmation:

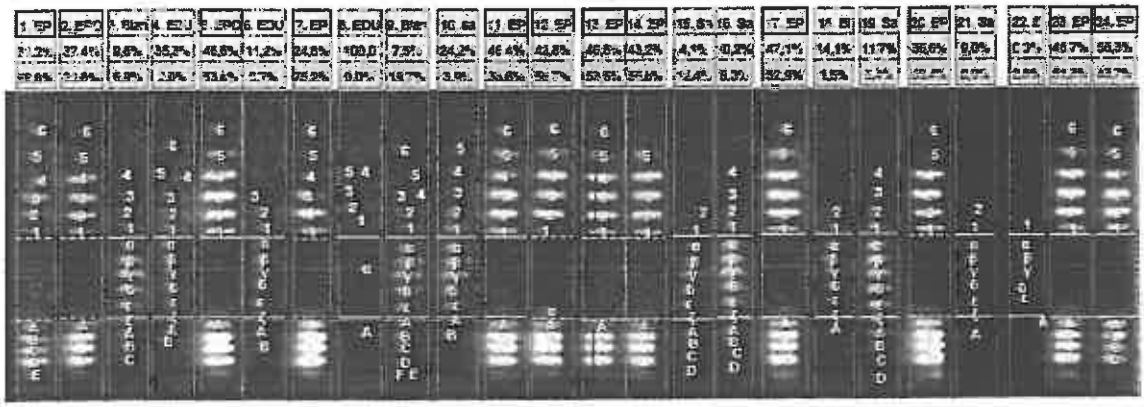




Laboratory 4

Experiment 1:

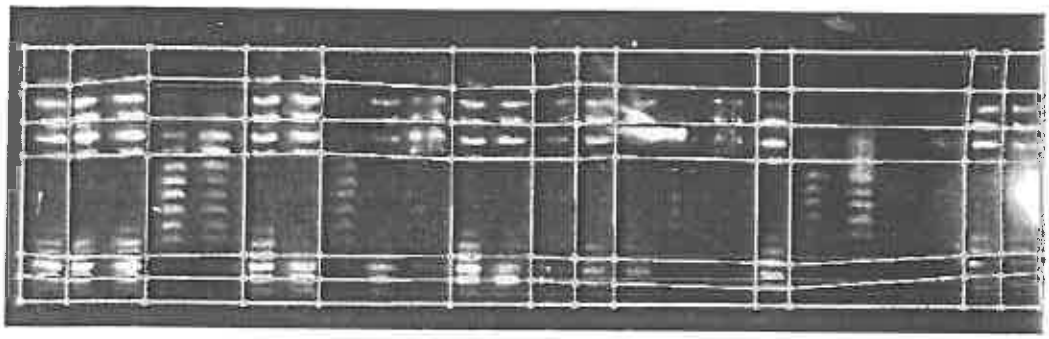


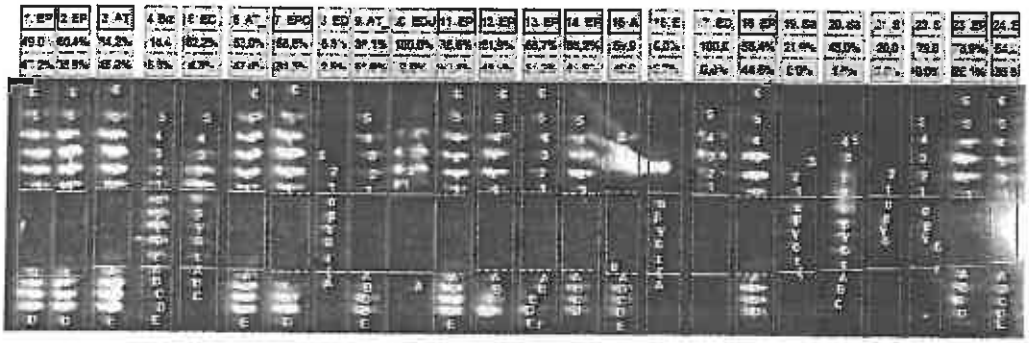


Lane	Name	Annotation
1,2,6,7, 11-14, 17, 20, 23,24	BRP+ARA	Standards rEPO (Biological Reference Preparation, BRP) and NESP (aranesp™, Amgen)
3	Blank	uEPO – blank urine control sample
18	Blank 2	uEPO – blank urine control sample
4	EDUC007_A1	WADA Educational EPO Test Sample 007, first Aliquot
6	EDUC008_A1	WADA Educational EPO Test Sample 008, first Aliquot
8	EDUC009_A1	WADA Educational EPO Test Sample 009, first Aliquot
10, 15, 16, 19, 21, 22	Sample	urine samples

Laboratory 4

Experiment 2:

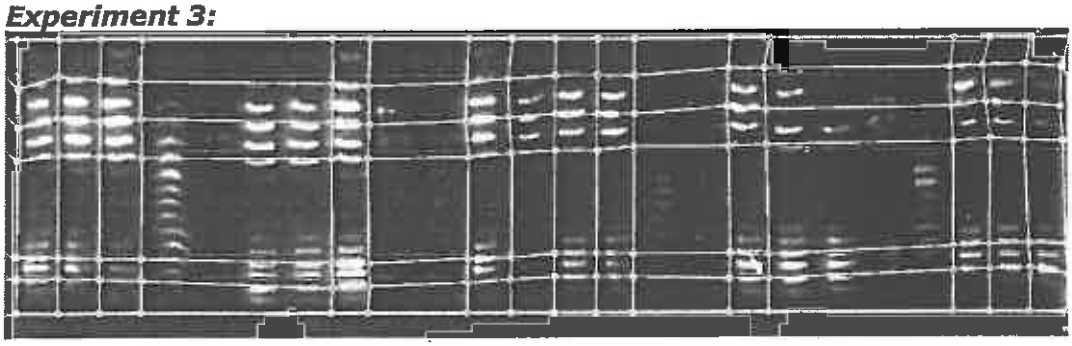


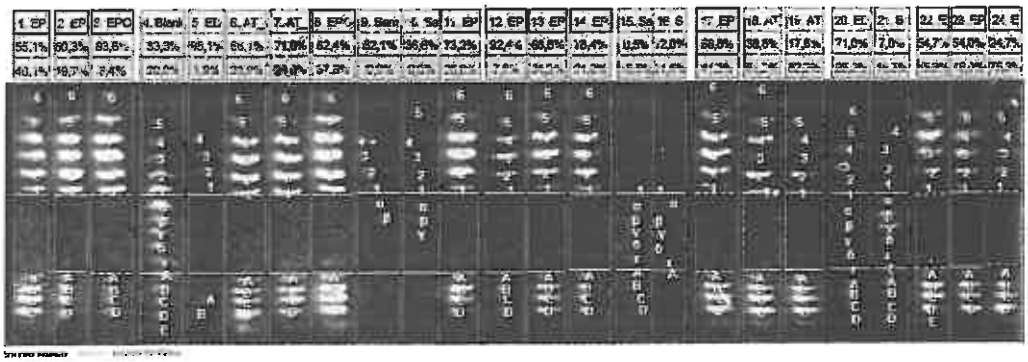


Lane	Name	Annotation
1,2, 7, 11-14, 18, 23, 24	BRP+ARA	Standards rEPO (Biological Reference Preparation, BRP) and NESP (aranesp™, Amgen)
4	Blank	uEPO – blank urine control sample
5, 16	EDUC007_A2	WADA Educational EPO Test Sample 007, second Aliquot
8	EDUC008_A2	WADA Educational EPO Test Sample 008, second Aliquot
10, 17	EDUC009_A2	WADA Educational EPO Test Sample 009, second Aliquot
3	AT_EDUC007	stability test of sample EDUC 007
6	AT_EDUC008	stability test of sample EDUC 008
9	AT_EDUC009	stability test of sample EDUC 009
19-22	Sample	urine samples

Laboratory 4

Experiment 3:

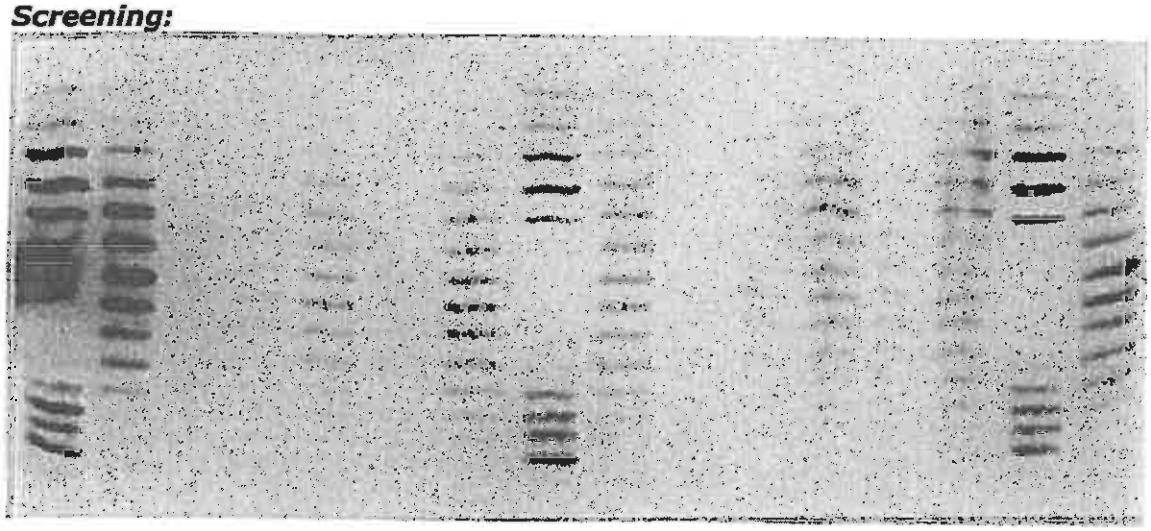


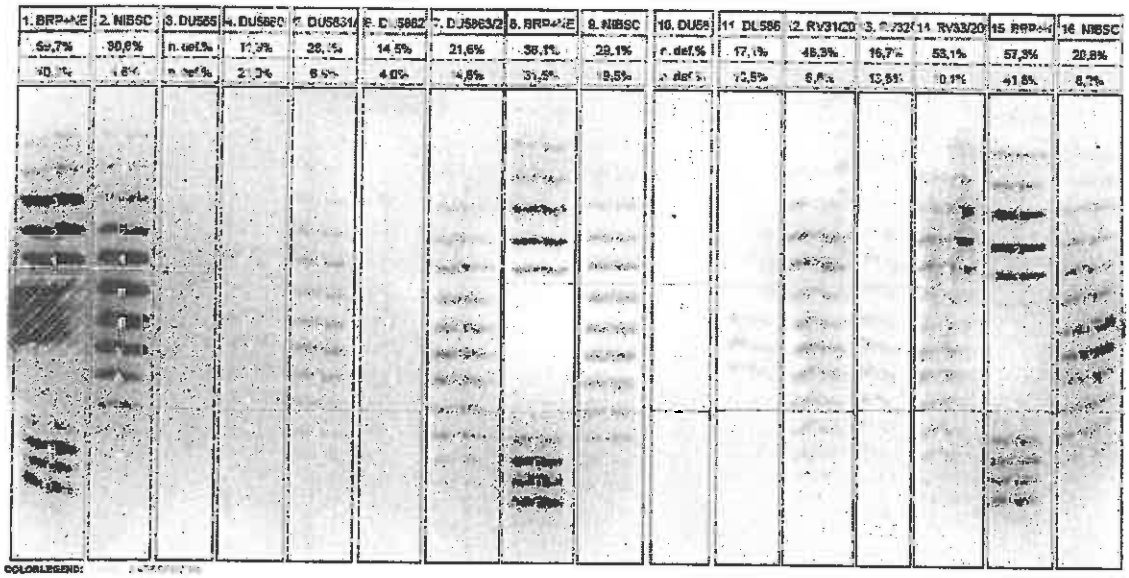


Lane	Name	Annotation
1-3, 8, 11-14, 17, 22-24	BRP+ARA	Standards rEPO (Biological Reference Preparation, BRP) and NESP (aranesp™, Amgen)
4, 21	Blank	uEPO – blank urine control sample
5	EDUC009_A1	WADA Educational EPO Test Sample 009, first Aliquot
20	EDUC009_A2	WADA Educational EPO Test Sample 009, second Aliquot
6, 19	AT_EDUC009_A1	stability test of sample EDUC 009, first Aliquot
7, 18	AT_EDUC009_A2	stability test of sample EDUC 009, second Aliquot
9, 10, 15, 16	Sample	urine samples

Laboratory 5

Screening:

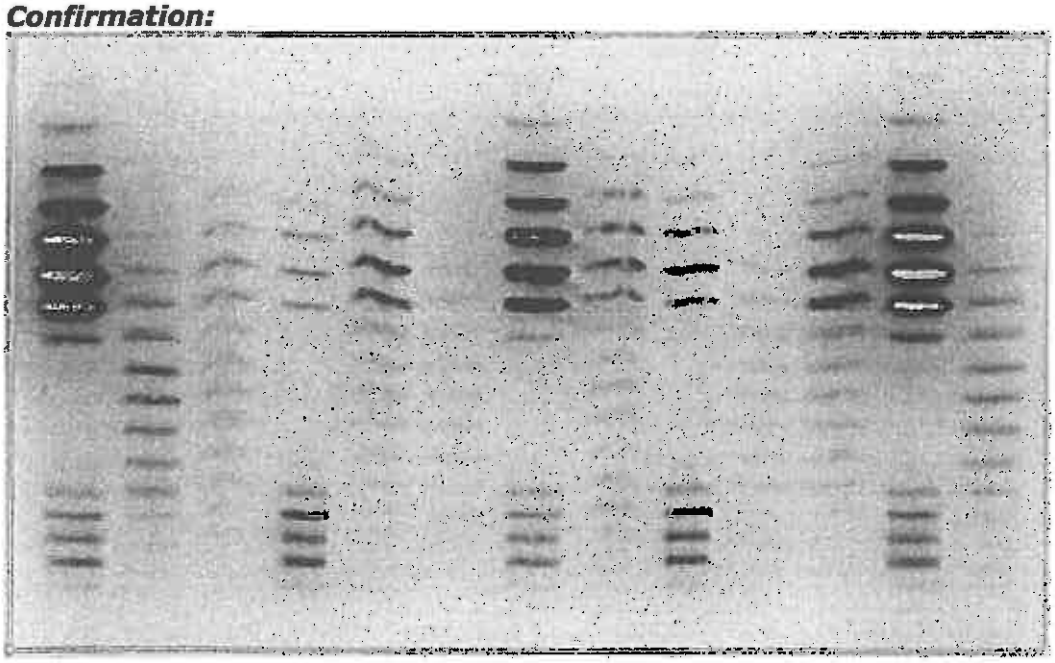


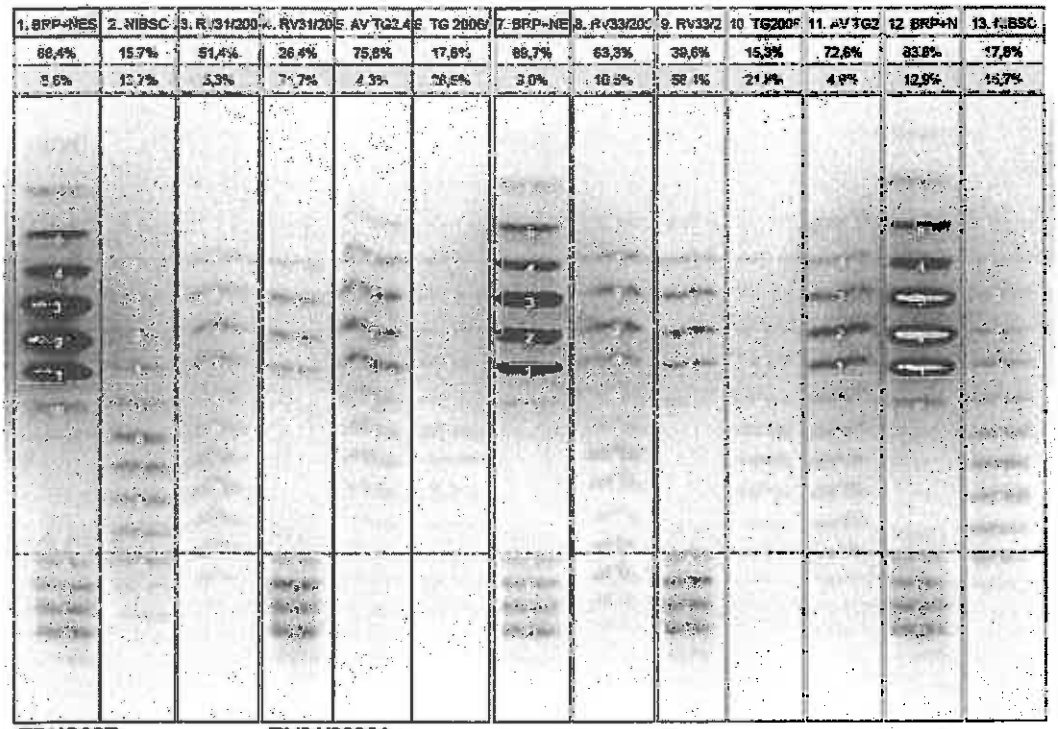


EDUC007 RV31/2006A
EDUC008 RV32/2006A
EDUC009 RV33/2006A

Laboratory 5

Confirmation:





EDJC007 RV31/2006A
EDJC008 RV32/2006A
EDJC009 RV33/2006A

Laboratory 7

Screening:

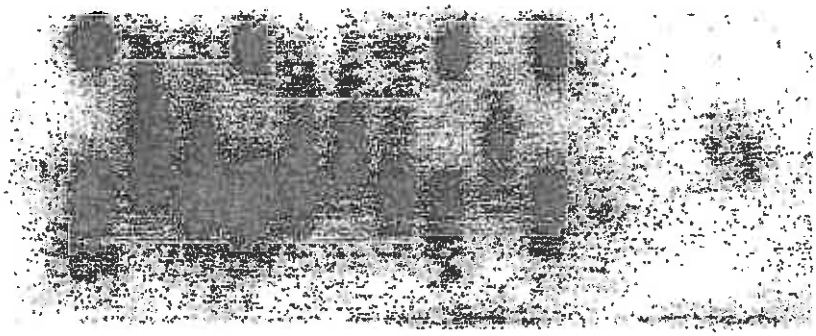


Figure 1

S - rHuEPO/NESP standard
-QC - Negative Quality Control
+QC - Positive Quality Control
1 - First WADA Sample: A7W03 (bottle number EDUC007)
2 - Second WADA Sample: A7W04 (bottle number EDUC008)
3 - Third WADA Sample: A7W05 (bottle number EDUC009)
4 - Fourth WADA Sample: A7W06 (bottle number EDUC010)

Confirmation:

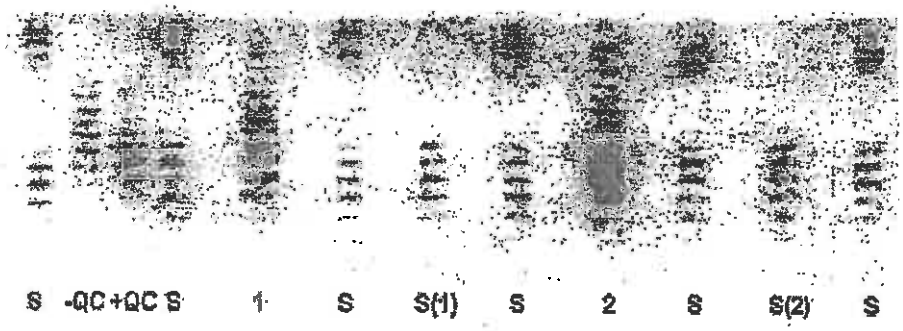
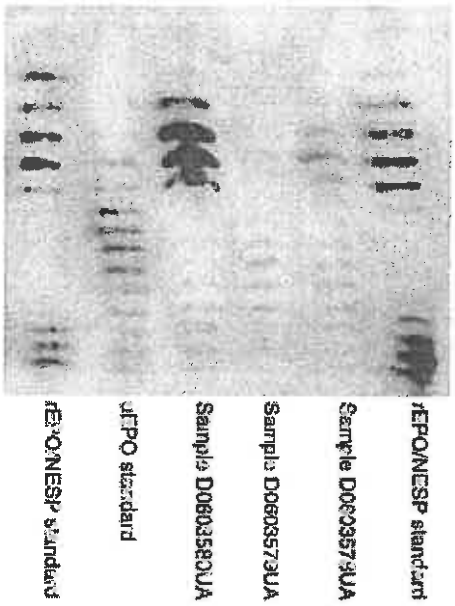


Figure 2

S - rHuEPO/NESP standard
-QC - Negative Quality Control
+QC - Positive Quality Control
1 - WADA Sample number A7W03 (bottle: EDUC007)
S(1) - Stability associated with the sample number referenced
2 - WADA Sample number A7W05 (bottle: EDUC009)
S(2) - Stability associated with the sample number referenced

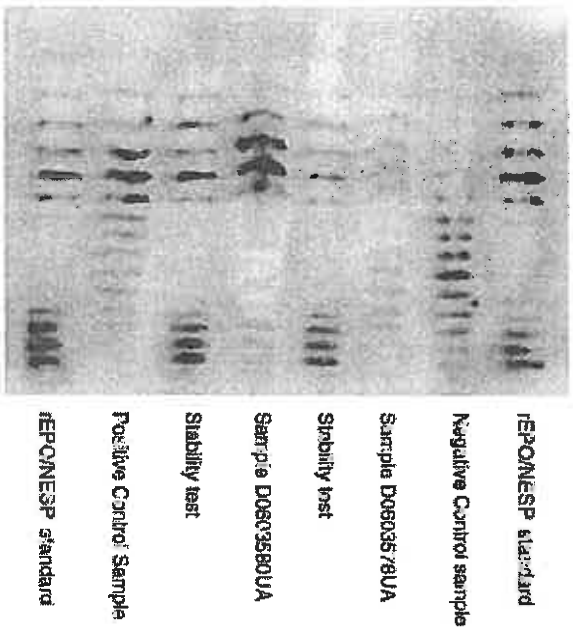
Laboratory 8

Screening:



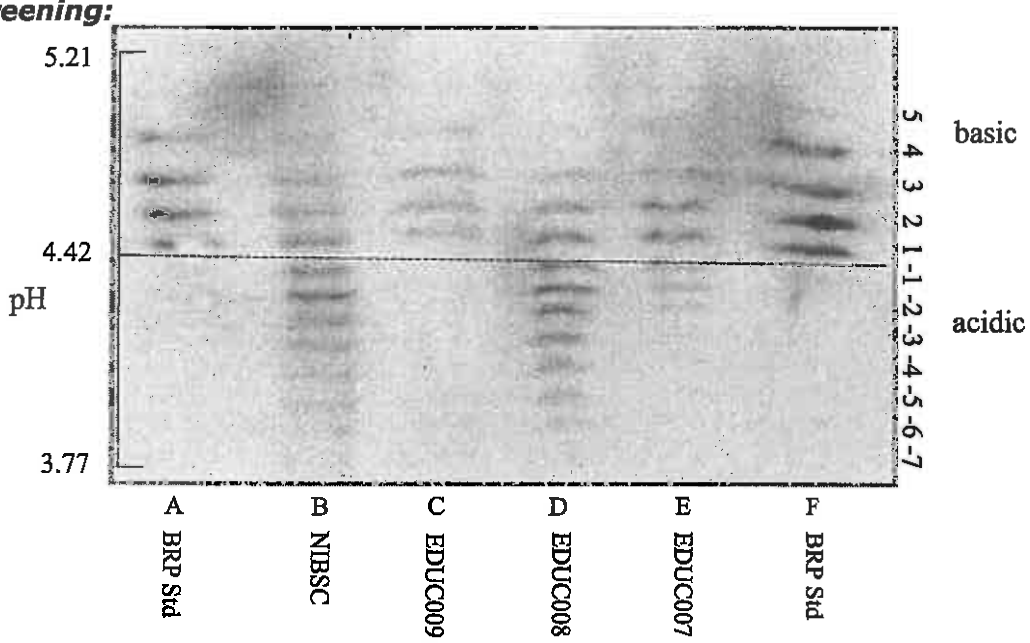
WADA EDUC 007:	D0603578UA
WADA EDUC 008	D0603579UA
WADA EDUC 009	D0603580UA

Confirmation:

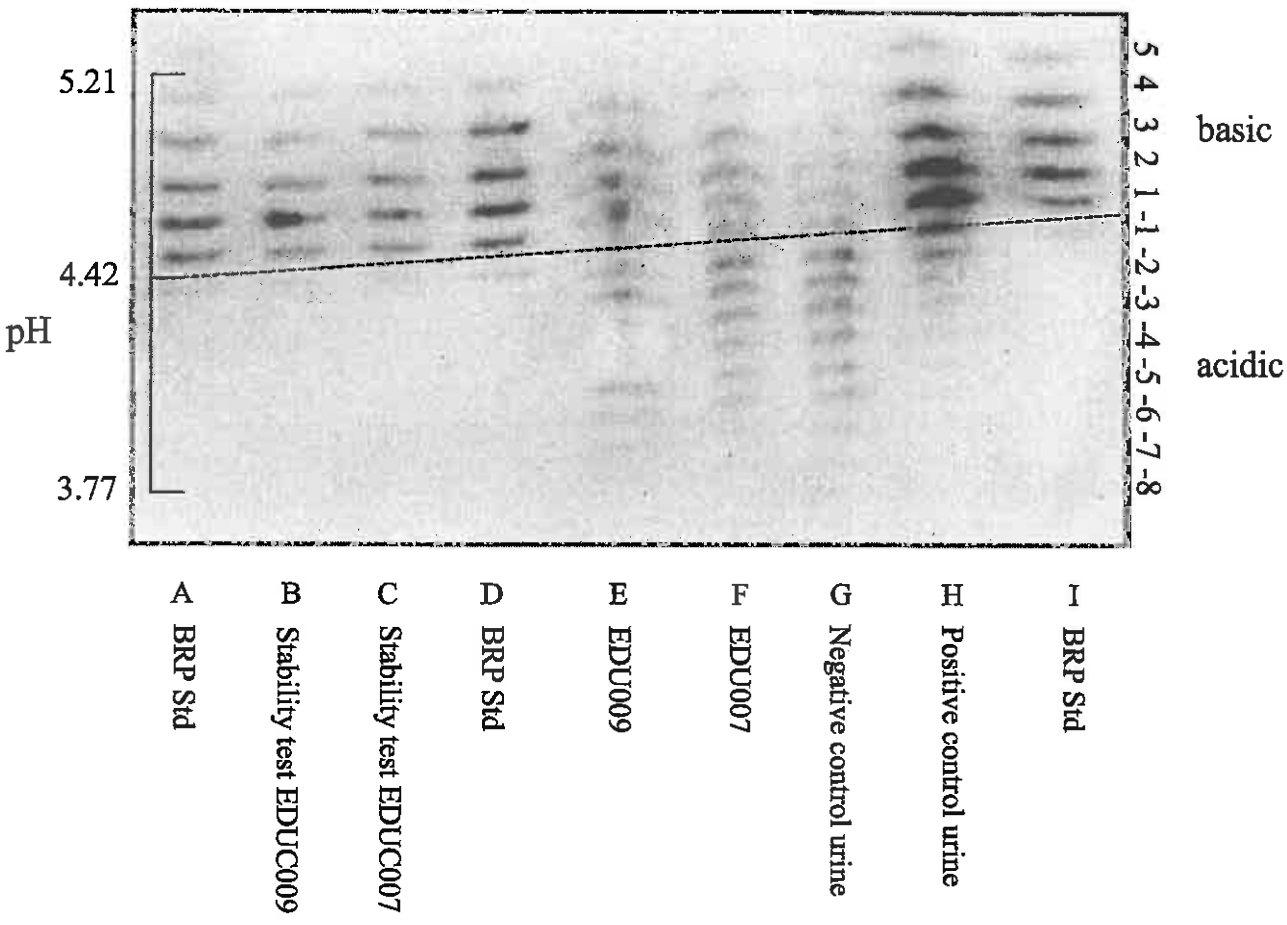


Laboratory 9

Screening:

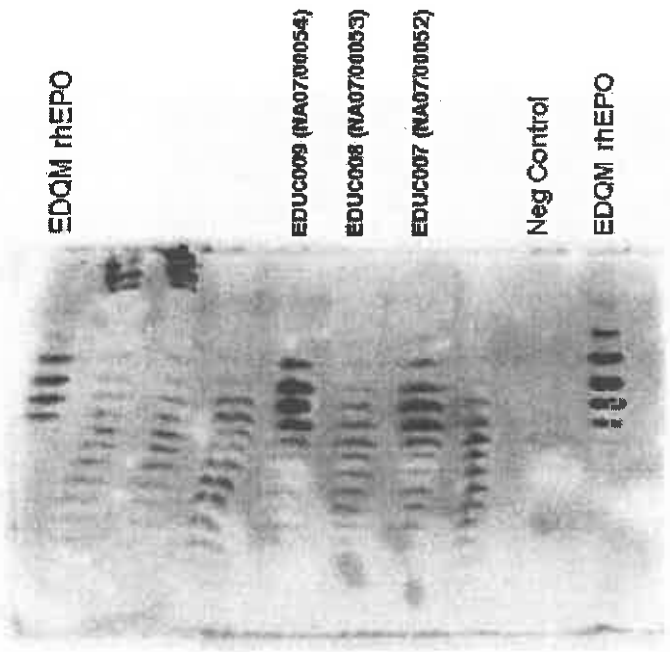


Confirmation:

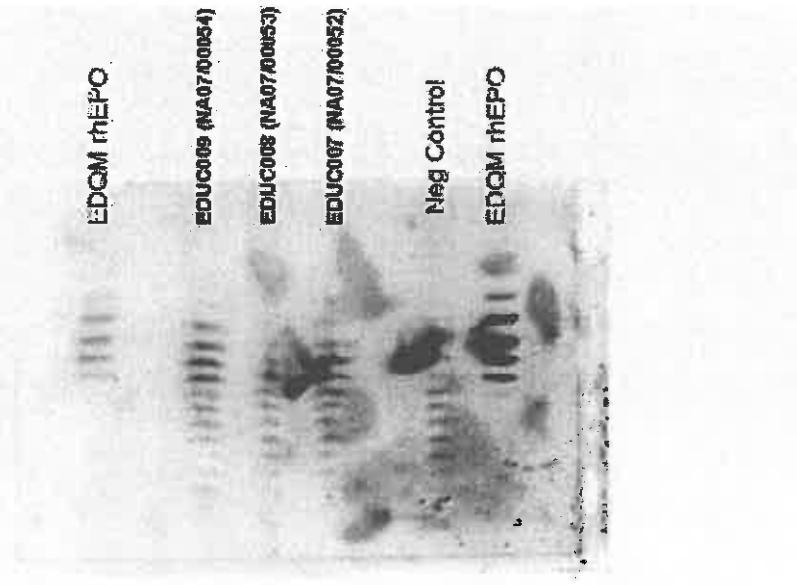


Laboratory 11

Screening:

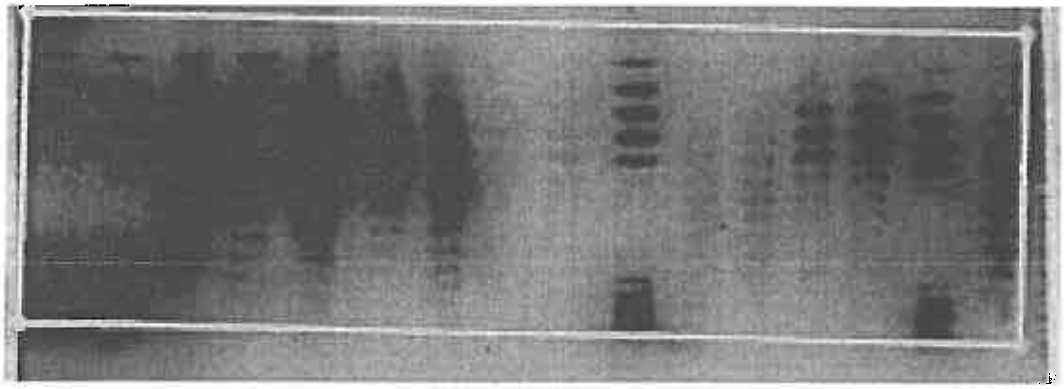


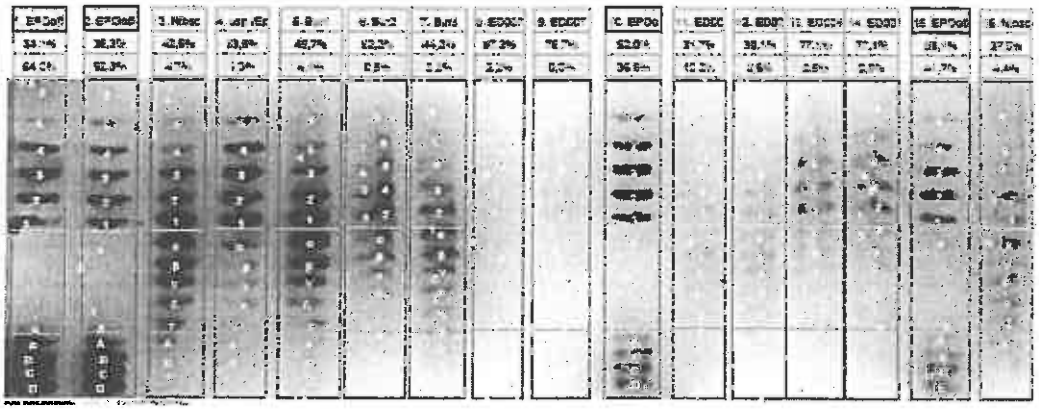
The analysis was repeated:



Laboratory 12

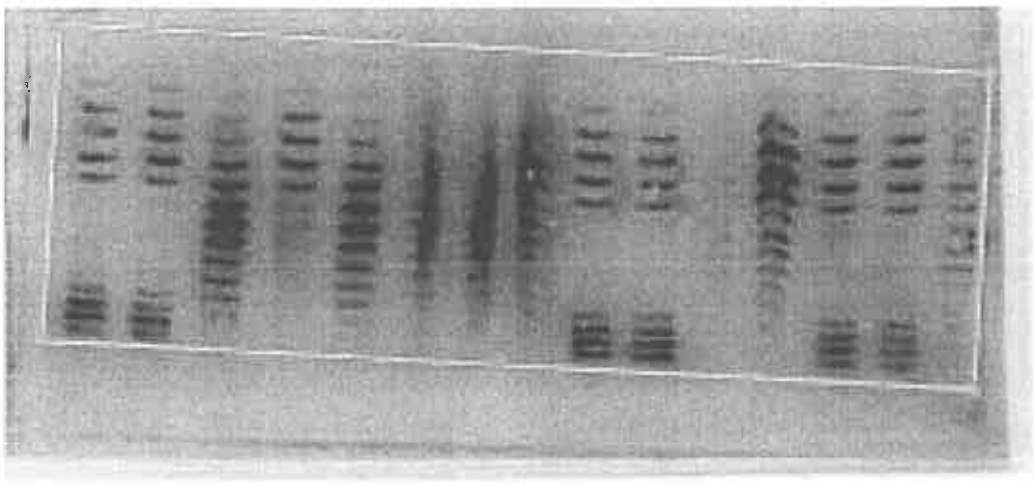
Screening:

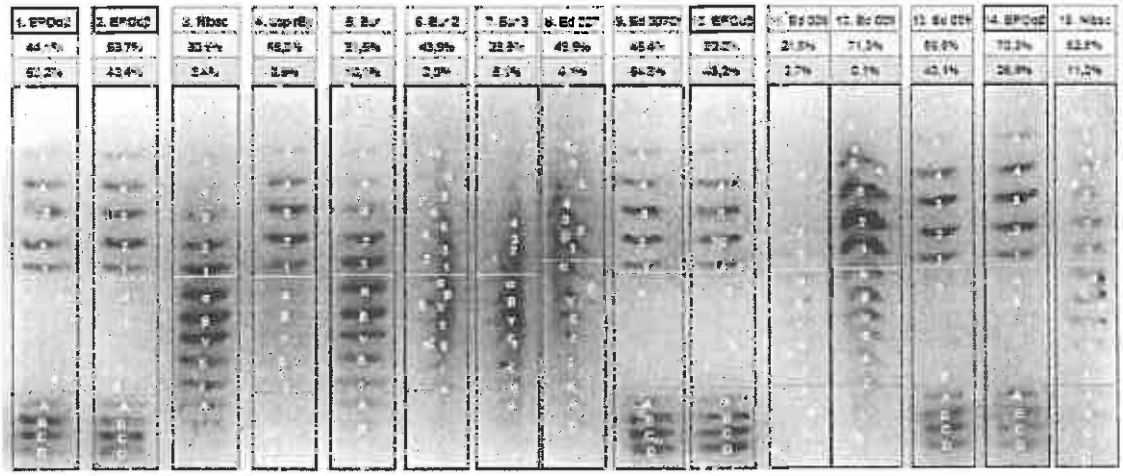




Laboratory 12

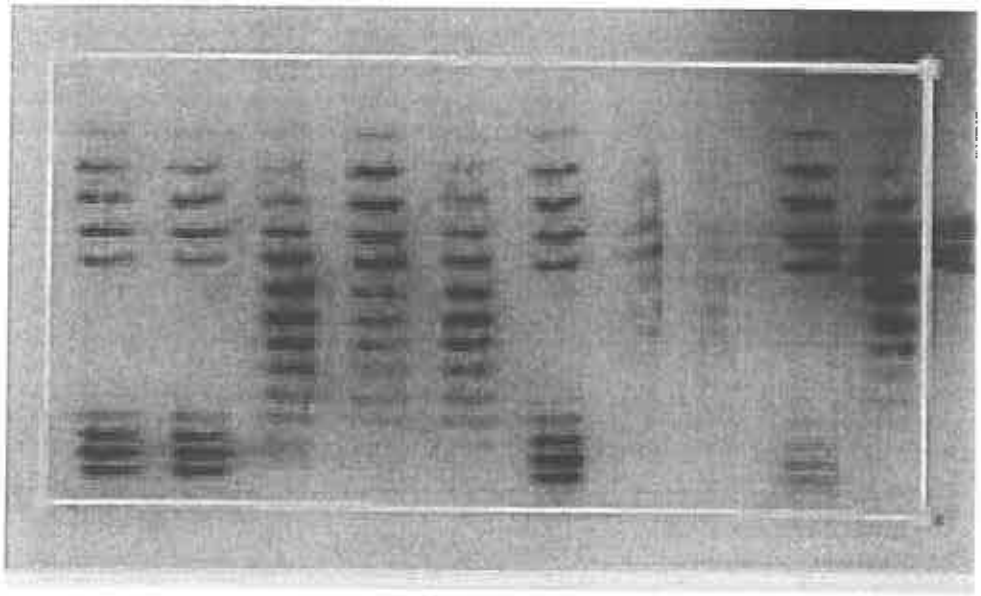
Confirmation:

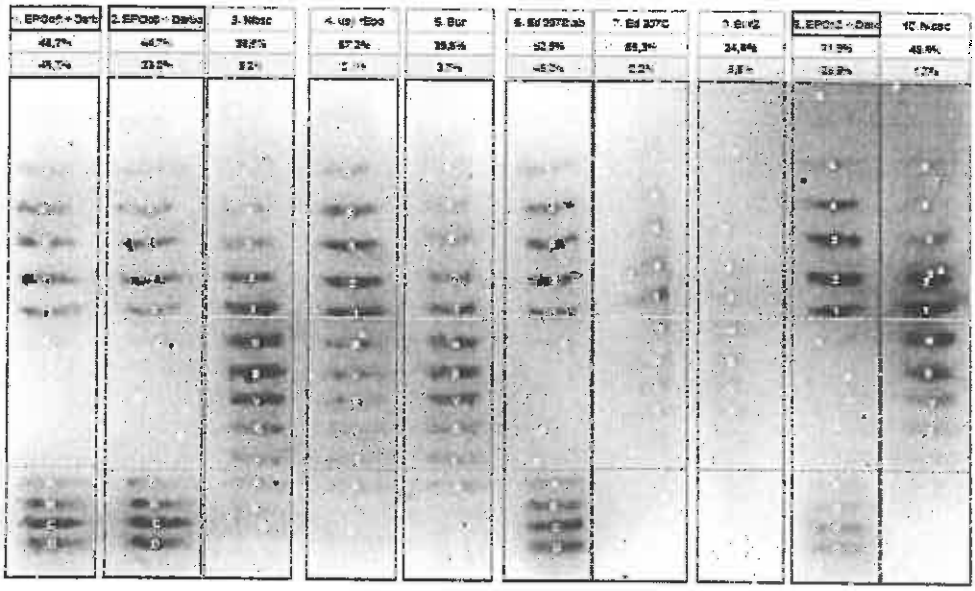




Laboratory 12

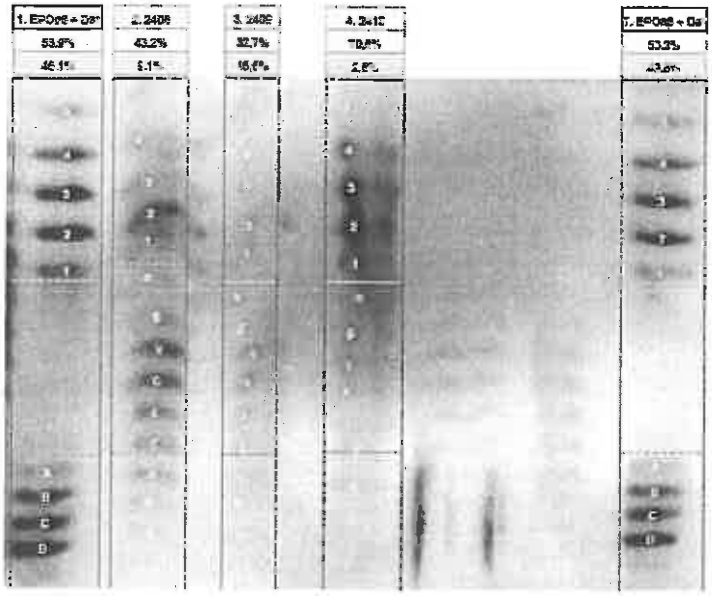
Confirmation repeated for sample EDUC 007:
(by processing a lower amount of retentate)





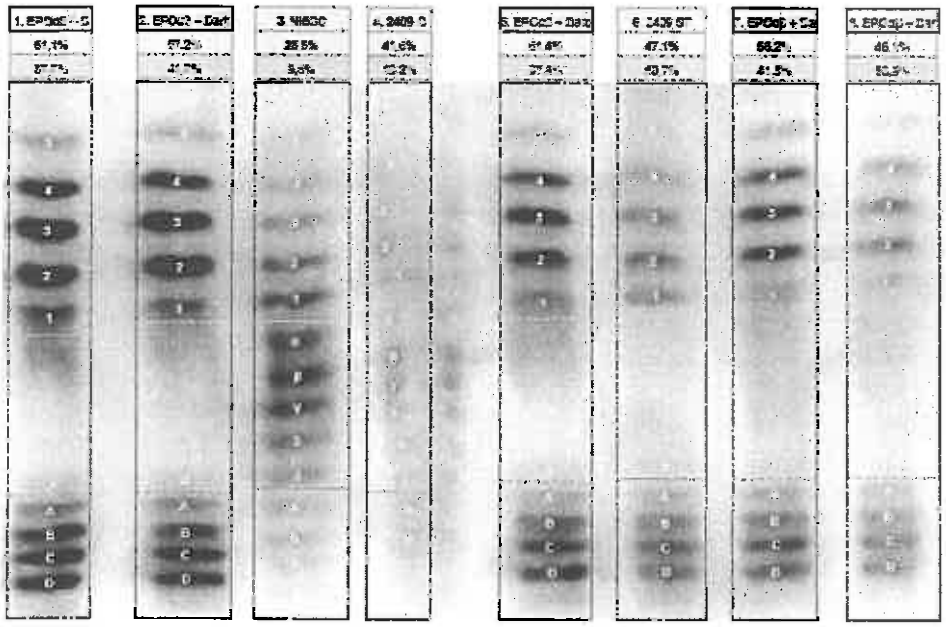
Laboratory 13

Screening:



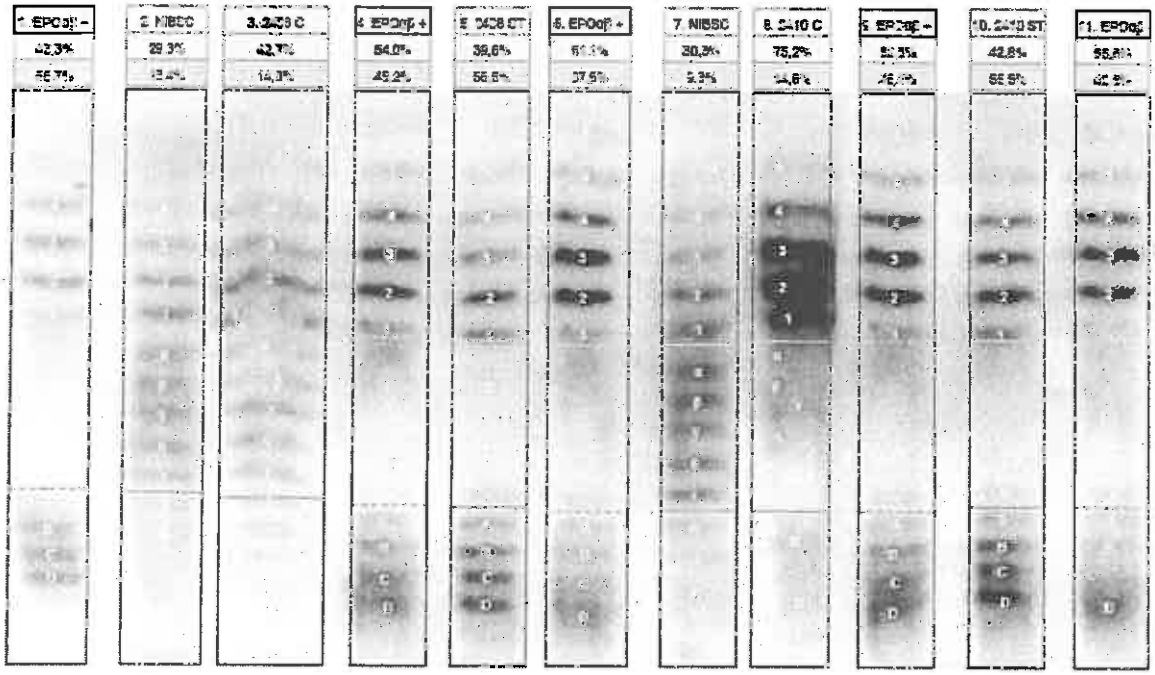
2408 → WADA EDUC 007;
2409 → WADA EDUC 008;
2410 → WADA EDUC 009.

Confirmation: 2409 (WADA EDUC 008)



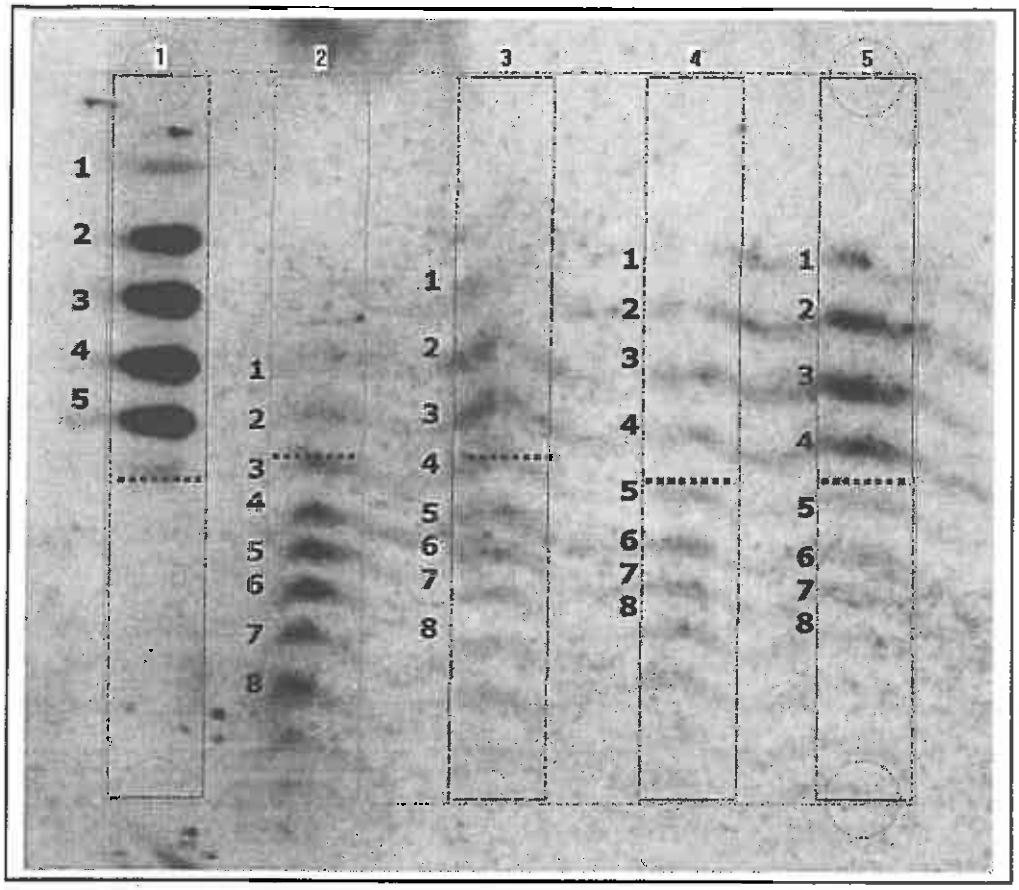
Laboratory 13

Confirmation: *2408 (WADA EDUC 007) & 2410 (WADA EDUC 009)*



Laboratory 15

pH 6

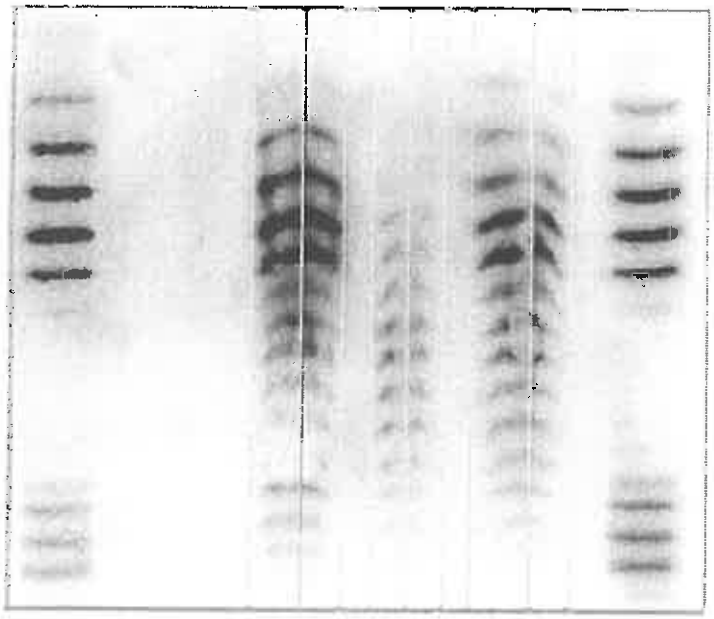


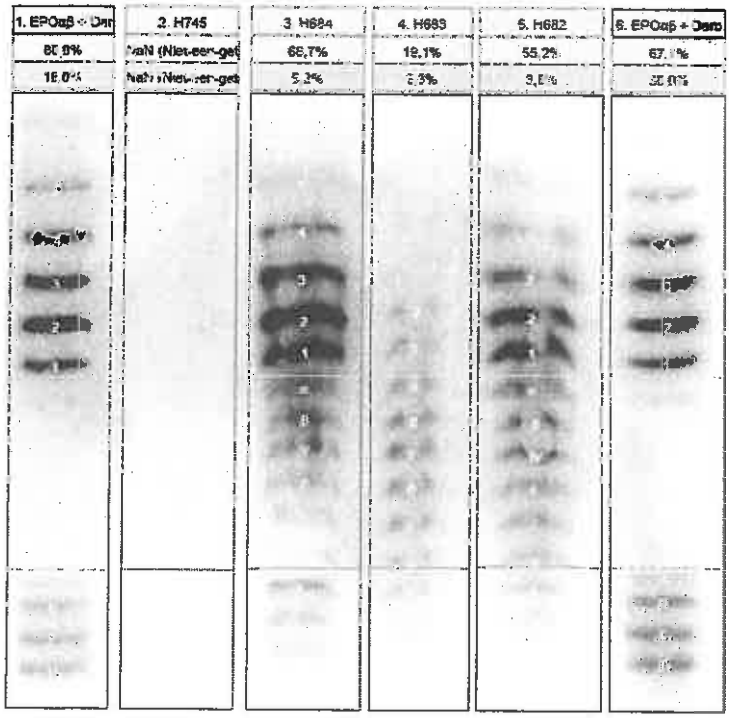
pH 2

Std BRP	Blk	545 (EDU 007)	546 (EDU 008)	547 (EDU 009)

Laboratory 17

Screening:

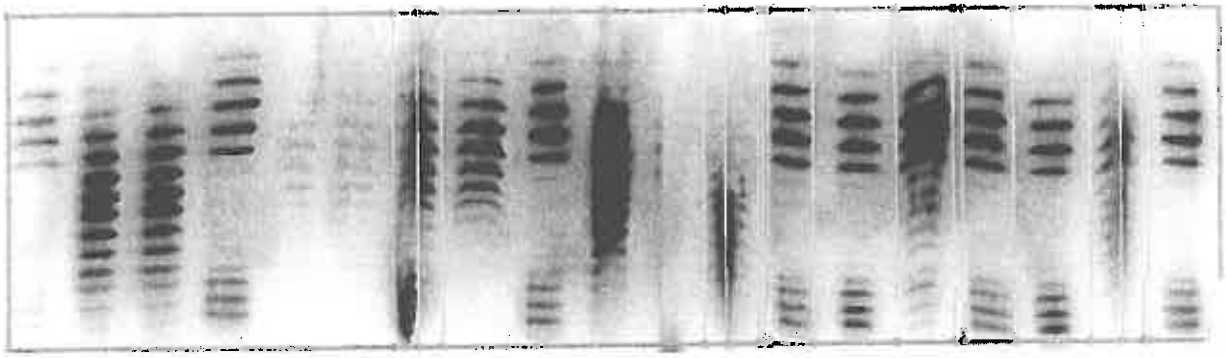


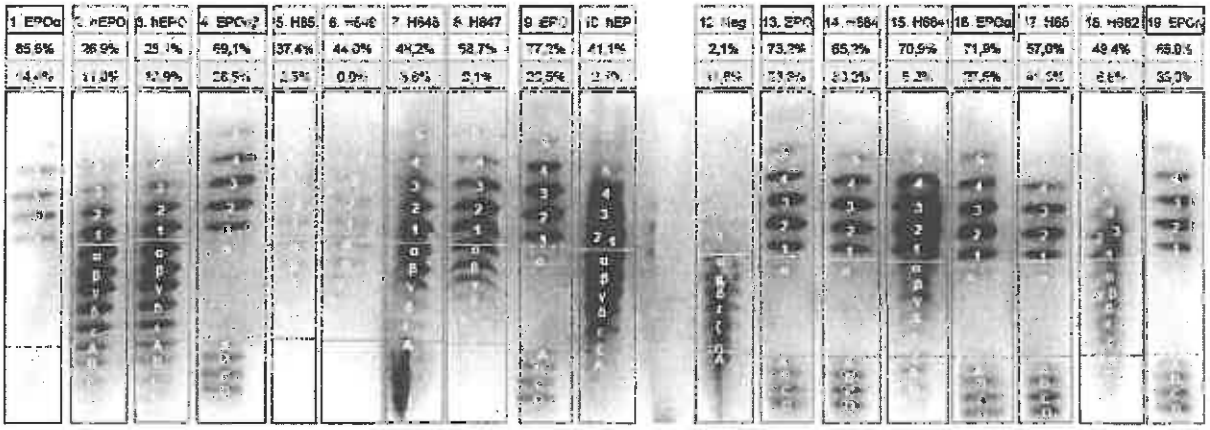


EDUC007 – laboratory code H682
EDUC008 – laboratory code H 683
EDUC009 – laboratory code H684

Laboratory 17

Confirmation:

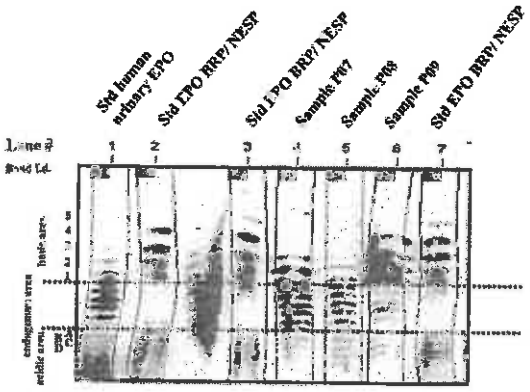




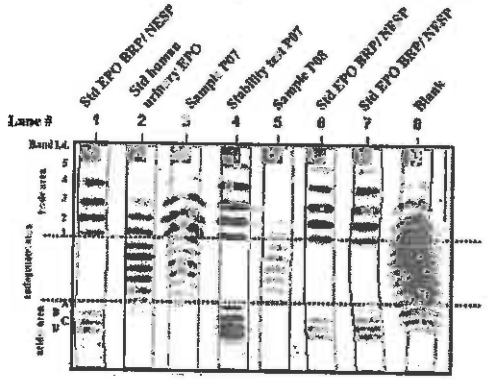
EDUC007 – laboratory code H682
EDUC008 – laboratory code H 683
EDUC009 – laboratory code H684-

Laboratory 18

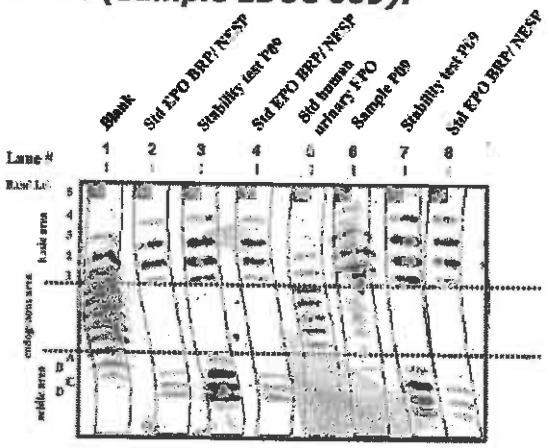
Screening:



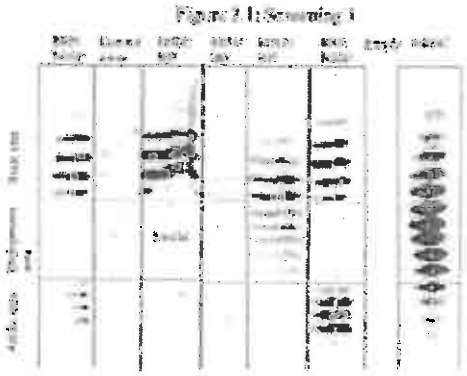
Confirmation (Sample EDUC 007):

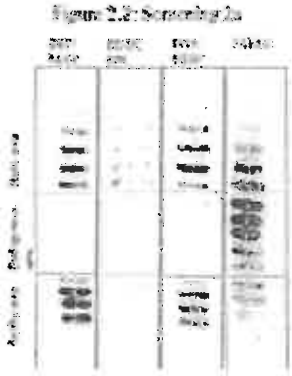


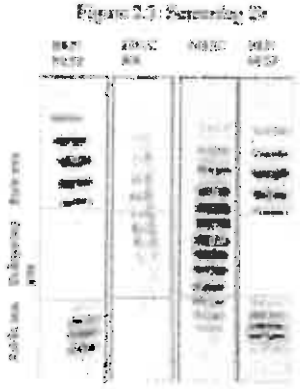
Confirmation (Sample EDUC 009):

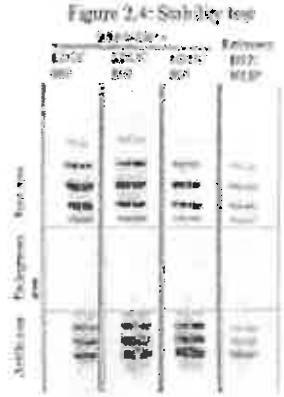


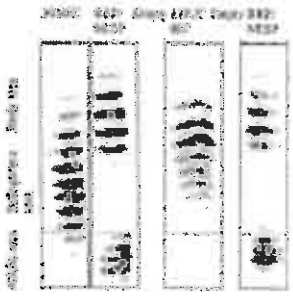
Laboratory 23

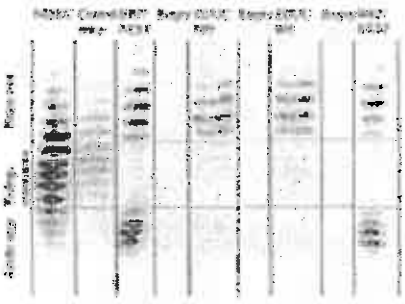






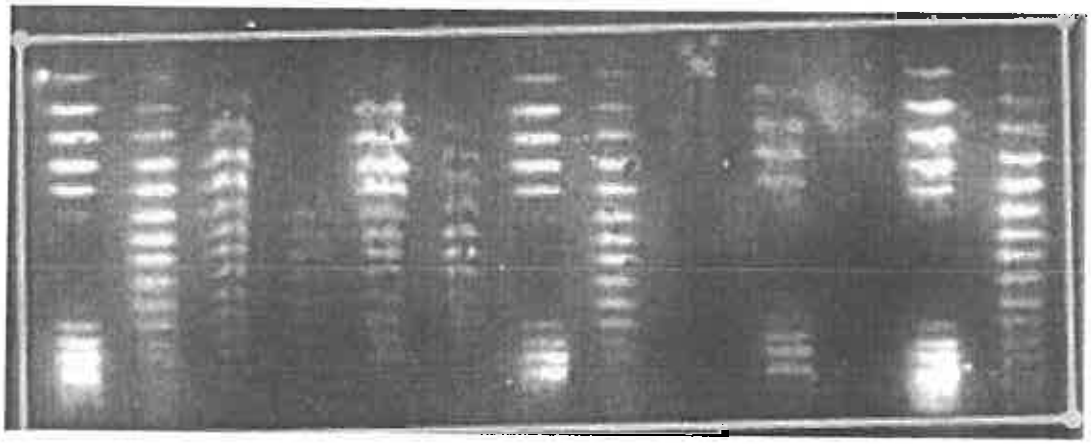


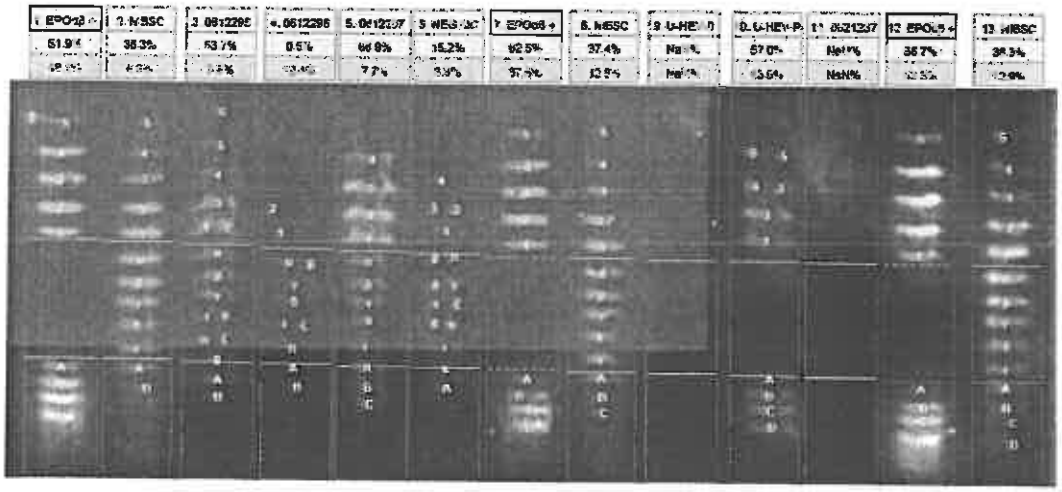




Laboratory 24

Screening:

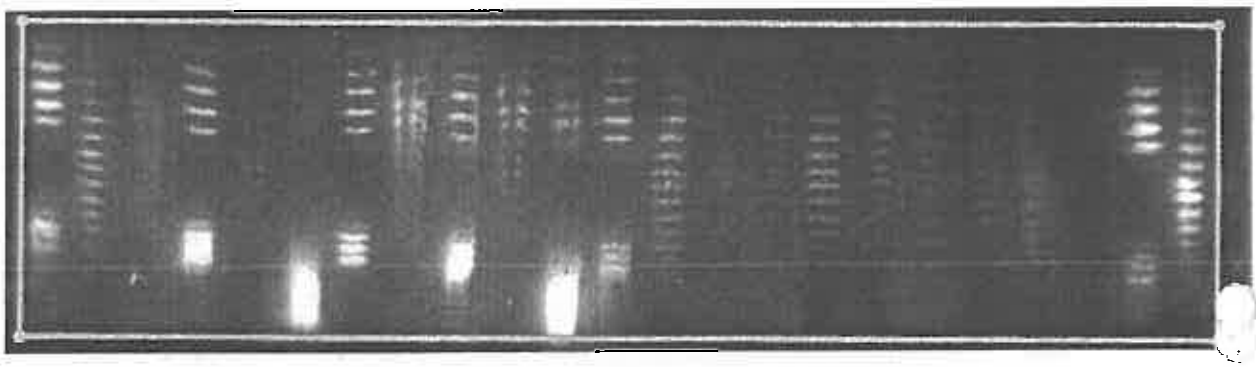


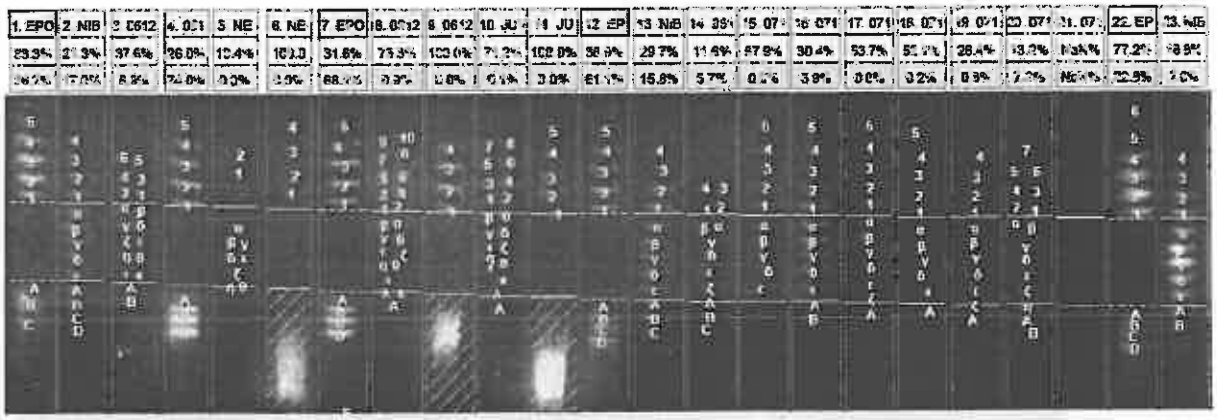


EDUC007 – laboratory code 0612295
EDUC008 – laboratory code 0612296
EDUC009 – laboratory code 0612297

Laboratory 24

Confirmation:

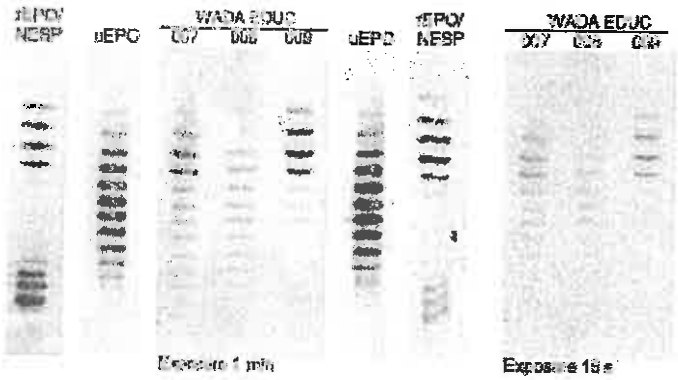




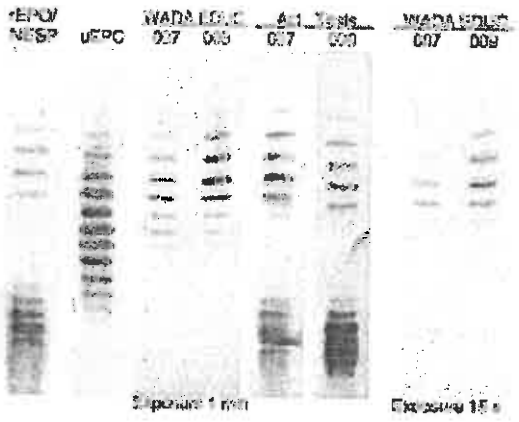
The sample EDUC007 – laboratory code 0612295
The sample EDUC008 – laboratory code 0612296
The sample EDUC009 – laboratory code 0612297

Laboratory 25

Screening:

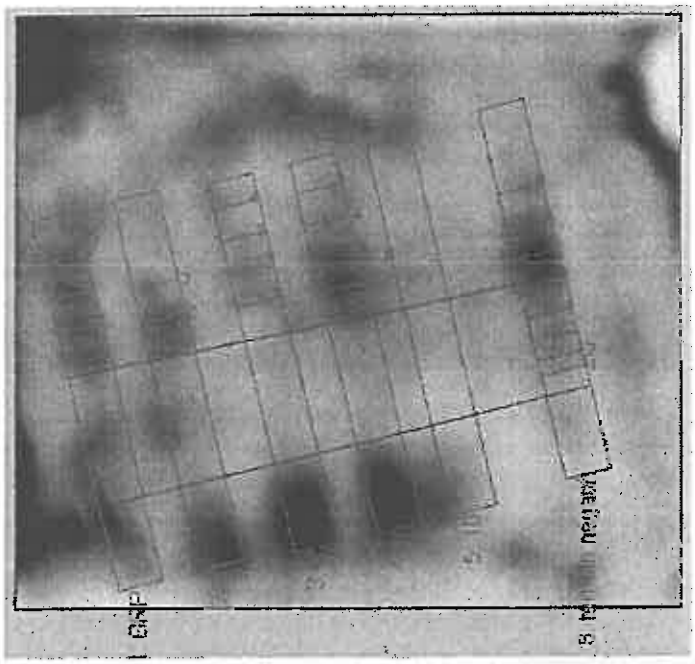


Confirmation:

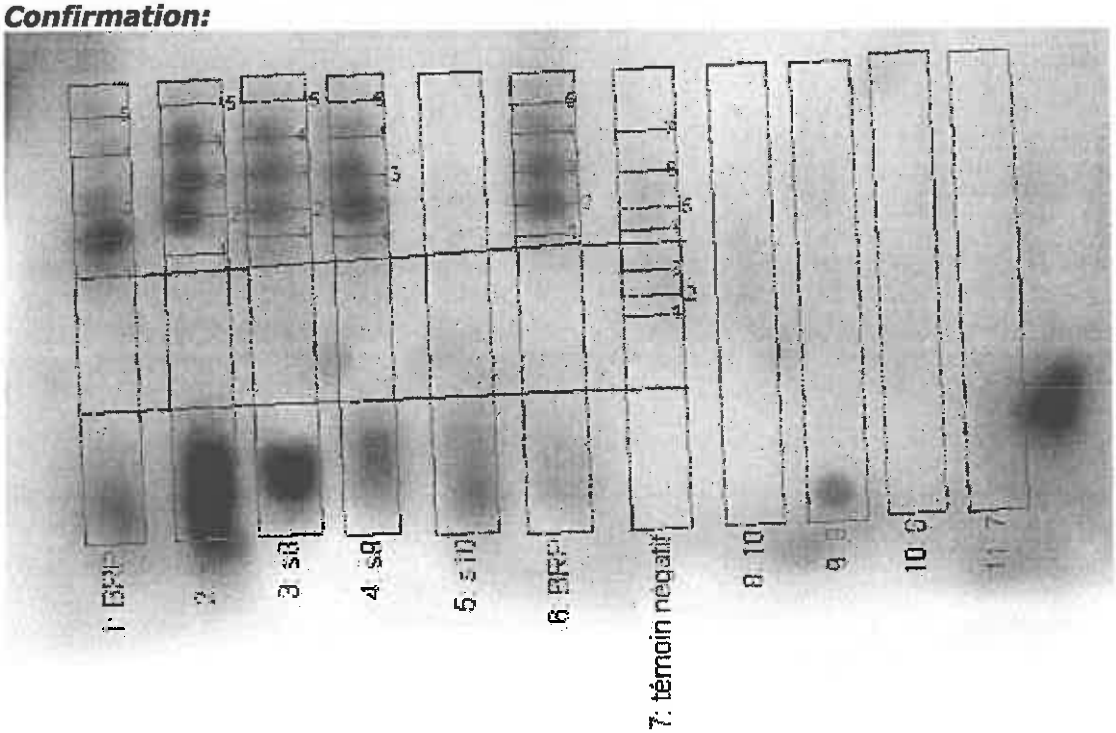


Laboratory 26

Screening:



Confirmation:



Laboratory 28

Screening:

Chemiluminescent acquisition by Fujifilm LAS 100 Plus Camera

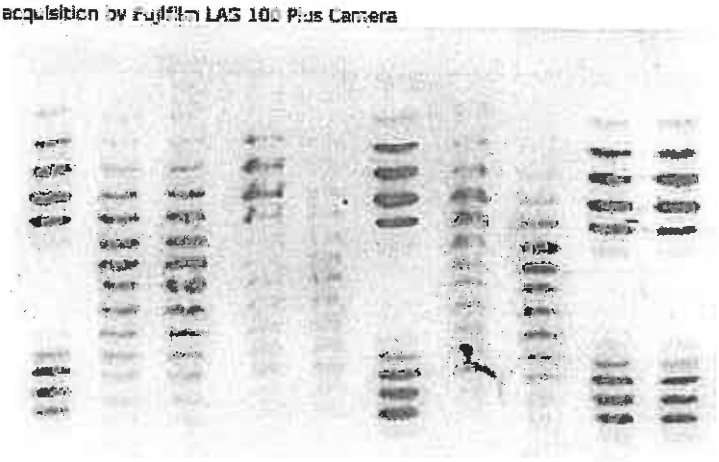
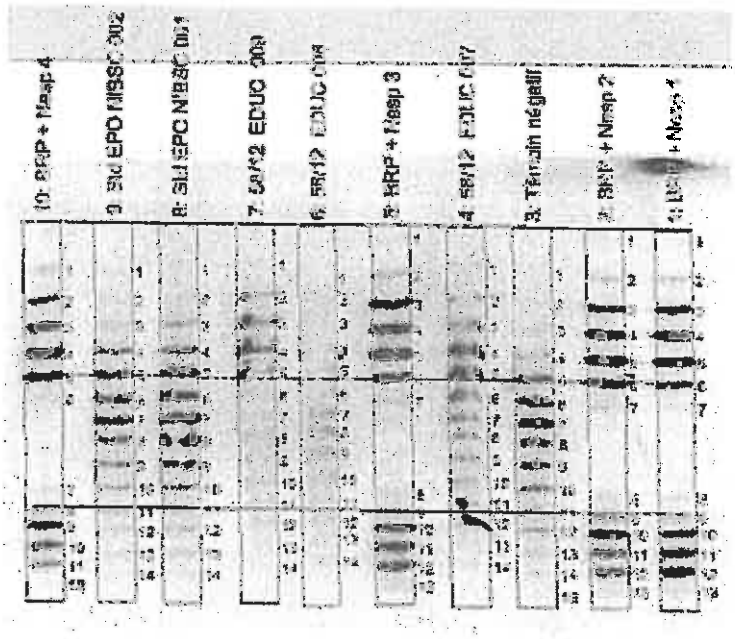
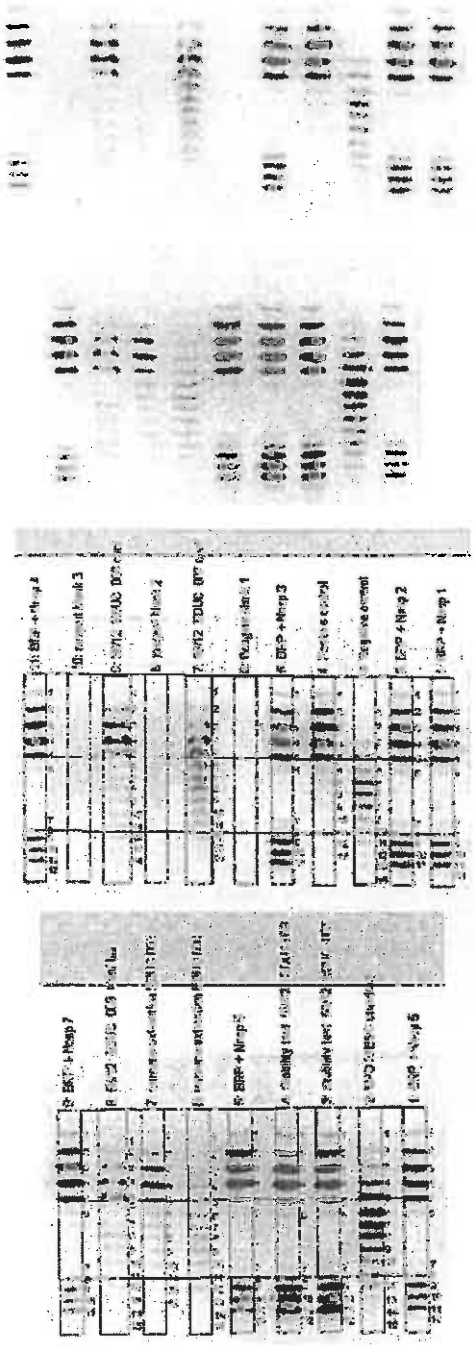


Image integration by AIDA software



Laboratory 28

Confirmation:

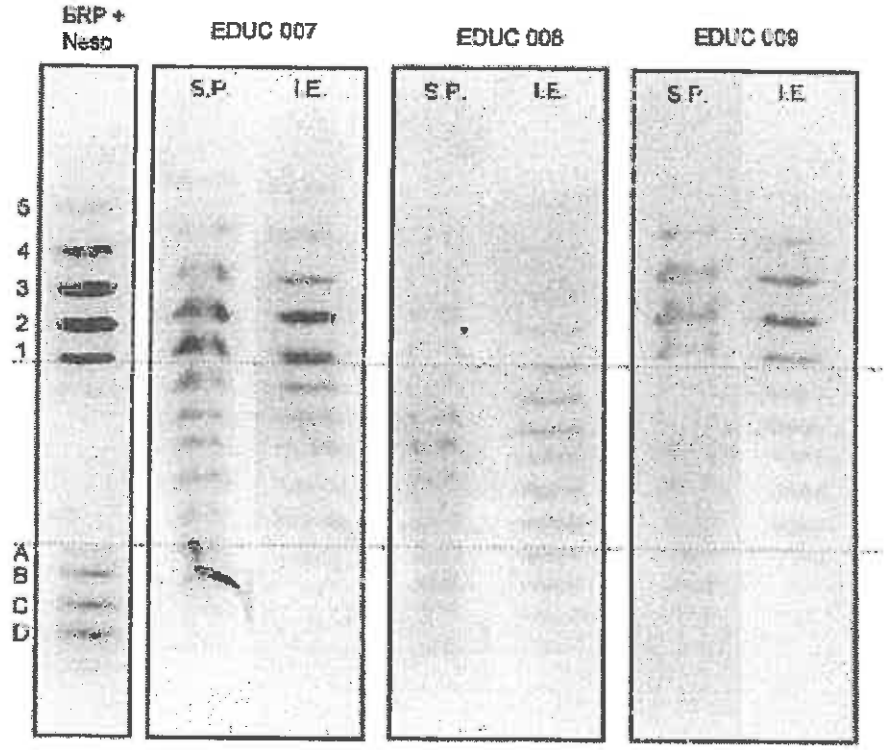


Laboratory 28

Additional step:

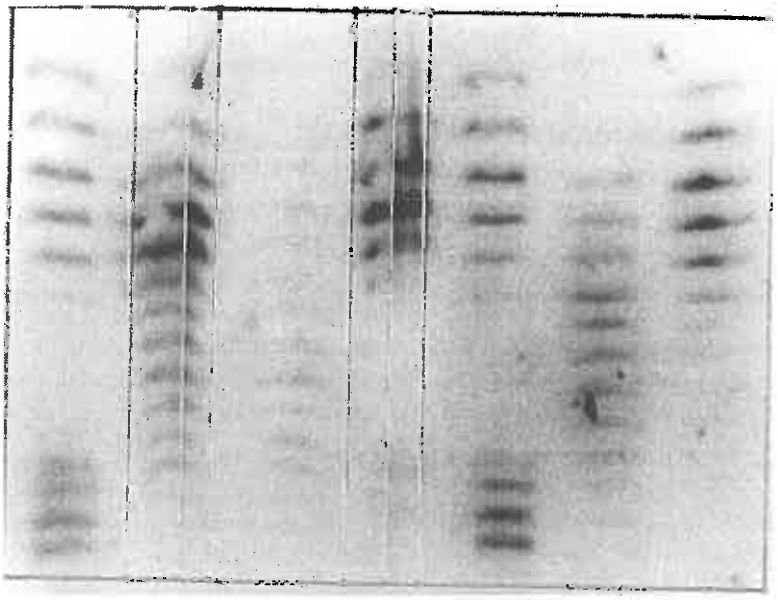
Extraction EPO from urinary matrix was performed by immunoaffinity method and consequently facilitating EPO migration. Finally, artefacts due to protein overload (smears, local signal reducing, isoforms distortion) were eliminated.

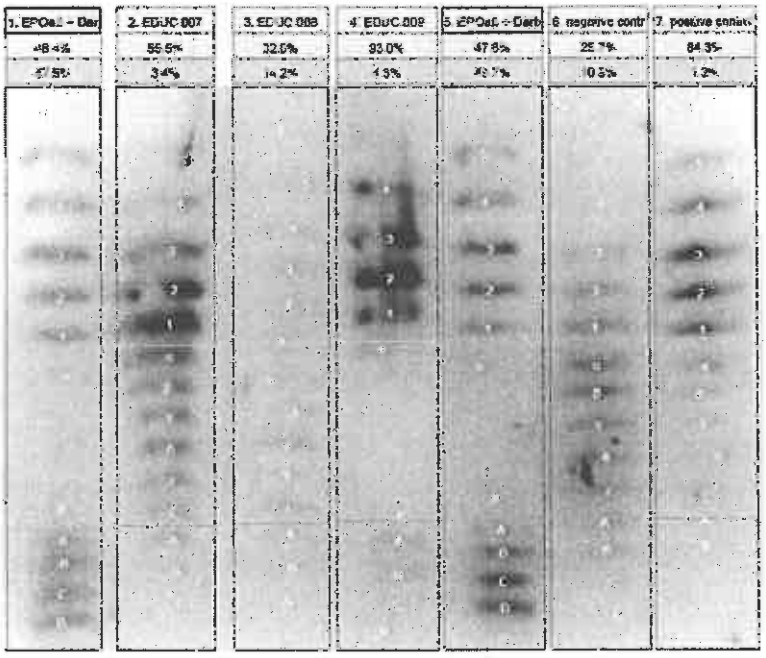
Isoelectric pattern samples comparison between standard method (S.P.) without Immunoextraction process and after Immuno extraction process (I.E).



Laboratory 30

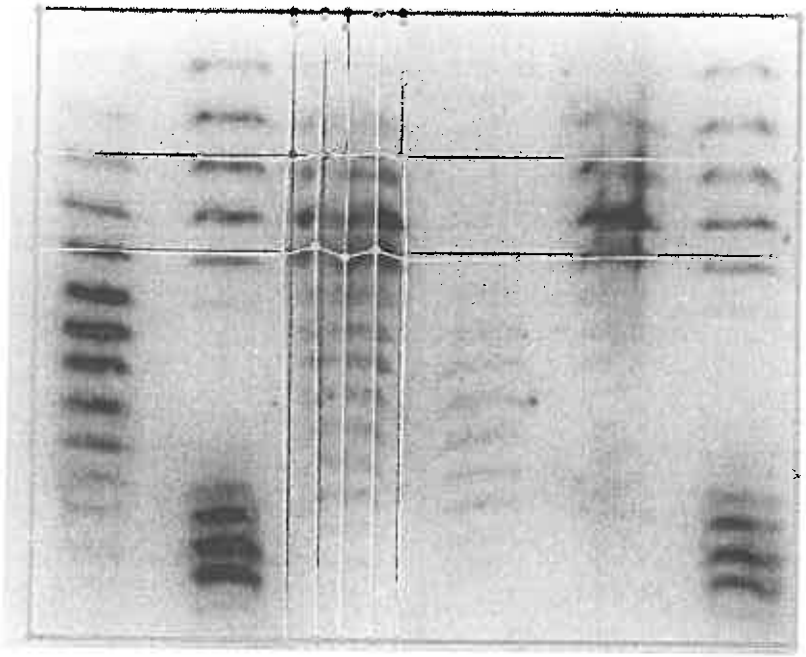
Screening:

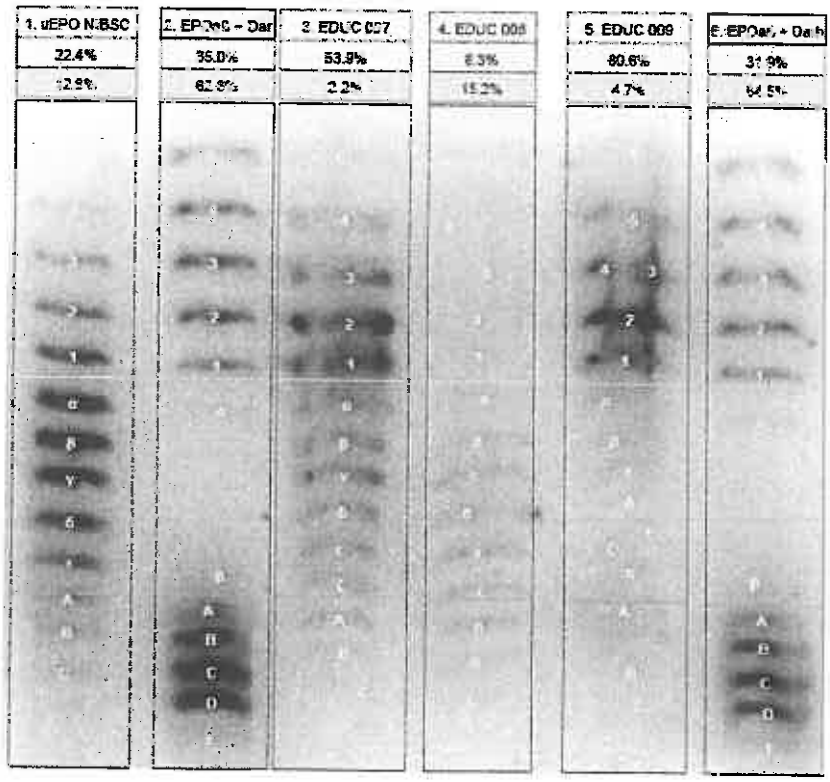




Laboratory 30

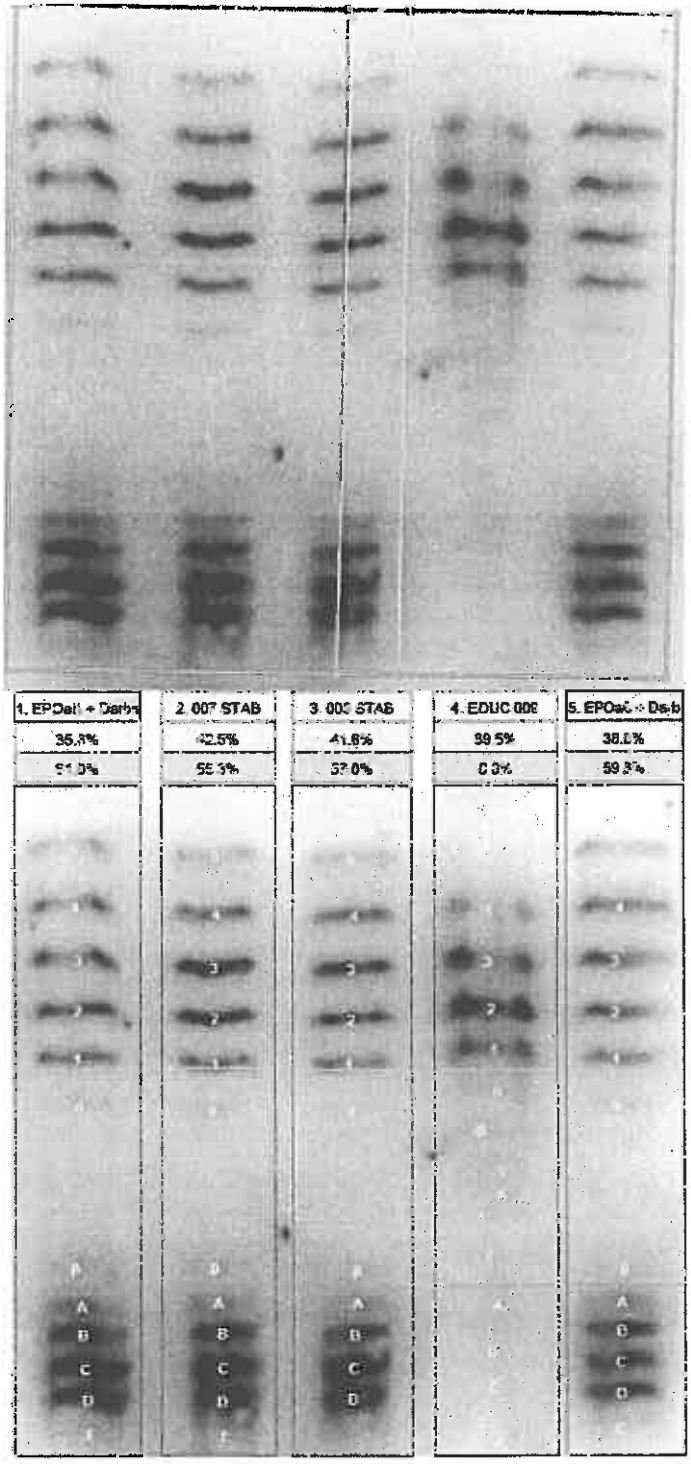
***Confirmation*:**





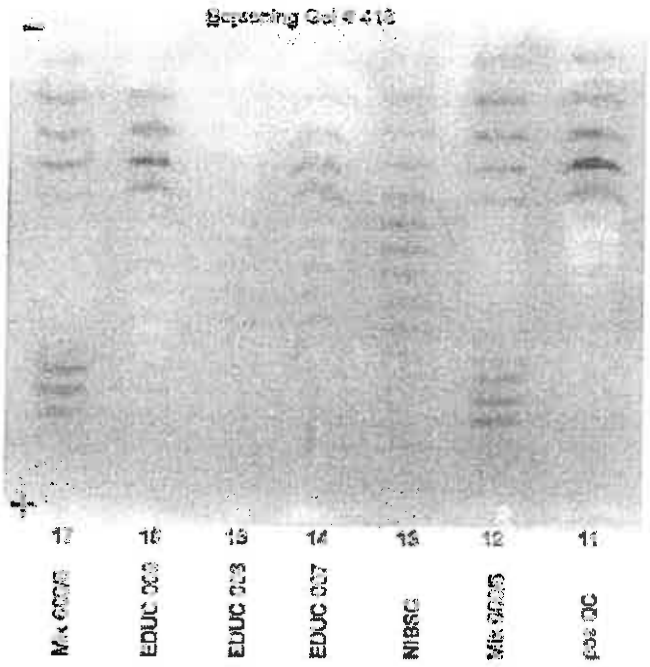
Laboratory 30

Confirmation 2(EDUC 009)/Stability test

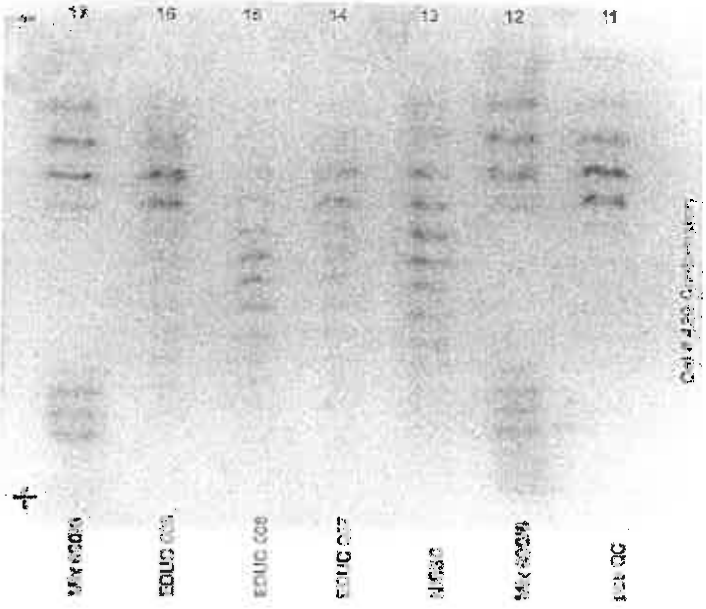


Laboratory 31

Screening:

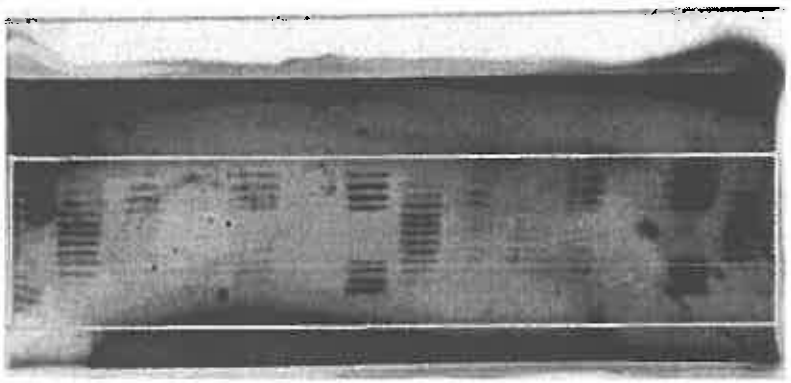


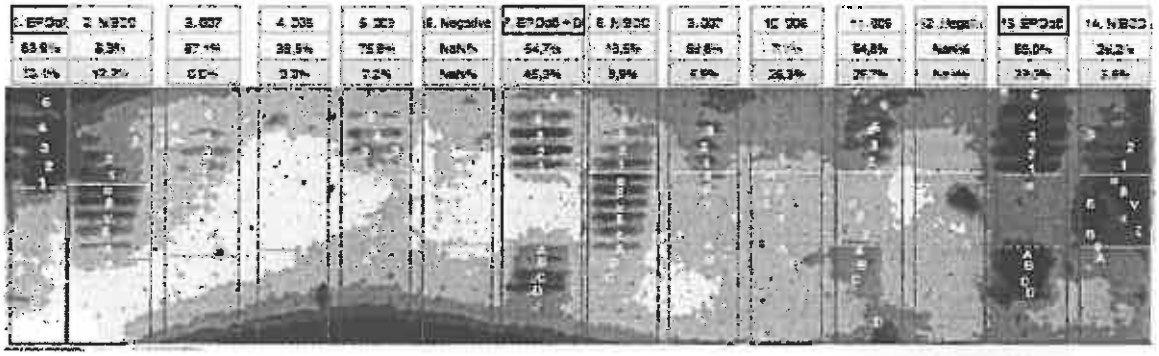
Confirmation:



Laboratory 32

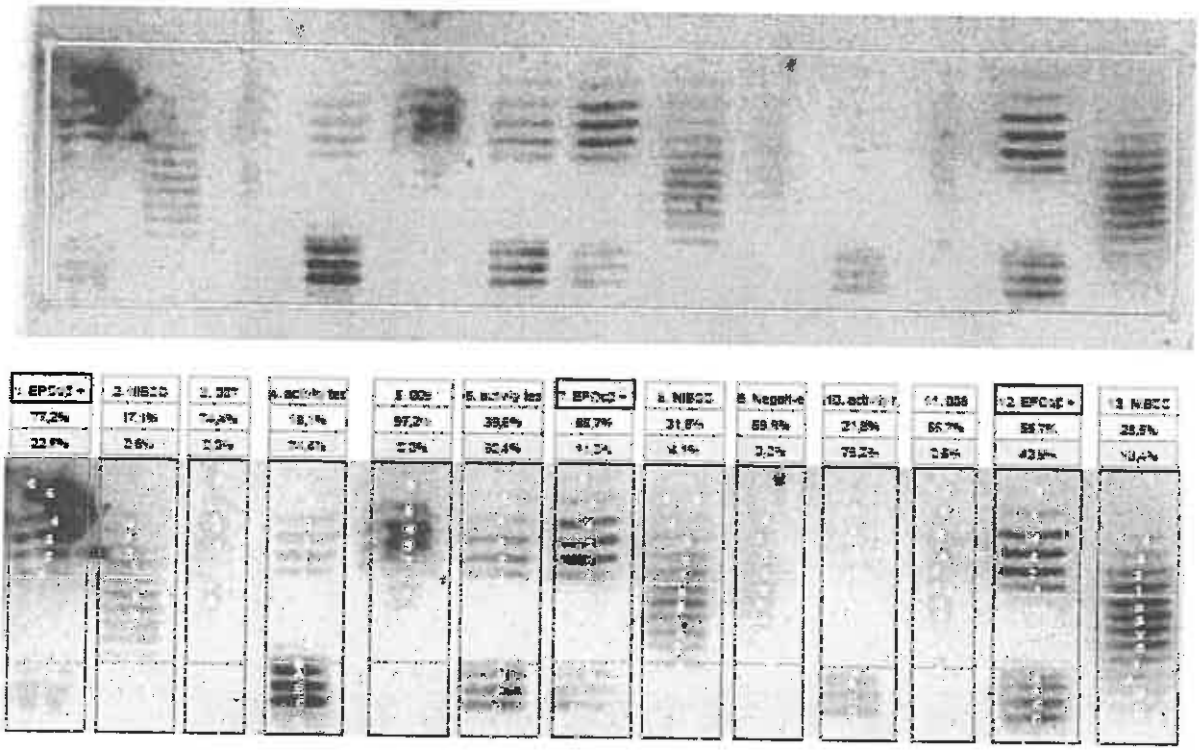
Screening:





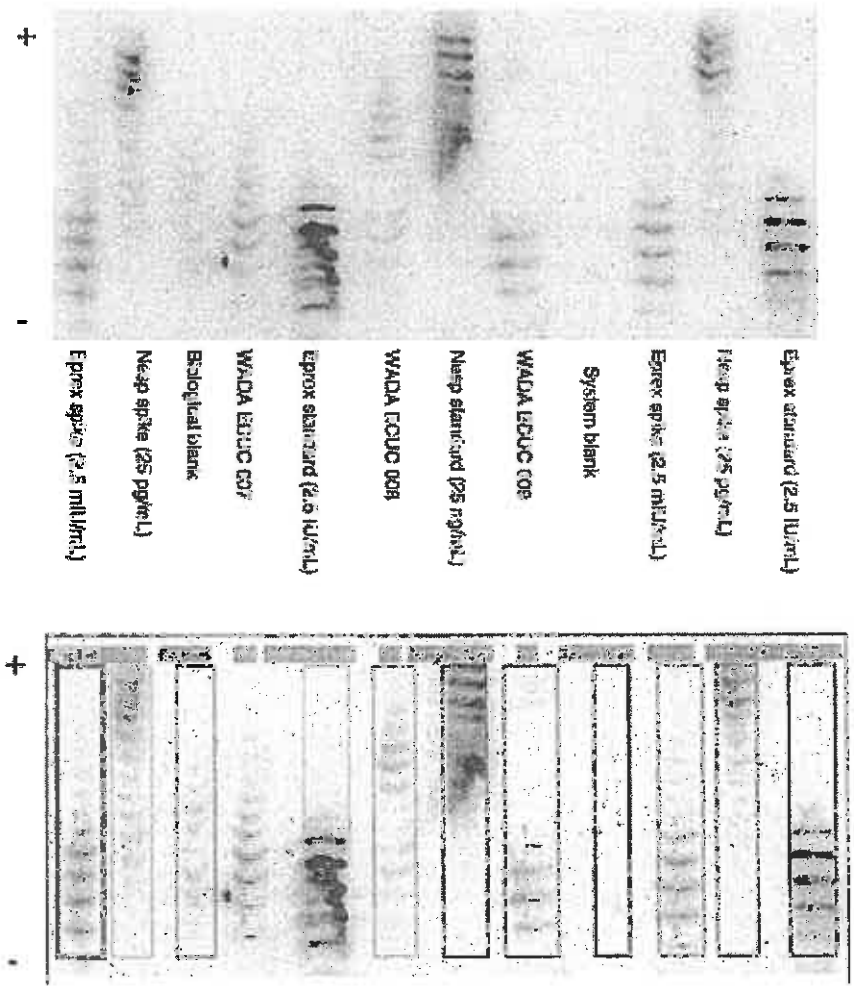
Laboratory 32

Confirmation:



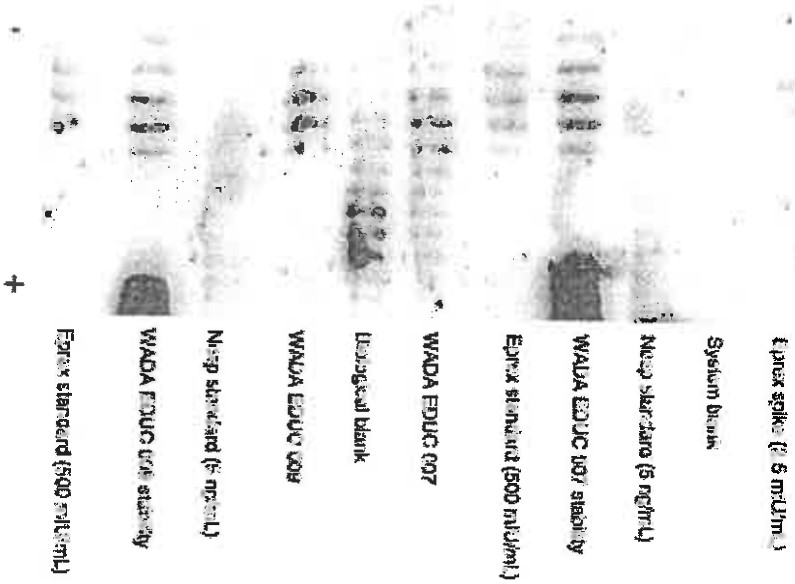
Laboratory 33

Screening:

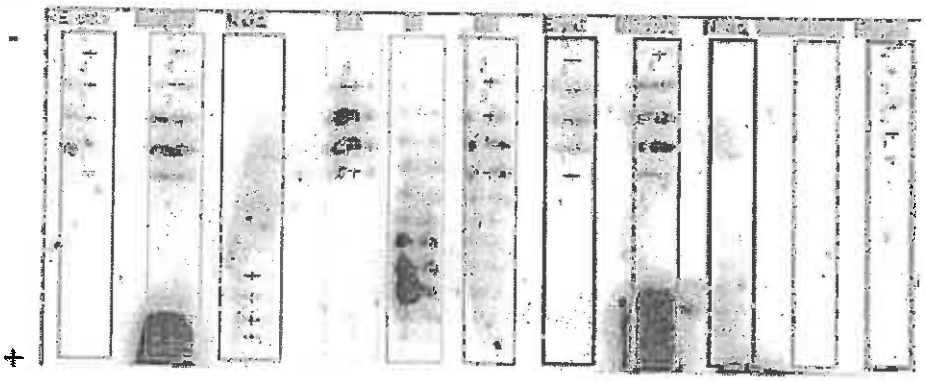


Laboratory 33

Confirmation:

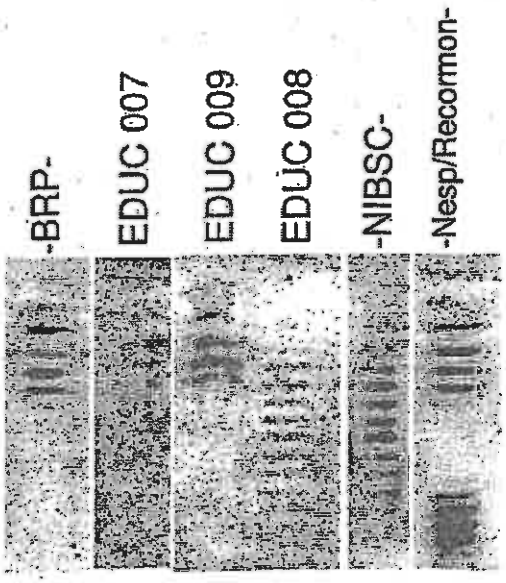


Confirmation:



Laboratory 34

Screening : 18.12.06



Confirmation: 09.01.07