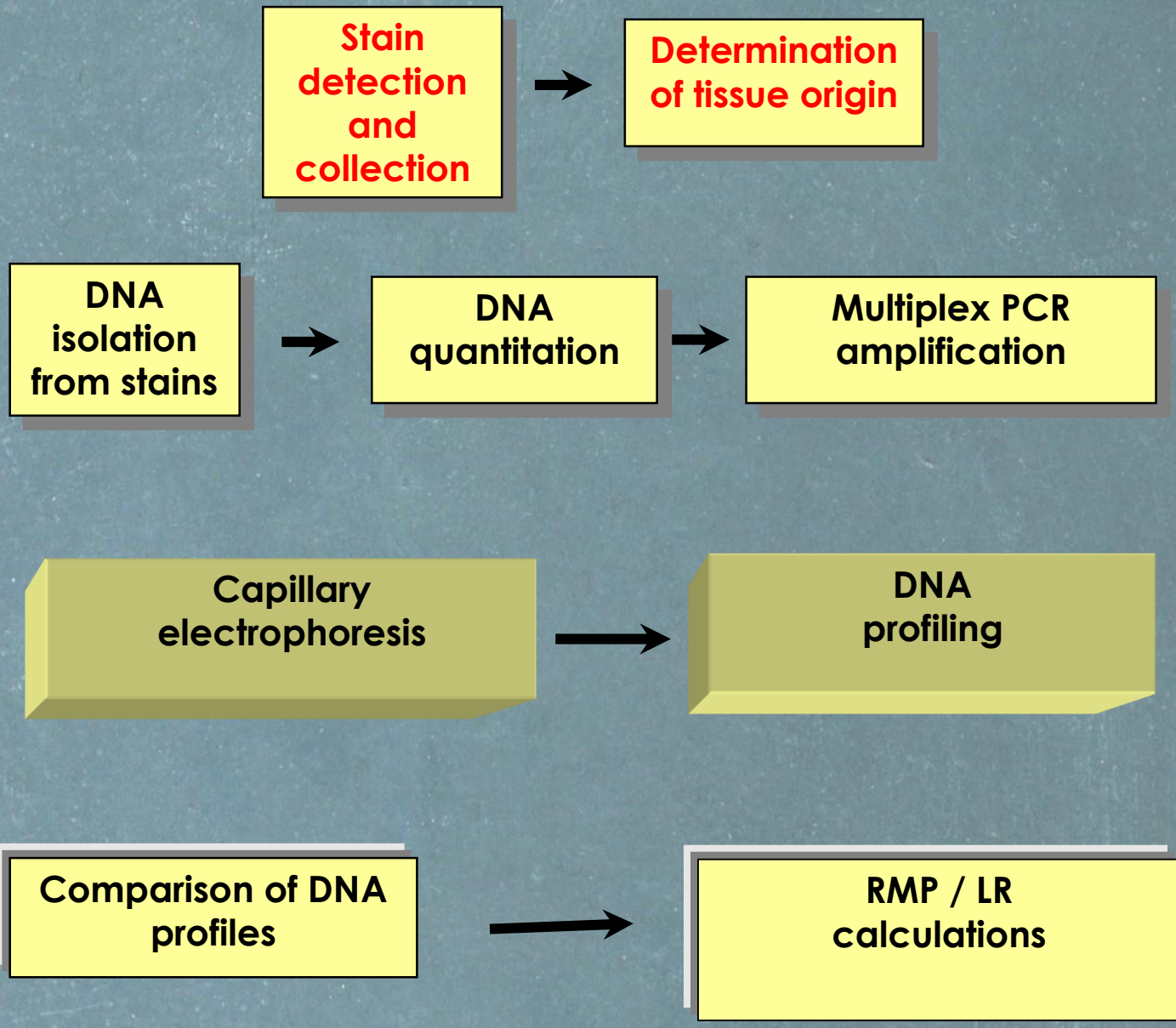
Forensic Genetics and Legal Medicine 2019-2020

18th May 2020

Stain detection and body fluid identification


Stain detection and collection
Determination of tissue origin
DNA isolation from stains
DNA quantitation
Multiplex PCR amplification
Capillary electrophoresis
DNA profiling
Comparison of DNA profiles
RMP / LR calculations

Stain detection

- ✓ Stains may be colorless (e.g. saliva)
- ✓ Stains may be colored but difficult to identify on a dark/colored backgroung (e.g. blood)
- ✓ Stains may be «latent» (e.g. washed blood)

Body fluid identification

- ✓ Presumptive tests (including stain detection methods): high sensitivity, low specificity
- Physical
- Chemical

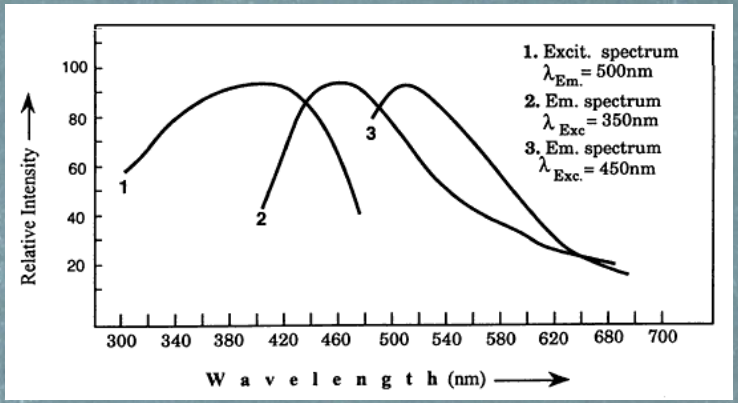
- ✓ Confermative:
- Immunological low sensitivity, higher specificity (compared to presumptive tests), limited range of targeted body fluids/tissues
- Molecular high sensitivity, higher specificity (compared to presumptive tests), broad range of targeted body fluids/tissues

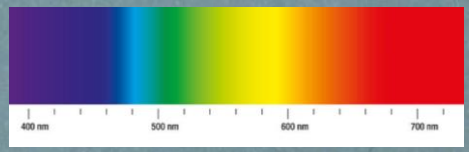
Biological evidences, such as semen, saliva and urine, absorb incident light at particular a wavelength and then re-emits the absorbed energy as light at a longer wavelength (fluorescence).

Alternate light sources (ALS) are equipments that emit concentrated light source of specifical wavelenght (monochromatic light).

A filter is required to screen out any reflected incident light or other competing light. To achieve this filtering effect, a series of different-colored goggles can be used.

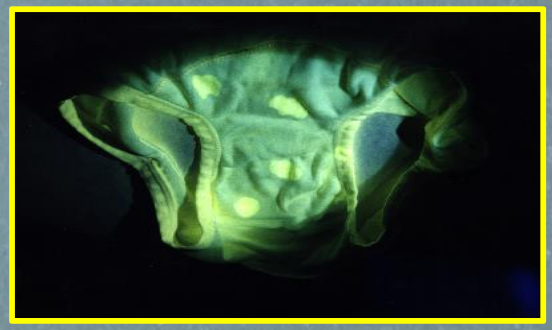
Semen





Excitation light	Goggles/Filters	Colour of the observed stain
UV	No goggles needed, but recommended to wear UV safety goggles	Blue
Violet	Yellow goggles	Yellow
Blue	Yellow goggles	Yellow
Green	Orange goggles	Orange
Green-yellow	Red goggles	Red
Green-yellow	Violet filters (425nm)	Black

Stoilovic. Forensic Sci Int 1991



Saliva

Excitation light	Goggles/Filters	Colour of the observed stain
Long UV [1,9,16]	No goggles needed, but recommended to wear UV safety goggles	White-bluish
415nm [4,15]	Yellow goggles/555nm interference filters	Not stated in literature
450nm [4,15]	Orange goggles/555nm interference filters	White (Orange goggles)
470nm [15]	530nm/555nm interference filters	Not stated in literature
490nm [15]	555nm interference filters	Not stated in literature
505nm [15]	555nm interference filters	Not stated in literature
532nm [9]	Goggles that block 532nm light	Yellow-orange

Notes: interference filters allow only the desirable wavelength pass through.

→ no specificity

Lee et al., Malay J Forensic Sci 2010

Urine

Excitation light	Goggles/Filters	Colour of the observed stain
UV [16]	No goggles needed, but recommended to wear UV safety goggles	Depends on abnormal substance presence
415nm [4]	Yellow goggles	Not stated in literature
450nm [4]	Orange goggles	White
505nm [4]	Red goggles	Not stated in literature
532nm [9]	Goggles that block 532nm light	Yellow-orange

→ no specificity

Lee et al., Malay J Forensic Sci 2010

False positives

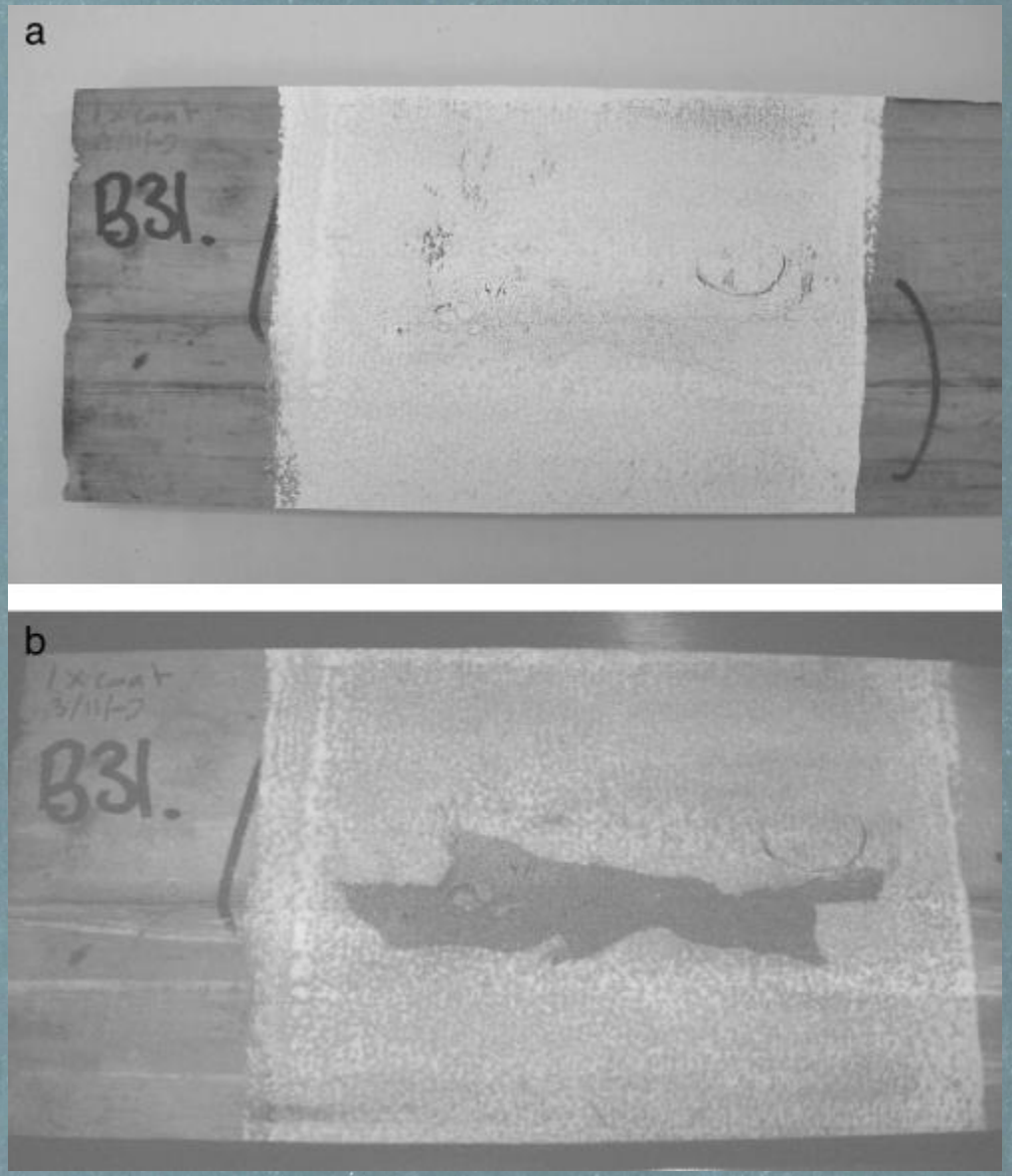
Examples of substances that fluoresce under ALS and may appear as stains on a garment under natural light include: laundry detergents, grease, lipstick, ink...

Vandenberg et al. J Forensic Sci 2006

Blood

dry blood does not show significant fluorescence, but it has a high absorption at 415 nm due to the presence of haemoglobin.

ALS, therefore, are able to enhance the contrast of bloodstains towards their background.



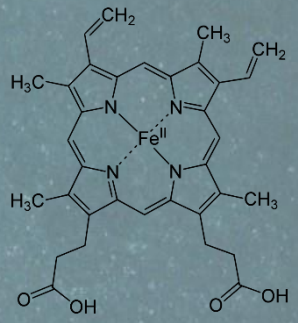
Bloodstain on wood under one coat of white acrylic-based paint: (a) natural light and (b) 415 nm excitation, viewed through yellow goggles.

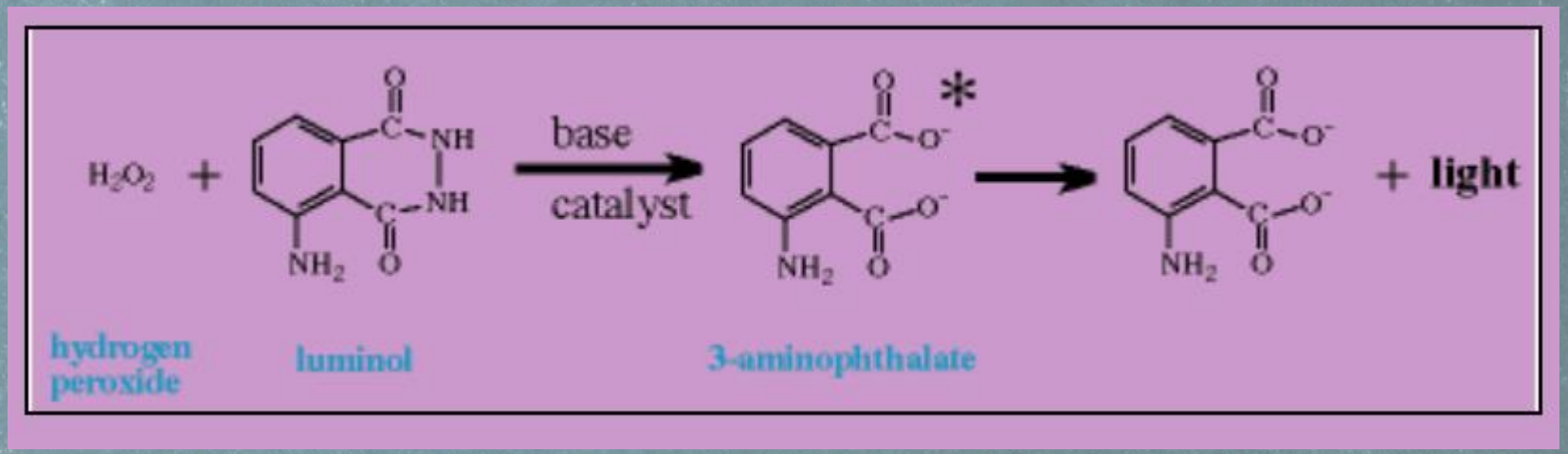
Vandenberg et al. J Forensic Sci 2006

Luminol (5-amino-2,3-dihydro-1,4-phthalazine-dione)

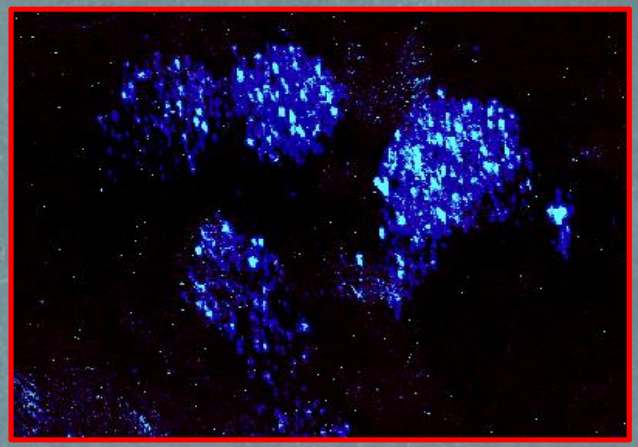
Chemiluminescence occurs after oxidation of luminol by an oxidant (e.g. hydrogen peroxide) in a basic aqueous solution.

Many metal cations (including iron in heme) catalyze the reaction increasing the speed of the oxidation and thus the onset/intensity of light production.





Detect latent stains



Select relevant visible stains

False positives (beside non-human blood)

Table 3. Spectral measurements showing the major interferences with the luminol test for blood detection‡. Errors shown are 95% confidence intervals in the mean values

Interfering substance	Mean peak wavelength shift from haemoglobin (nm)	Replicate peak intensities (arbitrary units)	Mean intensity (% of haemoglobin value)
Copper metal	2 ± 10	239, 254, 255, 255	106 ± 10
Enamel paint (Dulux®)	*9 ± 4*	*227, 235, 238, 245*	*100 ± 10*
125 g/L $NaClO_{(aq)}$ common household bleach	9 ± 4	174, 179, 210, 230	84 ± 22
Dark green spray paint (Taubman®)	22 ± 3	149, 180, 183, 255	81 ± 34
Turnip (pulp)	*3 ± 4*	*134, 166, 170, 230*	*74 ± 35*
Parsnip (pulp)	*8 ± 5*	*106, 112, 143, 170*	*56 ± 23*
Roof lining (1992 Ford Laser®)	13 ± 7	45, 45, 45, 70	22 ± 11
Horseradish (pulp)	*3 ± 4*	*33, 40, 60, 61*	*20 ± 12*
Wooden-furniture polish (Goddard's®)	11 ± 23	44, 47, 47, 53	20 ± 4

Creamer et al. Luminescence 2003

Bleach is volatile and evaporates quickly: its interference is negligible after 8-16 h
Bleach is less effective than water in removing blood chemiluminescence

Table 1. Effect of drying time on luminol chemiluminescence from bleach stains*

Drying time (h)	125 g/L NaClO (a.u.)	10 g/L NaClO (a.u.)
<1	80 ± 21	135 ± 38
2	184 ± 23	126 ± 32
8	–	11.7 ± 2.3

* No chemiluminescence was observed after 16 h for either bleach concentration.

Creamer et al. Luminescence 2005

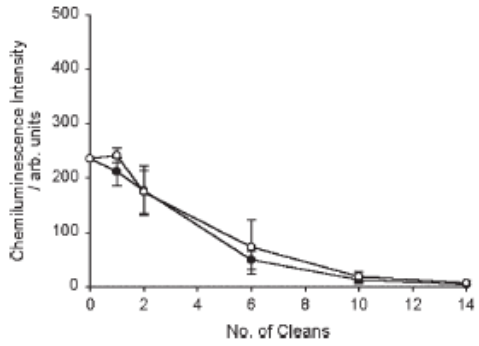


Figure 1. The effect of cleaning bloodstains on the resultant chemiluminescence from the luminol test. ●, wet blood and water; ○, dry blood and water.

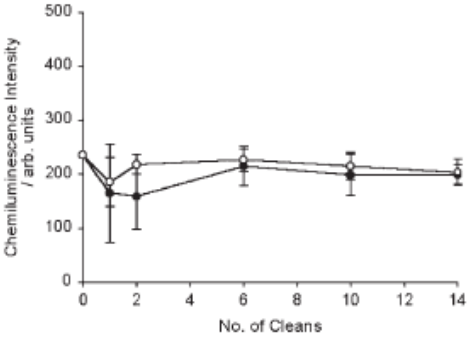


Figure 2. The effect of cleaning bloodstains on the resultant chemiluminescence from the luminol test. ●, wet blood and commercial bleach; ○, dry blood and commercial bleach.

Presumptive chemical tests

The stain is put against a substrate that can change in color in the presence of tissue-specific catalytic/enzymatic activity in the stain

Select visible stains

Blood

Iron in the heme group catalyzes peroxidation of (e.g.) tetramethylbenzidine in common urine sticks.

False positives

Urine sticks

TABLE 3—*Specificity results for the six different reagents.*

	Reagent					
Substance	Luminol	LMG	KM	Hemastix®	Hemident™	Bluestar©
Saliva	NR	NR	NR	1 (3)	NR	NR
Semen	NR	NR	1 (25)	NR	0 (25, white)	NR
Potato	NR	0 (6, green)	3 (7)	1 (25)	NR	1 (25)
Tomato	NR	NR	NR	1 (23)	NR	1 (25)
Tomato sauce	NR	NR	4 (6)	NR	NR	NR
Tomato sauce w/meat	NR	NR	NR	4 (6)	NR	1 (25)
Red onion	NR	0 (5, pink)	0 (25, yellow)	1 (21)	0 (6, pink)	1 (25)
Red kidney bean	NR	NR	2 (5)	NR	NR	1 (25)
Horseradish	NR	NR	4 (25)	NR	NR	1 (25)
1 M Ascorbic acid	NR	NR	0 (25, yellow)	NR	1 (11)	1 (25)
Bleach solution 5%	NR	NR	3 (2)	NR	1 (5)	1 (25)
10% Cupric sulfate	1 (25)	0 (25, blue)	0 (25, blue)	1 (25)	0 (25, blue)	1 (25)
10% Ferric sulfate	1 (25)	0 (25, orange)	0 (25, yellow)	1 (25)	0 (25, red/brown)	1 (25)
10% Nickel chloride	1 (25)	0 (25, blue)	0 (25, green)	1 (25)	0 (25, green)	NR

The shortest reaction time is shown here. Numbers in parentheses indicate the number of samples that reacted out of 25 and the color change observed if different from that of a reaction with blood.

A positive reaction was any sort of color change to the stain (or reagent strip in the case of Hemastix®); 0, a color change before all reagents were added; 1, indicates a color change within 1 min of all reagents being added; 2, color change within 1–2 min of all reagents being added; 3, color change within 2–3 min of all reagents being added; 4, color change within 3–4 min of all reagents being added; NR, indicates that there was no reaction within the 4 min of timed experimentation.

Tobe et al. J Forensic Sci 2007

…and many other fruits and vegetables (Cox J Forensic Sci 1991)

✓ non-human blood

Sensitivity of luminol vs other blood presumptive tests

Urine sticks

TABLE 2—*Sensitivity results for the six different reagents.*

	Reagent					
Dilution	Luminol	LMG	KM	Hemastix®	Hemident™	Bluestar©
1:10,000	1	1	1	1	1	1
1:100,000	1	NR	2	2	4	1
1:1,000,000	NR	NR	NR	NR	NR	NR
1:5,000,000	NR	NR	NR	NR	NR	NR
1:10,000,000	NR	NR	NR	NR	NR	NR

The shortest reaction time is shown here.

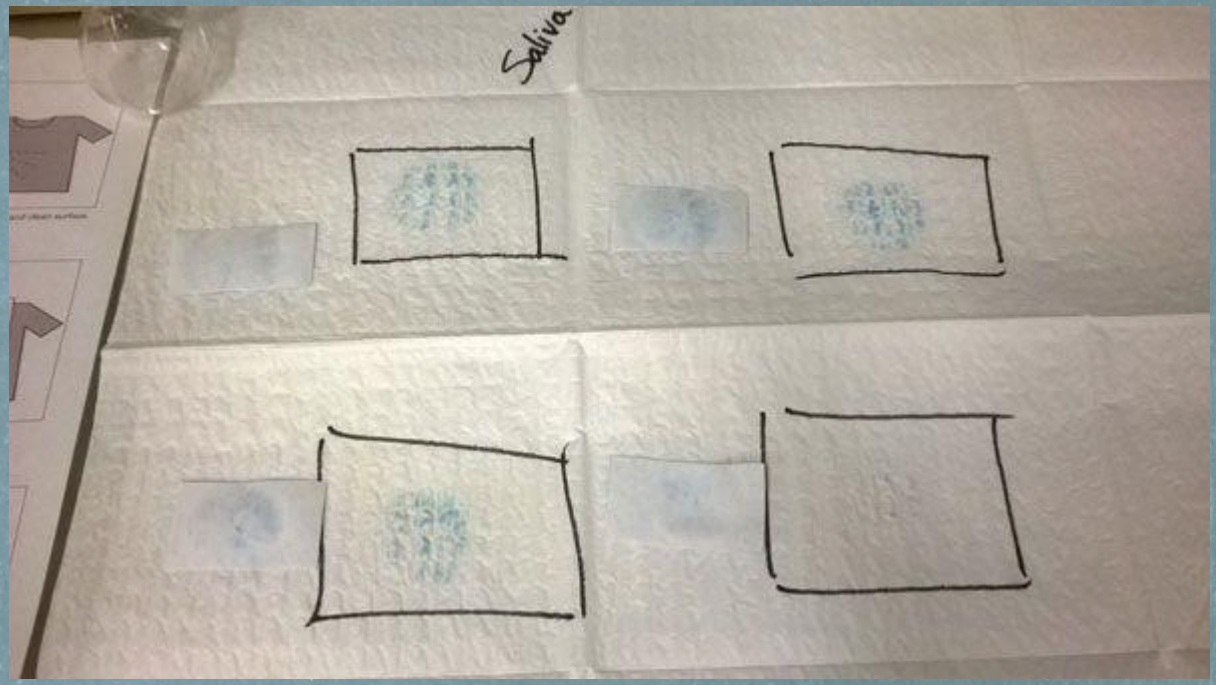
A positive reaction was any sort of color change to the stain (or reagent strip in the case of Hemastix®); 0, color change before all reagents were added; 1, color change within 1 min of all reagents being added; 2, color change within 1–2 min of all reagents being added; 3, color change within 2–3 min of all reagents being added; 4, color change within 3–4 min of all reagents being added;

NR, indicates that there was no reaction within the 4 min of timed experimentation; KM, Kastle–Meyer; LMG, leuchomalachite green.

Tobe et al. J Forensic Sci 2007

Saliva

Alpha-amylase is produced by salivary glands and starts the digestion of starch in saliva. Presence of alpha-amylase can be highlighted by means of a substrate made up of an unsoluble chromogenic molecule linked to polisaccaridic chains. Cleavage of polisaccaridic chains by alpha-amylase frees the chromogenic molecule in solution.



False positives

Non body fluid compounds

Many human body fluids different from saliva contain alpha amylase.
Alpha-amylase secreted by salivary and pancreatic glands in the digestive tract is partly reabsorbed and therefore it can be present in: serum, urine, sweat, vaginal secretions, seminal fluid, tears, milk... It is also eliminated with faeces.
Several bacteria (including those found in vaginal mucosa) can also synthetize alpha-amylase.

Sample Type	Product name	Red starch result Neat/1 in10/1 in 100
Washing powder*	Persil biological powder (Lever Faberge)	+ / + / -
Washing powder*	Persil non-biological (Lever Faberge)	+ / - / -
Washing powder*	Tesco non-biological	+ (P) / - / -
Hand cream	Vaseline intensive care dry skin hand & body lotion (Elida Faberge)	+ / - / -
Face lotion	Garnier Pure antiblemish pen	(+/-) / - / -

Martin et al Sci Just 2006

Table 2
Phadebas® Forensic Press Test results for saliva and other forensically relevant body fluids examined at 23 °C and 37 °C.

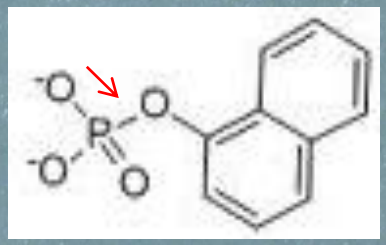
Sample	23 °C									37 °C								
	Time of observation (min)									Time of observation (min)								
	1	2	3	4	5	10	20	30	40	1	2	3	4	5	10	20	30	40
Control																		
Positive	M	M	M	S	S	VS	VS	VS	VS	M	M	S	S	S	VS	VS	VS	VS
Negative	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Saliva																		
1	M	M	M	S	S	VS	VS	VS	VS	S	S	S	VS	VS	VS	VS	VS	VS
2	W	W	M	S	S	VS	VS	VS	VS	W	W	M	M	S	VS	VS	VS	VS
3	W	M	S	S	S	S	VS	VS	VS	W	M	S	S	S	VS	VS	VS	VS
4	S	S	S	S	S	S	S	VS	VS	S	S	S	S	S	VS	VS	VS	VS
5	M	M	S	S	S	VS	VS	VS	VS	M	M	S	S	S	VS	VS	VS	VS
6	S	S	S	S	S	VS	VS	VS	VS	S	S	S	S	S	VS	VS	VS	VS
7, 8	M	S	S	S	S	VS	VS	VS	VS	M	S	S	VS	VS	VS	VS	VS	VS
Blood																		
1	N[a]	N[a]	N[a]	N[a]	N[a]	N[a]	N[a]	N[a]	N[a]	N[a]	N[a]	N[a]	N[a]	N[a]	N[a]	N[a]	N[a]	N[a]
Faeces																		
1	N[b]	N[b]	N[b]	N[b]	N[b]	VW[b]	W[b]	M[b]	S[b]	N	N	N	N	N	N	W	M	S
2	N[b]	N[b]	N[b]	N[b]	N[b]	VW[b]	VW[b]	VW[b]	W[b]	N[b]	N[b]	N[b]	N[b]	N[b]	N[b]	N[b]	N[b]	VW[b]
3	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
4	N	N	N	N	N	N	N	VW	VW	N	N	N	N	N	N	VW	VW	M
5	N[b]	N[b]	N[b]	N[b]	N[b]	N[b]	N[b]	VW[b]	VW[b]	N[b]	N[b]	N[b]	N[b]	N[b]	N[b]	VW[b]	VW[b]	W[b]
6, 7	N[b]	N[b]	N[b]	N[b]	N[b]	VW[b]	VW[b]	M[b]	M[b]	N[b]	N[b]	N[b]	N[b]	N[b]	N[b]	W[b]	M[b]	S[b]
8	N[b]	N[b]	N[b]	N[b]	N[b]	N[b]	N[b]	VW[b]	W[b]	N[b]	N[b]	N[b]	N[b]	N[b]	N[b]	VW[b]	W[b]	S[b]
Nasal secretions																		
1, 2, 4–7	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
3	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	M	M	M
8	N	N	N	N	N	N	N	N	VW	N	N	N	N	N	N	N	N	N
Perspiration																		
1	N[c]	N[c]	N[c]	N[c]	N[c]	N[c]	N[c]	N[c]	N[c]	N[c]	N[c]	N[c]	N[c]	N[c]	N[c]	N[c]	N[c]	N[c]
2	N[c]	N[c]	N[c]	N[c]	N[c]	N[c]	N[c]	N[c]	N[c]	N[c]	N[c]	N[c]	N[c]	N[c]	N[c]	N[c]	N[c]	VW[c]
3, 4	N[c]	N[c]	N[c]	N[c]	N[c]	N[c]	N[c]	N[c]	N[c]	N[c]	N[c]	N[c]	N[c]	N[c]	N[c]	VW[c]	W[c]	M[c]
Semen																		
1	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	VW[d]	W[d]
2	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]
3, 5	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	VW[d]
4	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	N[d]	W[d]
Tear fluid																		
1, 2	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Urine																		
1,2, 4–8	N[e]	N[e]	N[e]	N[e]	N[e]	N[e]	N[e]	N[e]	N[e]	N[e]	N[e]	N[e]	N[e]	N[e]	N[e]	N[e]	N[e]	N[e]
3	N[e]	N[e]	N[e]	N[e]	N[e]	N[e]	N[e]	N[e]	N[e]	N[e]	N[e]	N[e]	N[e]	N[e]	N[e]	N[e]	VW[e]	W[e]
Vaginal secretions																		
1, 2	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N

Colour intensity score: N = negative; VW = very weak; W = weak; M = moderate; S = strong; VS = very strong.
[a] Red-brown stain visible.
[b] Green-brown stain visible.
[c] White area visible.
[d] Clear area visible.
[e] Yellow stain visible.

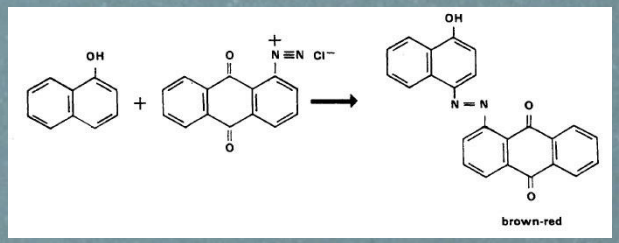
Wornes et al. Forensic Sci Int 2018

Semen

Acid phosphatase produced by the prostate (PAP) is abundant in seminal fluid. PAP can hydrolize a colorless substrate to free a chromogen molecule.



Alpha-Naphthyl acid phosphate



Alpha-Naphthol + chromogen molecule

False positives

Other human body fluids can contain (though at < concentrations) acid phosphatase.

Indirect test:

- pressing dampened filter paper onto the item to collect a proportion of any seminal fluid present
- paper is then tested with the chemical reagent

Direct test:

- Item sprayed with chemical reagent

Table 5. Direct aerosol AP testing semen-free vaginal swabs — reaction times/colours.

Age	Donor Time in cycle	Initial reaction	Reaction at 2 min	Reaction at 10 min
20s	Middle	1 min V v weak pink (grey)	Occasional v weak pink	Some pink/grey/purple
	End	1 min V v weak pink	Some weak pink	Mottled purple (start at 3 – 4 min)
30s	Beginning	Neg	Neg	Neg
	Middle	Neg	Neg	One area grey
40s	Beginning	Neg	Neg	One area weak pink
	Middle	Neg	Neg	Neg
50s	N/A	15 sec Pink	Some weak pink	Some weak pink
	N/A	1 min v v weak pink	Weak pink	Weak pink
Negative control		Neg	Neg	One area v weak pink
Positive control		Purple	Purple	Purple

Neg = no colour change observed.

Table 6. Direct aerosol AP testing of semen-free knickers worn for a day.

Age	Donor Time in cycle	Initial reaction	Reaction at 2 mins	Reaction at 10 mins
20s	End	25 sec pink 1 min 15 sec, some purple, front of crotch	Pink. Purple spreading	Purple edge of visible staining and front of crotch
30s	Middle	1 min 45 sec Pink/purple edge of visible staining	Pink/purple edge of visible staining	Some purple
40s	Middle	24 sec pink 45 sec purple at back	Purple outside visible stain and up back	Purple outside visible stain and up back
50s	N/A	Immediate strong pink	Some faint purple especially on back	V strong purple
Neg ative control		Neg	Neg	Neg
Positive control		Purple	Purple	Purple

Neg = no colour change observed.

Table 7. Comparison direct and indirect AP testing, body fluids.

Body fluid	Indirect AP testing				Direct aerosol AP testing			
	Initial reaction	2 min	5 min	10 min	Initial reaction	2 min	5 min	10 min
Blood	Neg	NC	NC	NC	Neg	NC	NC	NC
Saliva	Neg	NC	NC	NC	Very Weak 1 min 25 sec	Weak 2 min	Purple	NC
Urine	Neg	Very weak 3 min 42 sec	NC	Slightly darker 10 min	Very weak 1 min 25 sec	Weak 2 min	NC	NC
Faeces	Weak 27 sec	NC	NC	NC	Could not determine, stain masked any colour. Halo appears 1 min 52 sec			
Semen	Strong 6 sec	Very strong 1 min 23 sec	NC	NC	Strong 2 sec V strong 35 sec	NC	NC	NC

Neg = no colour change. NC = no change in colour seen.

All positive reactions were purple in colour.

Table 8. Comparison of direct and indirect AP testing, household products.

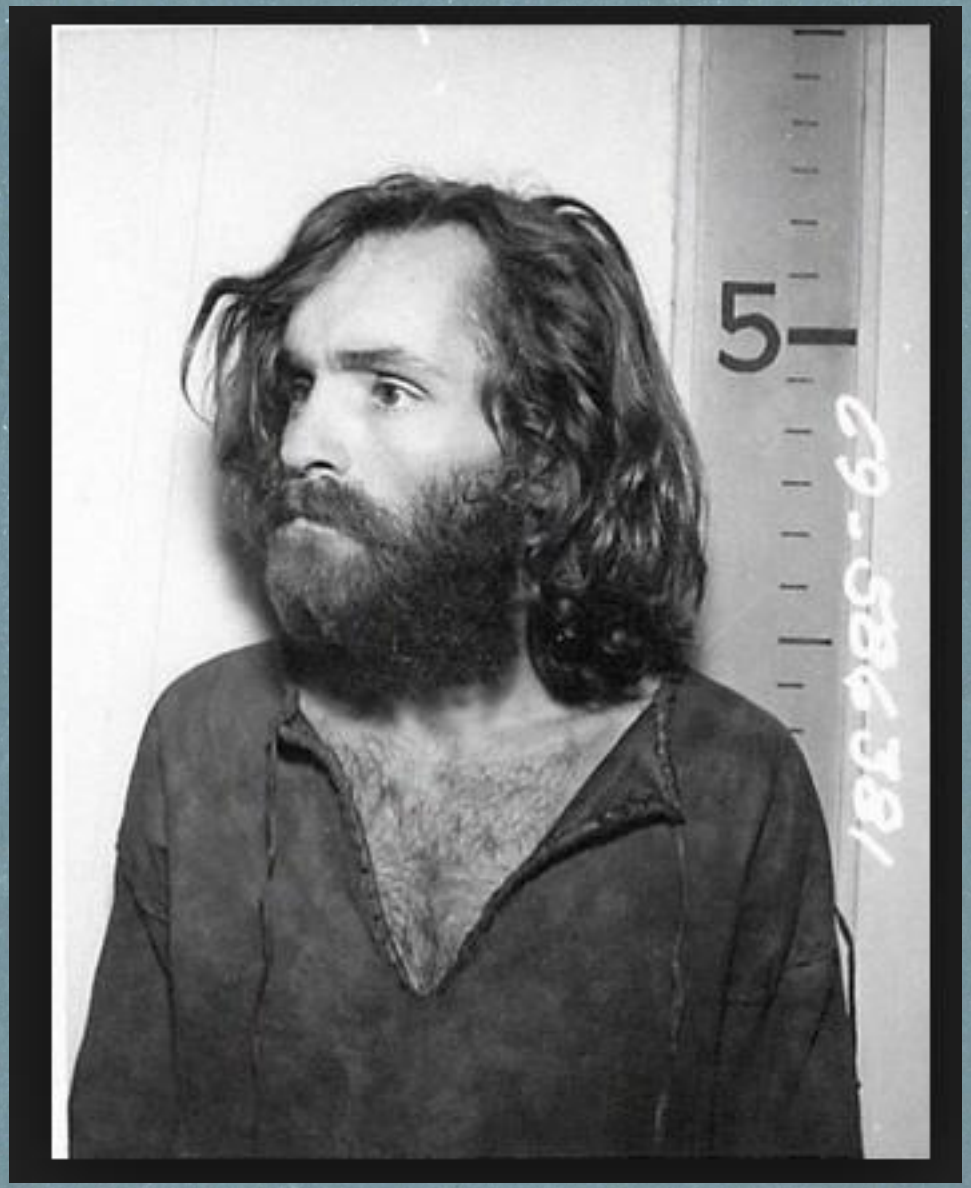
Substance tested	Indirect AP testing	Direct aerosol AP testing
Green beans	Weak 1min 30sec	Weak halo 1min 7sec
Broccoli	Negative	Weak 1min 9sec
Carrot	Weak halo 7min 30sec	Weak halo 4min
Beetroot	Weak halo 7min 30sec	Weak halo 6min
Potato	Weak halo 2min 30sec	Weak 2min
Pineapple	Pink 15sec	Pink 1–2sec
Pea	Weak pink 6min	Weak pink 2min 20sec
Grape	Weak 1min	Could not determine due to colour
Mushroom	Weak halo 1min 30sec	Weak halo 1min 5sec
Red pepper	Pink 5 -10sec	Pink 1min 40sec
Horseradish	Pink 30sec	Pink halo 10sec
Celery	Weak halo 2min	Weak halo 8min
Apple	Negative	Purple 30sec
Chocolate	Weak halo 1min 30sec	Purple halo 2 min
Onion	Pink 1min 45sec	Pink 15sec
Toilet cleaner	Weak 2min 30sec	Weak 1min 35sec
Laundry soap	Negative	Pink 1min 17sec
Tea	Purple 30sec	Purple 7sec
Lemon juice	Pink 15sec	Pink 8sec
Orange juice	Pink 20sec	Pink 10sec
Red wine	Weak 35sec	Weak 15sec
Durex Proforma Condom	Negative	Pink 42sec

Unless otherwise stated the positive reaction was purple in colour.

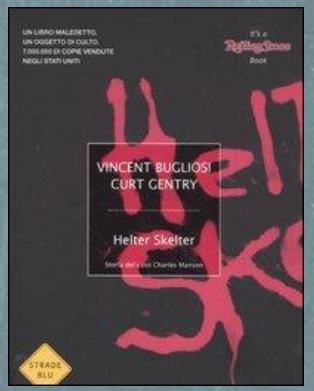
Over 40 other foodstuffs/drinks/condoms/lubricants were all direct aerosol and indirect AP tested and found to be AP negative at 10 min.

Some non body fluid componds can react as semen

Lewis et al. Sci & Justice 2013



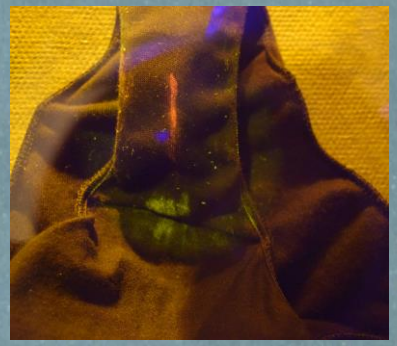
“The bloodstains? Well they are not exactly bloodstains. They are benzidine reactions”
Charles Manson

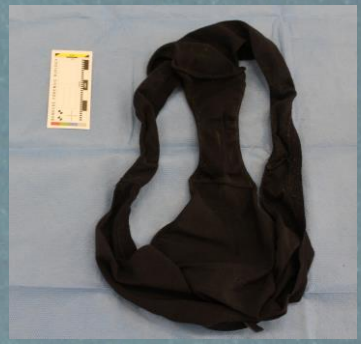


Stain collection

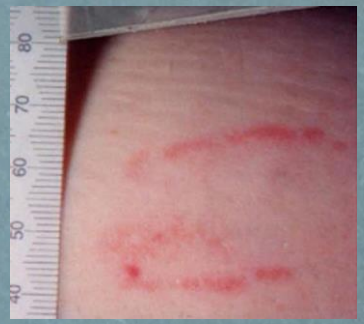
Visible stains or stains detected by means of ALS, luminol or selected consequently to a presumptive test (which is normally non desctructive) will have to be transferred to the laboratory for confirmation and, eventually, DNA analysis.

- Often the whole evidence can be easily transferred to the lab





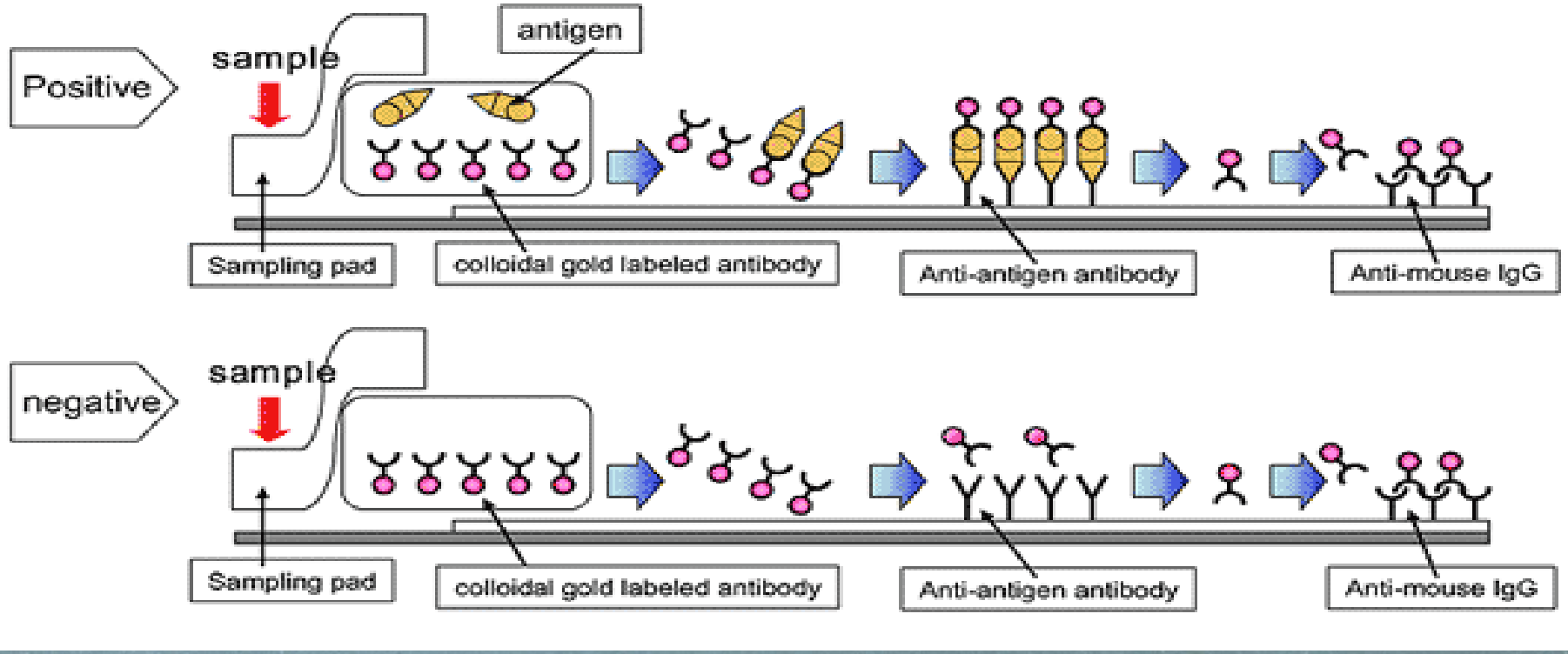
- Sometimes this is uneasy or impossible



Double swab technique: surface first brushed with a moistened swab, than brushed again with a second dry swab that collects celular material rehydrated by first swab. Both swabs are collected and combined in following DNA extraction.

Confirmatory immunological tests

✓ “sandwich immunoassay”



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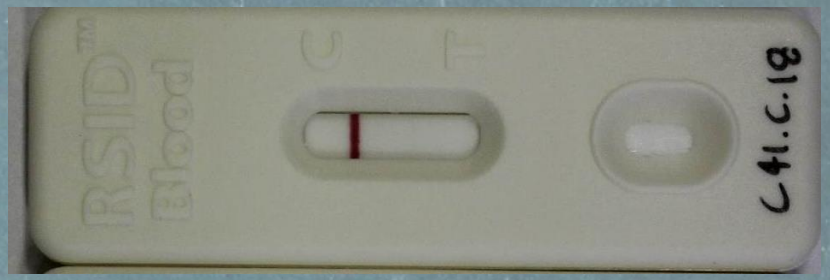

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Confirmatory immunological tests

✓ Most commonly targeted antigens

• blood: haemoglobin, glycophorin A (membrane protein bearing antigens for the MNS blood group)

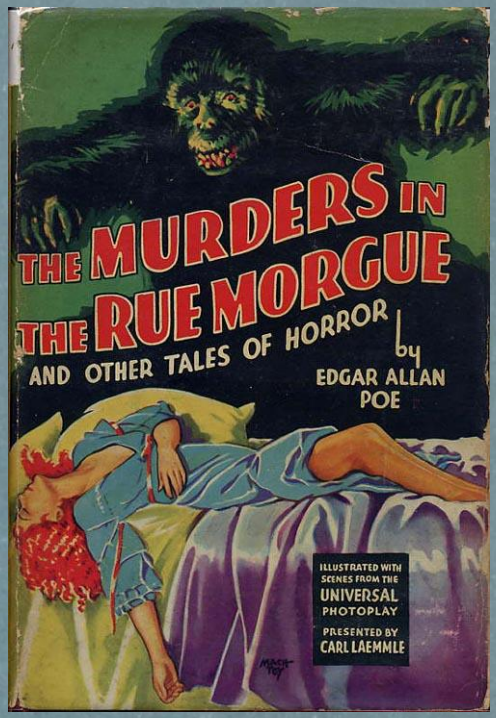
• saliva: alpha amylase

• semen: prostatic specific antigen (PSA), semenogelin (synthetized in seminal vescicles); positive results also in azospermic males

✓ Species-specific

• false positives mostly limited to primates →

NB Cross-reactivity between human and ferret haemoglobin



✓ Problems of cross-reactivity with other human body fluids clearly persist

Summary: PSA concentrations in the forensically most important female body fluids

Body fluid (female)	Maximum value found in literature ng/ml[a]	Preparation of Samples Dilution factor for swabs: 0,15[h] Dilution factor for stains: 0,01[i]	Expected test result with the PSA SEMIQUANT Assay
Saliva	0.34 [b]	Extraction of swabs	Negative
		Extraction of stains	Negative
Blood (serum)	< 0.6 [c]	Extraction of swabs	Negative
		Extraction of stains	Negative
Urine	1.239 [d] (using oral contraceptives)	undiluted	Positive results possible[j]
		Extraction of swabs	Negative
		Extraction of stains	Negative
NB 800 ng/ml in urine of >12 years old males	7.36 (average)[e]	undiluted	Positive results possible[j]
		Extraction of swabs	Weak positive results possible
		Extraction of stains	Negative
Vaginal fluid	1.5 [f]	Extraction of swabs	Weak positive results possible[k]
	< 10 [g]	Extraction of stains	Negative
		Extraction of swabs	Weak positive results possible[k]
		Extraction of stains	Negative

a: respective maximum values, if an average value is given it will be listed in the table
b: Manello et al., 1996 (values from Breul et al. 1993 are not considered)
c: Fillela et al., 1996
d: Manello et al., 1998
e: this high PSA concentration from Breul et al. (1993) is not in line with the PSA concentrations found in the majority of the studies
f: Macaluso et al., 1999, extracted supernatant from vaginal swabs, estimated dilution factor 1:3
g: Graves et al., 1985 (Detection limit of the assay for vaginal fluid was 10 ng/ml, all samples were negative under these conditions)
h: estimated capacity of liquid uptake for the swab: 150 µl, volume used for extraction: 1000 µl (Dale + Custis, 2004)
i: estimated that 10 µl correlate with a 1 cm^2 stain, volume used for extraction: 1000 µl (Dale + Custis, 2004)
j: no reported positive results for female urine with the PSA SEMIQUANT assay have been found in the literature
k: so far one female volunteer has been found whose vaginal swabs showed positive test results with the PSA SEMIQUANT assay dependent on the menstrual cycle (Denison et al., 2004)

Category	Bodily Fluids/ Materials	No. of Samples	RSID-Saliva Test
1	Semen (neat)	7	–
2	Vaginal swab without semen	8	–
3	Breast milk stain	2	–
		1	+
4	Blood (neat)—male	3	–
	Blood (neat)—female	3	–
5	Urine (neat)—male	4	+
	Urine (neat)—female	2	+
		1	–
	Urine stain—male	4	–
	Urine stain—female	3	–
6	Sweat swab—male	4	–
	Sweat swab—female	3	–
7	Fecal swab	3	+
Total		43	

Pang et al. J Forensic Sci 2008

"...some of the extracts of saliva, urine, stool, vaginal secretions, and semen yielded a positive reaction with the assay. These results were anticipated since hemoglobin may be present at low concentration in various body fluids"

Hochmeister MN et al. J Forensic Sci. 1999 May;44(3):597-602. Validation studies of an immunochromatographic 1-step test for the forensic identification of human **blood**. Hochmeister MNJ Forensic Sci. 1999

Sensitivity of presumptive chemical tests vs immunological confirmatory tests

Immunoassay

Urine sticks

TABLE 1—*Comparison of the sensitivity of four presumptive blood test kits on 50 µL of dilute blood.*

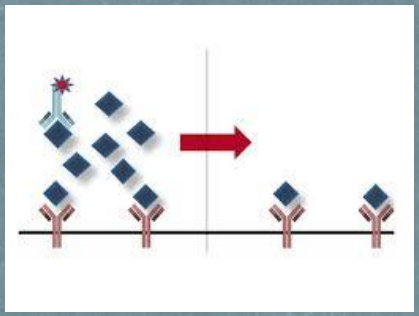
Blood Dilution	KM	LMG	Hemastix®	Hexagon OBTI
Neat	+	+	+	−
1 in 10	+	+	+	−
1 in 50	+	+	+	+
1 in 100	+	+	+	+
1 in 250	+	+	+	+
1 in 1×10^3	+	+	+	+
1 in 5×10^3	+	+	+	+*
1 in 1×10^4	+	+	+	+*
1 in 5×10^4	−	−	+	−
1 in 1×10^5	−	−	−	−

KM, Kastle-Meyer; LMG, Leucomalachite green; +, positive result; −, negative result.

*Result after reducing the volume of buffer in collection bottle from 2 mL to 200 µL.

Johnston et al. J Forensic Sci 2008

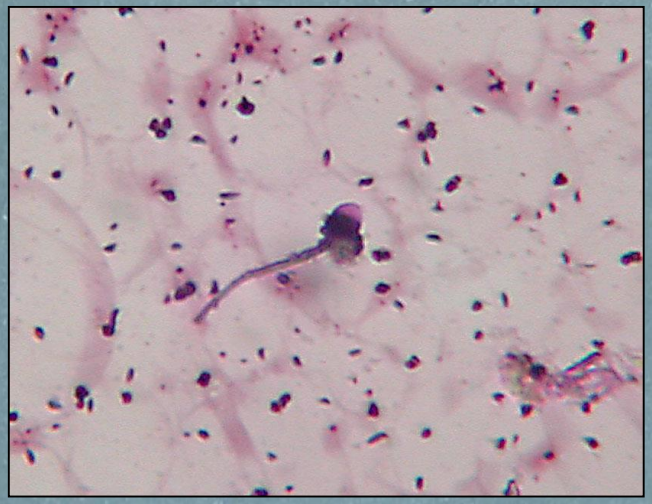
High dose hook effect



Excess free antigen saturates fixed anti-antigen antibodies

Confirmatory microscopic test (semen)

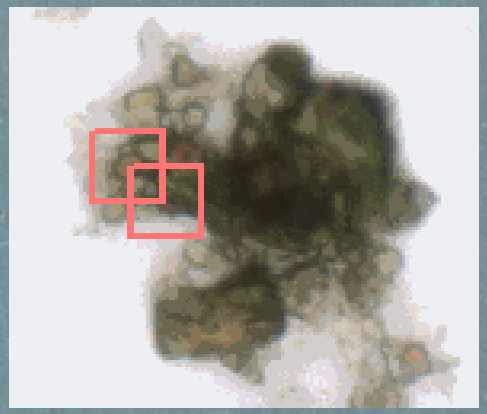
✓ Vaginal smears in sexual assault cases



"I have divers times examined human semen from a healthy man, not a sick man, not spoiled by keeping for a long time and not liquefied after the lapse of some time; but immediately after ejaculation before six beats of the pulse had intervened, and I have seen so great a number of living animals in it that sometimes more than a thousand were moving about in an amount of material of the size of a grain of sand... . Antoni van Leuweenhoek, 1677

✓ For dry stain on clothes or other substrates special staining techniques to highlight spermatozoa may be necessary

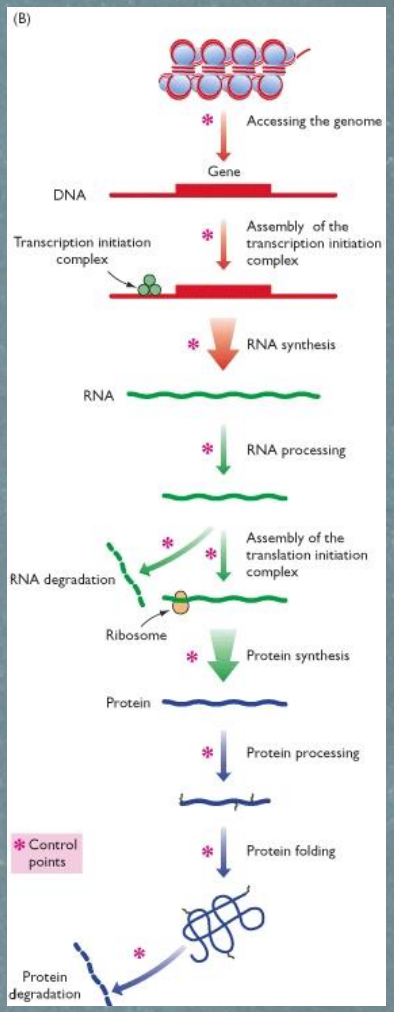
- Baecchi staining (red heads, blue tails): acid fuchsin degrades DNA Dimo-Simonin et al. J Forensic Sci 1997
- Christmas tree staining (red heads, green tails)



NB according to literature, in up to 30% of sexual assault cases male DNA is found in absence of visible spermatozoa (negative citology)! Sibille et al. Forensic Sci Int 2002

Confirmatory molecular methods

- ✓ RNA: conversion of genetic information into proteins and the regulation of this process
- genetic information is transcribed into **mRNA** which is then transferred to the ribosome. Each specialised cell type expresses only a subset of the 22,000 coding genes that constitute the full human genome and this mRNA subset can be used as a chemical fingerprint for cell type identification
- mRNA expression information for different tissue types is available through microarray and whole transcriptome shotgun deep-sequencing (RNA-Seq)
- Regulatory RNAs and especially **miRNAs** also contribute to cell type differentiation
- miRNAs base pair to mRNAs (primarily their 3'-UTR) to establish mRNA decay or inhibition of translation
- a miRNA can have several, up to hundreds, of targets
- genes encoding miRNAs are located throughout the genome but especially within introns, and thus often expressed co-transcriptionally, under control of common regulatory sequences. This can result in tissue specific expression patterns.



Confirmatory molecular methods

- ✓ RNA stability: different RNA classes have different half-lives in vivo (mRNA is readily degraded in the cell by omnipresent, highly reactive ribonucleases; miRNA are highly stable due to association with RNA-binding proteins), but ex vivo…
- RNA is more prone to degradation than DNA especially in the single–stranded regions
- RNA degradation in a deceased individual or body parts occurs predominantly due to the enzymatic activity of cellular Rnases
- In dried stains, RNase activity is significantly reduced and RNA degradation occurs mostly due to physical and chemical factors

Hydrolysis, favored by cations, metals, alkali conditions… ←

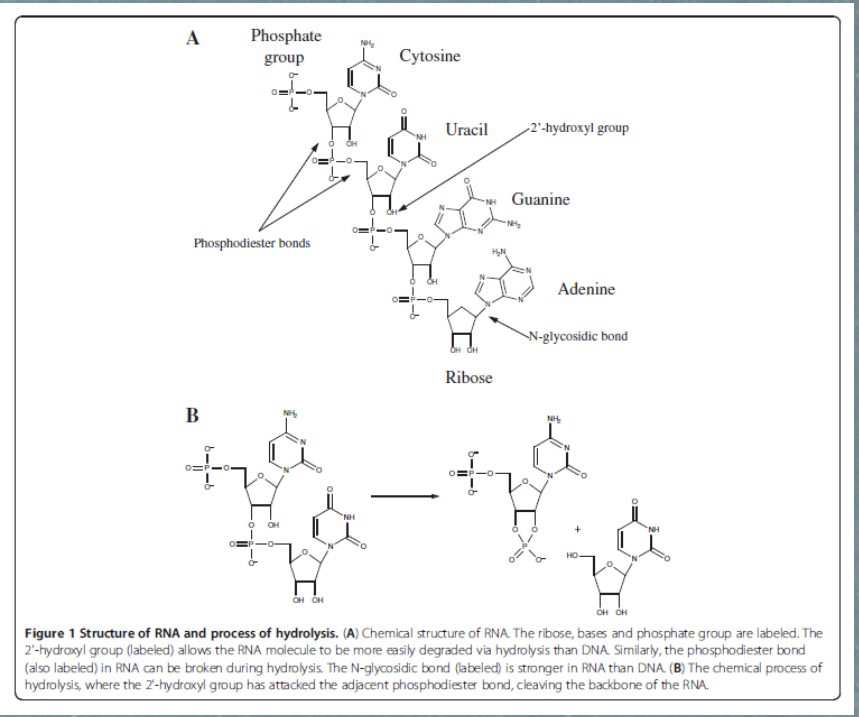


Figure 1 Structure of RNA and process of hydrolysis. (**A**) Chemical structure of RNA. The ribose, bases and phosphate group are labeled. The 2'-hydroxyl group (labeled) allows the RNA molecule to be more easily degraded via hydrolysis than DNA. Similarly, the phosphodiester bond (also labeled) in RNA can be broken during hydrolysis. The N-glycosidic bond (labeled) is stronger in RNA than DNA. (**B**) The chemical process of hydrolysis, where the 2'-hydroxyl group has attacked the adjacent phosphodiester bond, cleaving the backbone of the RNA.

Fordyce et al. Invest Genet 2013

In the last ~ 10 years studies have started to investigate the possibility to recover RNA from forensic stains

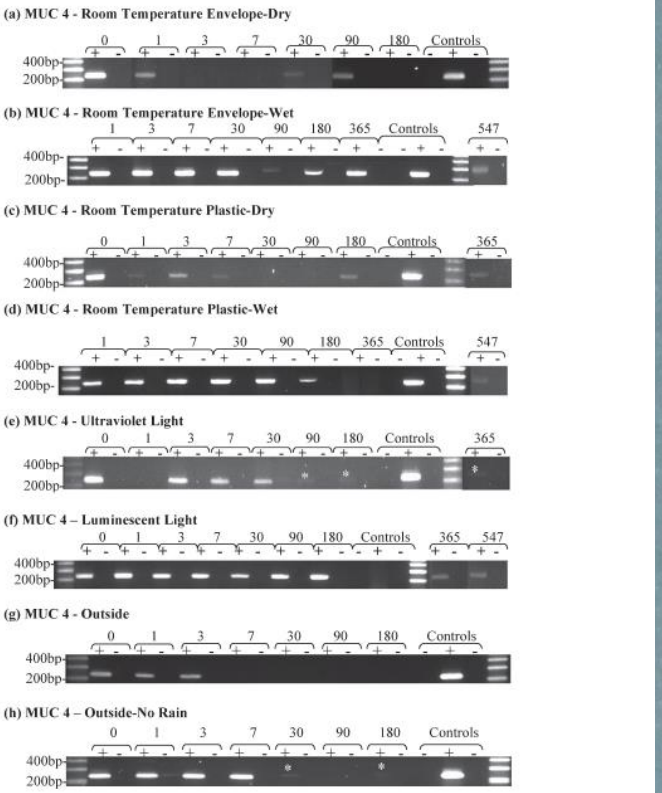


FIG. 4—*Example of mRNA stability studies using a variety of environmental conditions: Mucin 4. The gel images show RT-PCR products for MUC4 using total RNA recovered from vaginal swabs exposed to a variety of environmental conditions. Each sample is shown with (+) and without (−) the use of reverse transcriptase (RT). Additional controls for each gene tested (shown on the extreme right of each panel) include no RNA added (−) and amplification of the gene using a well-characterized sample with (+) and without (−) RT. PCR products were separated on a 2.5% agarose gel and visualized with SYBR® Gold. The asterisk (*) indicates the presence of a difficult-to-visualize amplimer.*

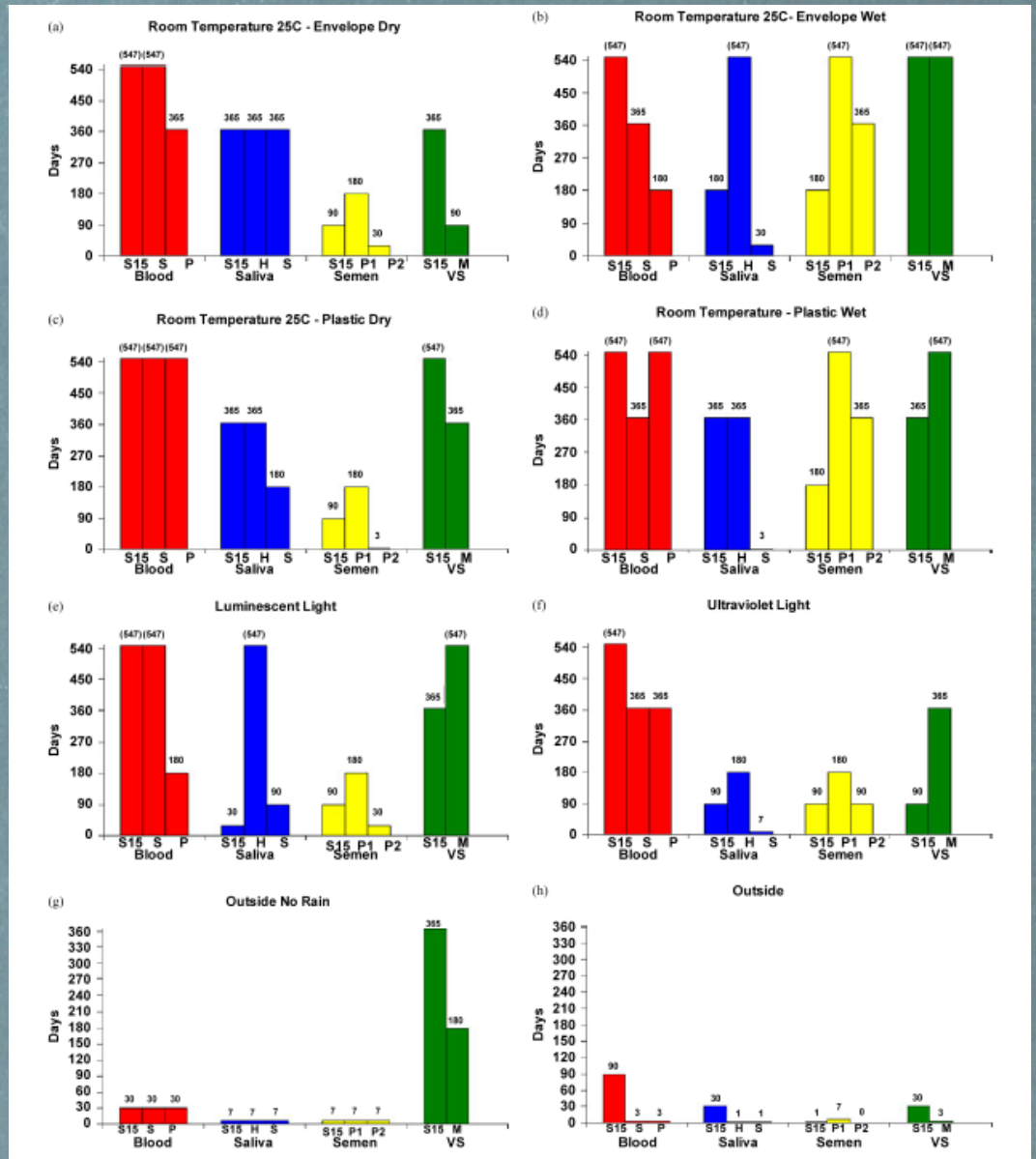
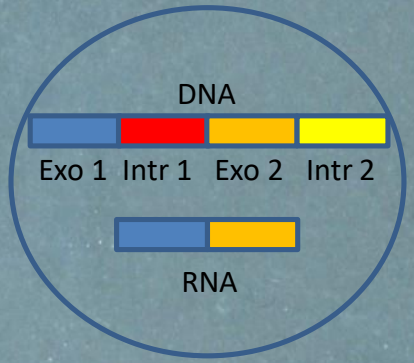


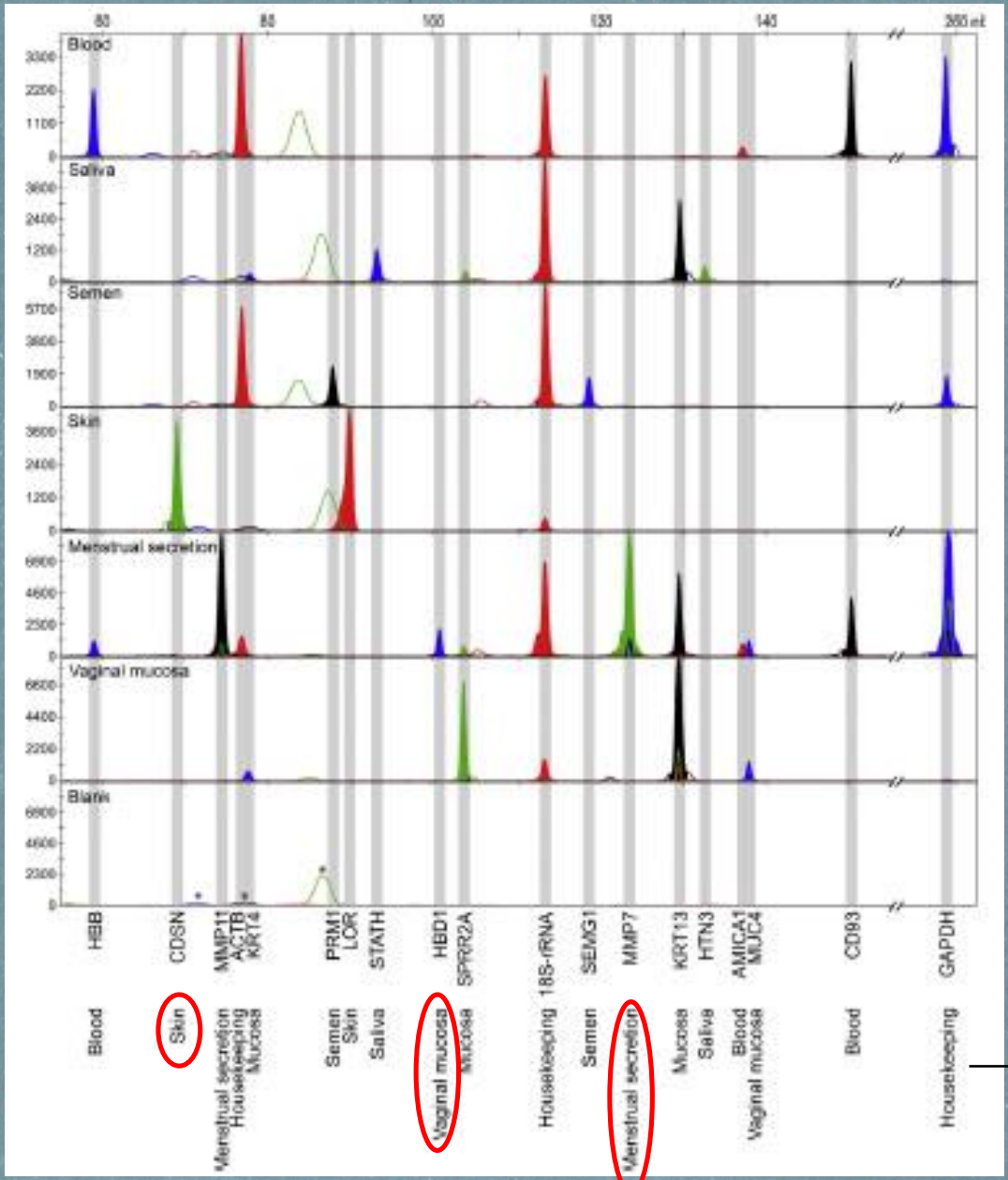
FIG. 3—*mRNA stability under a variety of environmental conditions. The graphs indicate the limits (in days) after which particular mRNA species were detected in blood, saliva, semen, and vaginal secretions after exposure to a variety of environmental conditions. These include (a) envelope (dry); (b) envelope (wet); (c) plastic (dry); (d) plastic (wet); (e) luminescent light; (f) UV light; (g) outside exposed to the elements; and (h) outside, as in (g), but protected from the rain. For each body fluid, the first gene shown is the housekeeping gene S15. The second and third genes represent the tissue-specific genes: SPTB (S) and PBGD (P) for blood, HTN3 (H) and STATH (S) for saliva, PRM1 (P1) and PRM2 (P2) for semen, and MUC4 (M) for vaginal secretions. Numbers in parentheses indicate the time of sampling but that the detection limit had not yet been reached.*

Setzer et al. J Forensic Sci 2008

In particular, several **mRNA profiling** assays compatible with standard capillary electrophoresis instruments available in forensic labs have been developed

- RNA/DNA coextraction
- DNase tratment of RNA
- synthesis of cDNA from mRNA through reverse transcription PCR (RT PCR) using random deca/hexamers (since polyA tails may be fragmented and lacking, use of oligo (dT) is not recommended)
- Amplification of cDNAby multiplex PCR using fluorescent-labelled tissue-specific and housekeeping primers. Primers are either designed as to span exon-exon junctions or complementary to sequences on different exons to guarantee specificity





Lindenbergh et al. Forensic Sci Int Genet 2012

Interpretation

a) Recurrence of tissue-specific peaks in replicate analysis

→ mRNA profiling assays can target body fluids / tissues not included in commercial immunoassays

Interpretation

b) numerical scoring method in which values are assigned to each of the used mRNA markers based on correct and incorrect expression in samples of known origin

cervico vaginal fluid ←

Sample type	No. of samples analyzed	HBB	ALAS2	PRF1	GlycoA	PF4	SPTB	PBGD	UCE	TEF
Blood	25	100	100	100	100	100	92	84	100	92
Menstrual blood	33	97	76	82	36	76	36	3	94	91
Saliva	50	0	0	2	0	0	0	0	68	70
CVF	49	16	2	37	0	4	4	0	90	88
Semen	28	0	0	4	11	4	4	0	89	100
Sweat	12	0	0	0	0	0	0	0	0	8
Skin	12	0	0	0	0	0	0	0	0	0

Table 4 Scoring values for mRNA markers

Target body fluid	Marker	Marker value	Scoring threshold for body fluid identification
Semen[a]	PRM1	100	300
	PRM2	100	
	KLK3	100	
	SEMG1	100	
	TGM4	100	
Saliva	STATH	98	250
	HTN3	100	
	PRB4	100	
	MUC7	96	
	SMR3B	92	
CVF	HBD1	78	300[b]
	MUC4	82	
	Lcris	95	
	Ljen	64	
	Lcris2	96	
	Lgas	95	
Blood[c]	HBB	88	400
	ALAS2	98	
	PRF1	72	
	GlycoA	93	
	PF4	94	
	SPTB	92	
	PBGD	100	
MB	MSX1	43	670[d]
	LEFTY2	89	
	SFRP4	45	
	MMP7	40	
	Hs202072	83	
	MMP10	67	
	MMP11	44	

Roeder et al. Int J Legal Med 2013

Confirmatory molecular methods

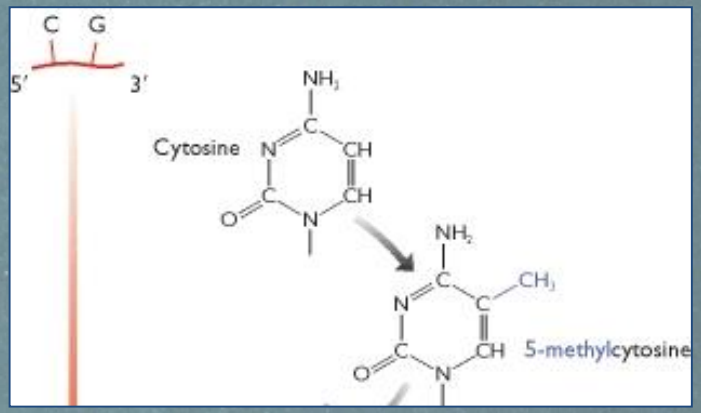
✓DNA methylation

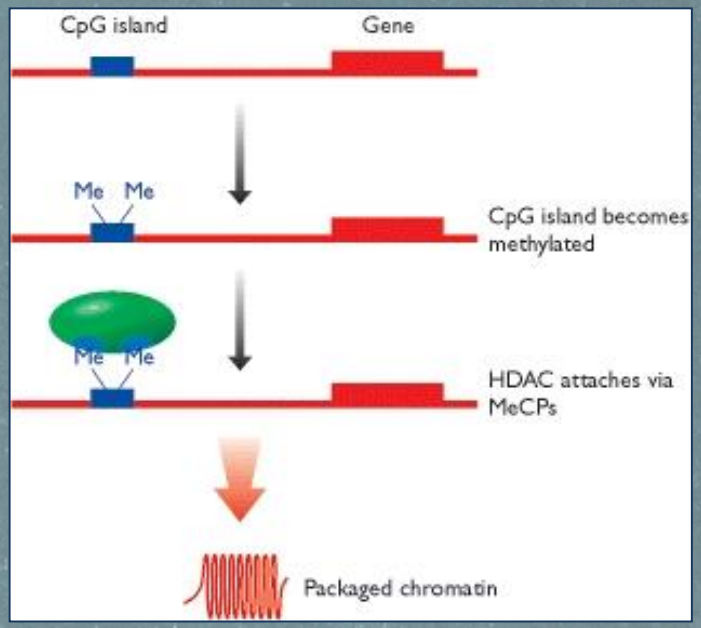
Epigenetics (inheritable pattern affecting gene expression without any modification of the DNA sequence: X chromosome inactivation; genetic imprinting…)

- in vertebrates ~ 10% cytosines are methylated
- methylation is not random but limited to 5' C-G 3' sequences
- about 50% of our genes lay close to G and C rich regions (CpG islands)
- hypo/hypermethylation of CpG islands regulates gene expression through chromatin changes

✓ different tissue types have different methylation patterns

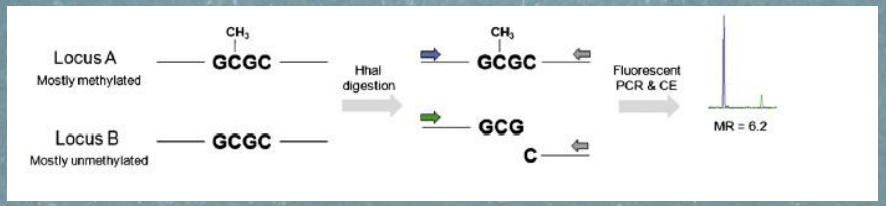
✓ <u>DNA methylation patterns can be analyzed even in archival DNA (cold cases)</u>



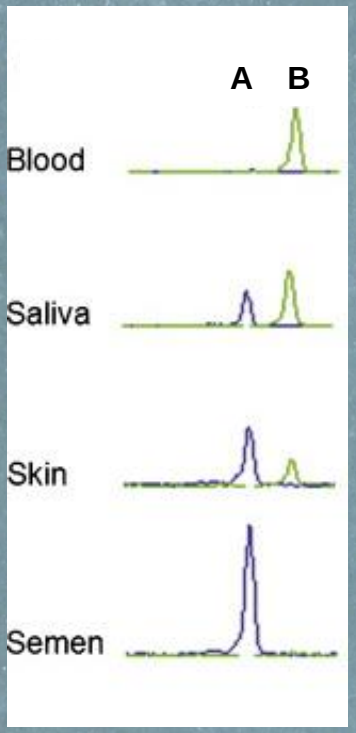


Methylation detection techniques

a) Restriction enzyme analysis

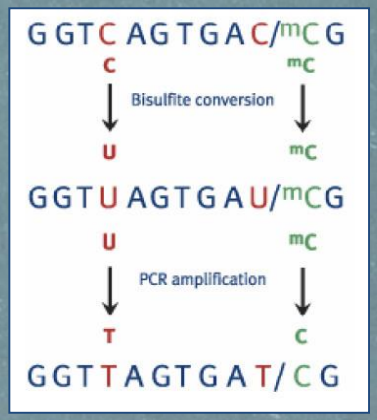


Methylated DNA is protected from cleavage and amplified by specific fluorescent primers



Frumkin et al. Forensic Sci Int Genet 2011

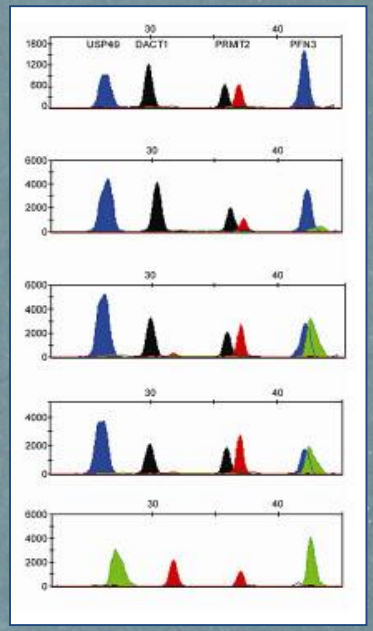
b) Bisulfite conversion



Sodium bisulfite converts unmethylated cytosine to uracil while keeping methylated cytosines unchanged

Uracil becomes thymine in PCR

Tissue-specific methylation (presence of T/C in the amplified DNA sequence can then be detected by SBE and capilary electrophoresis



Blood

Saliva

Menstrual blood

Vaginal secretion

Semen

An et al. Int J Legal Med 2013