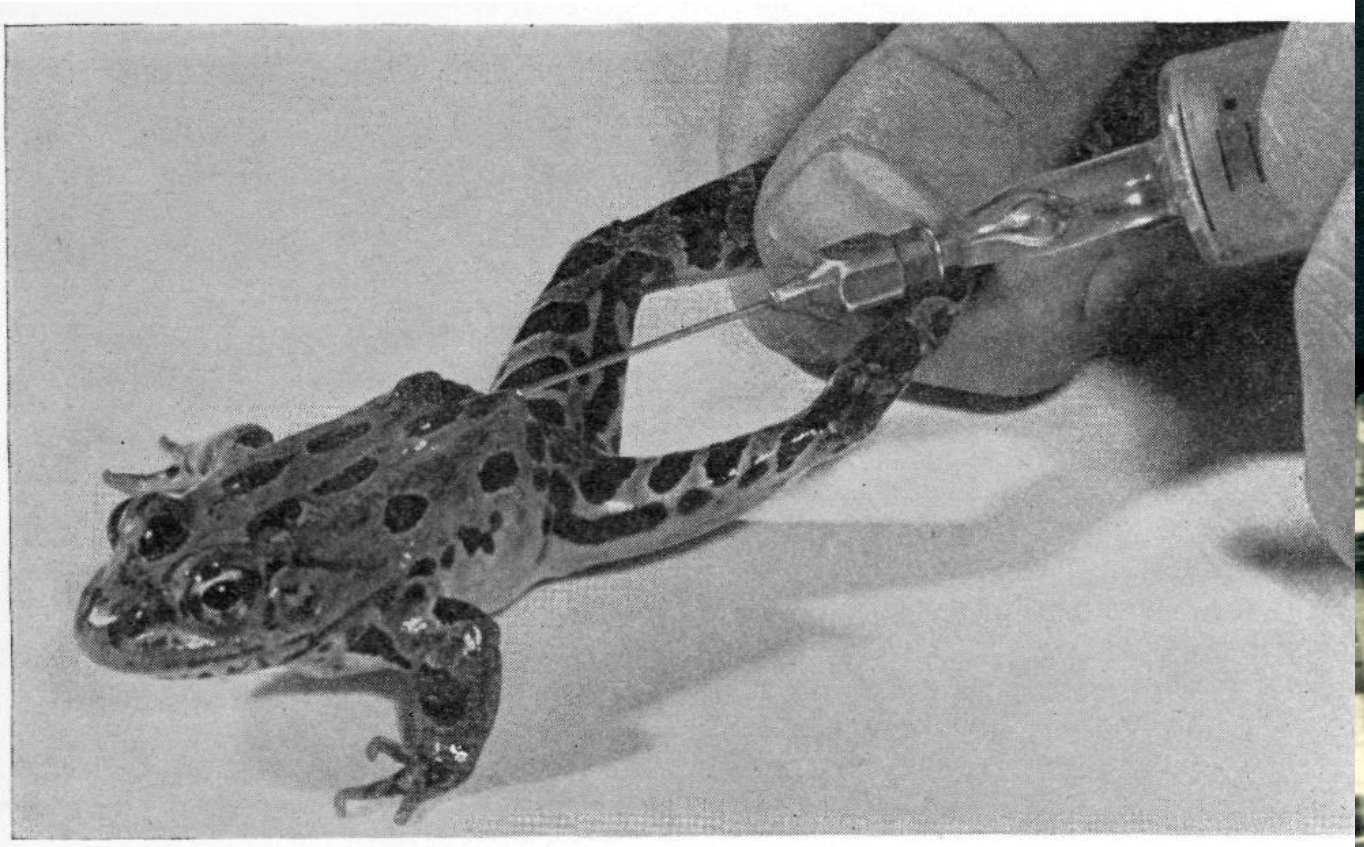
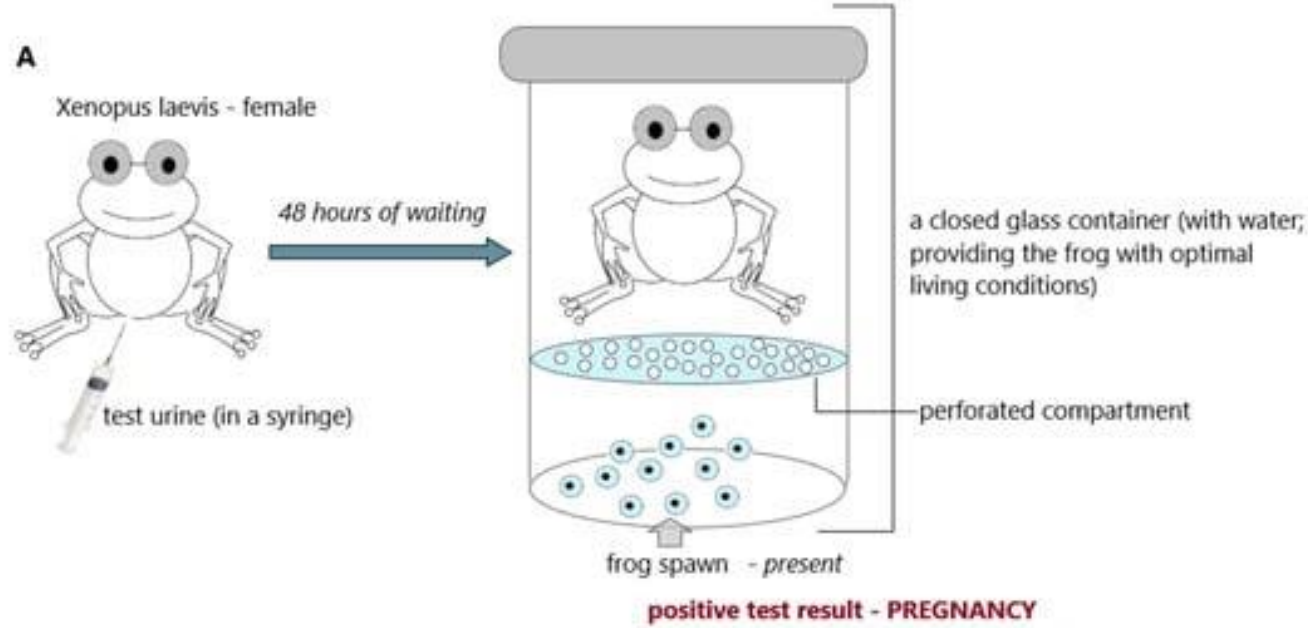
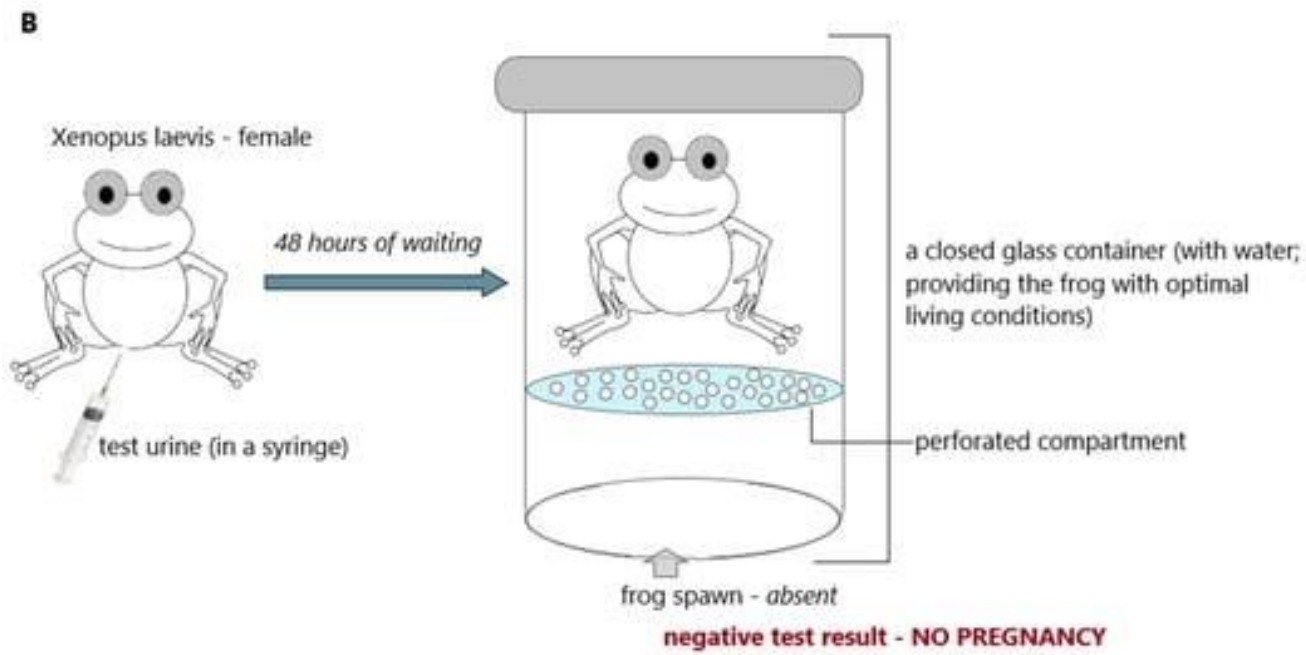
The Endocrine Laboratory

Jaak Billen

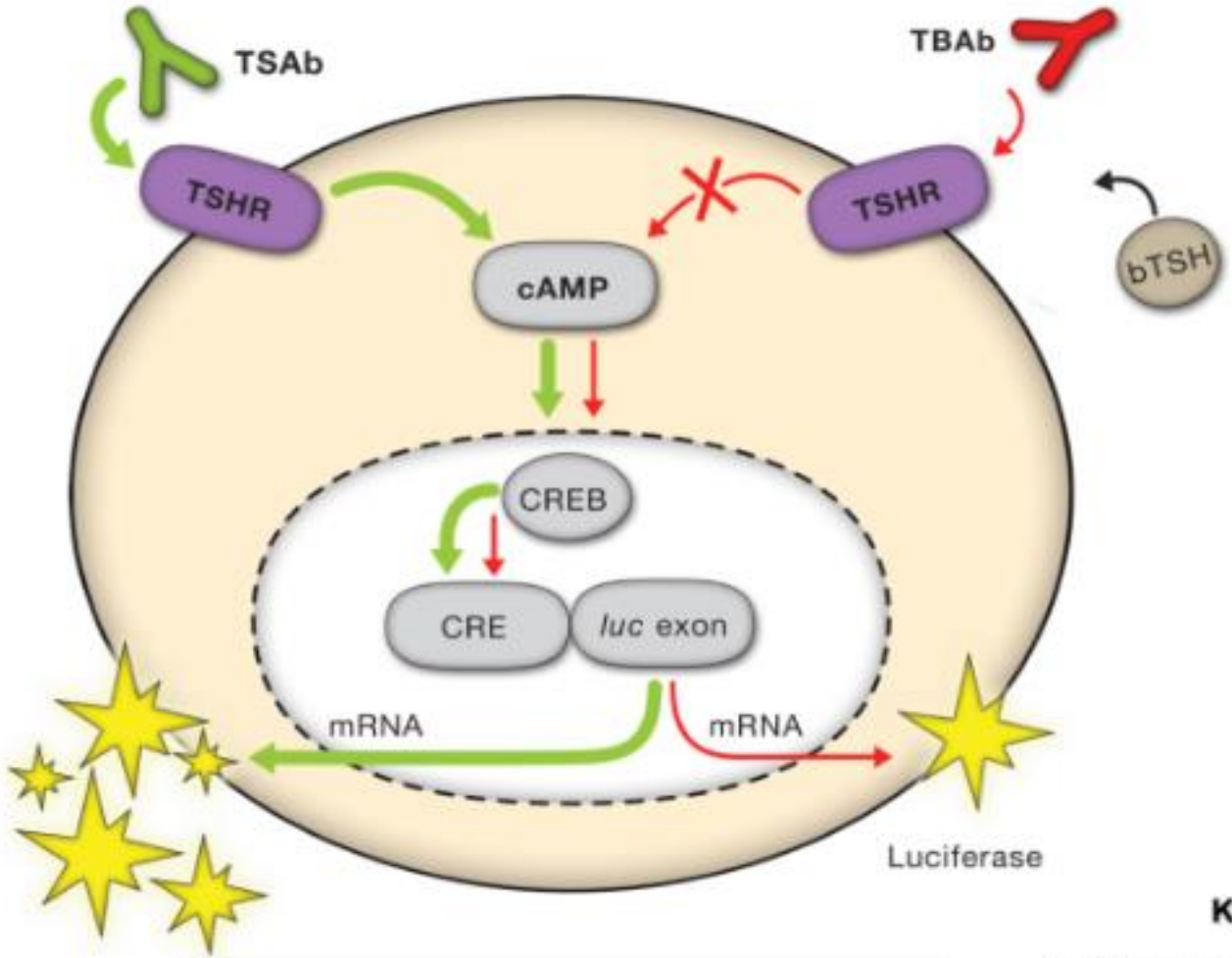
Old school “bio-assay”




A
Xenopus laevis - female
48 hours of waiting
test urine (in a syringe)
a closed glass container (with water; providing the frog with optimal living conditions)
perforated compartment
frog spawn - present
positive test result - PREGNANCY


B
Xenopus laevis - female
48 hours of waiting
test urine (in a syringe)
a closed glass container (with water; providing the frog with optimal living conditions)
perforated compartment
frog spawn - absent
negative test result - NO PREGNANCY

Bioassays for TSH-R Stimulating and Blocking Antibodies



TSHR Stimulating Autoantibody (TSAb)

TSHR Blocking Autoantibody (TBAb)

Luciferase

Blocked

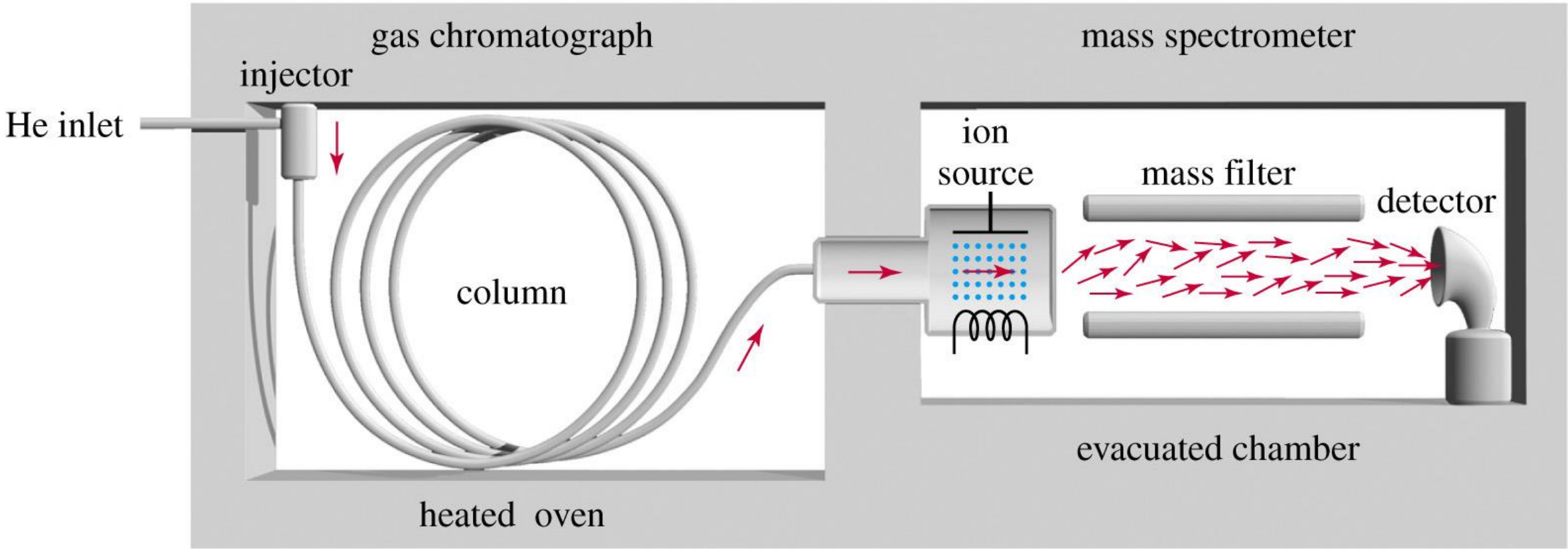
JG|U

Kahaly & al.,

JCEM 2010, ***Autoimmunity Rev*** 2010, ***Thyroid*** 2011, ***Ophthalmology*** 2012, ***Am J Clin Pathol*** 2013, ***Clin Exp Immunol*** 2013, ***JCEM*** 2014, ***Thyroid*** 2015, ***JCEM*** 2016

CHROMATOGRAPHY AND MASS SPECTROMERY

GC-MS (steroïds)
1930-today



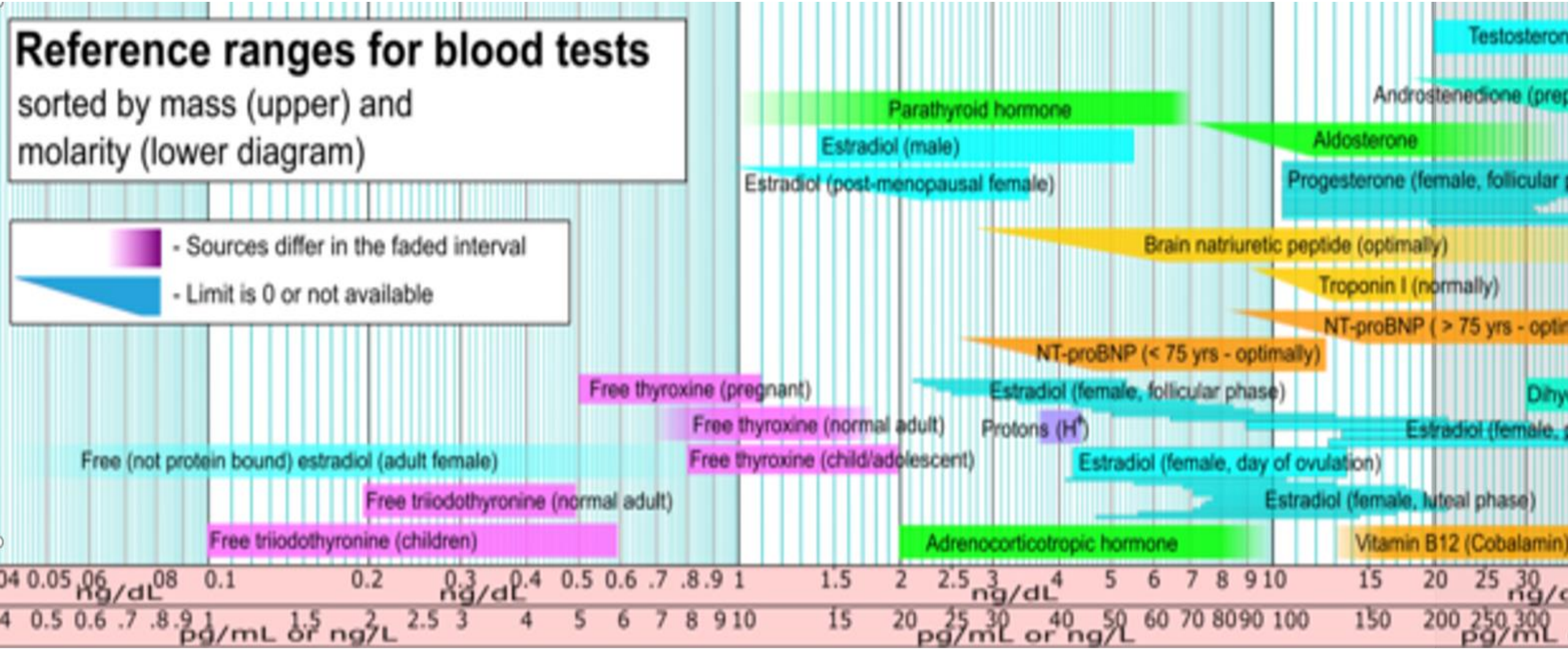
- Laborious (derivatisation)
- Time consuming (long run times)
- But still considered reference method

Rosalyn Yalow

RIA

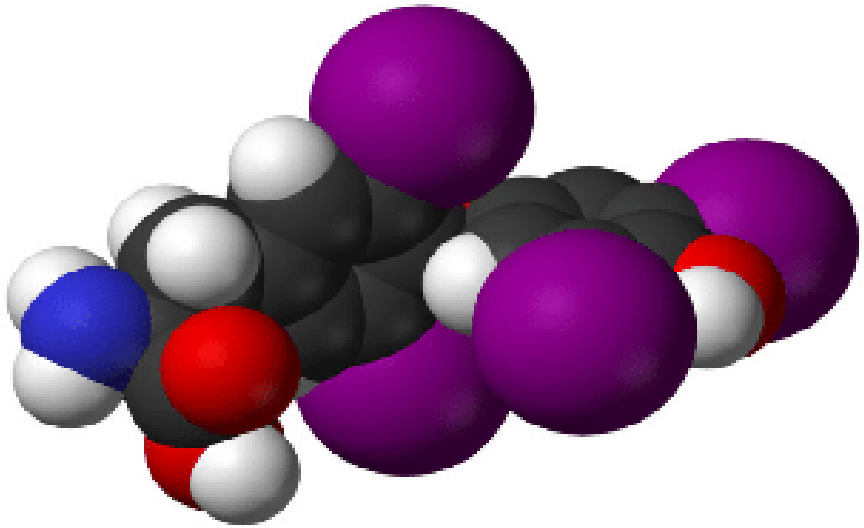
1960-today

Concentration range of analytes in blood



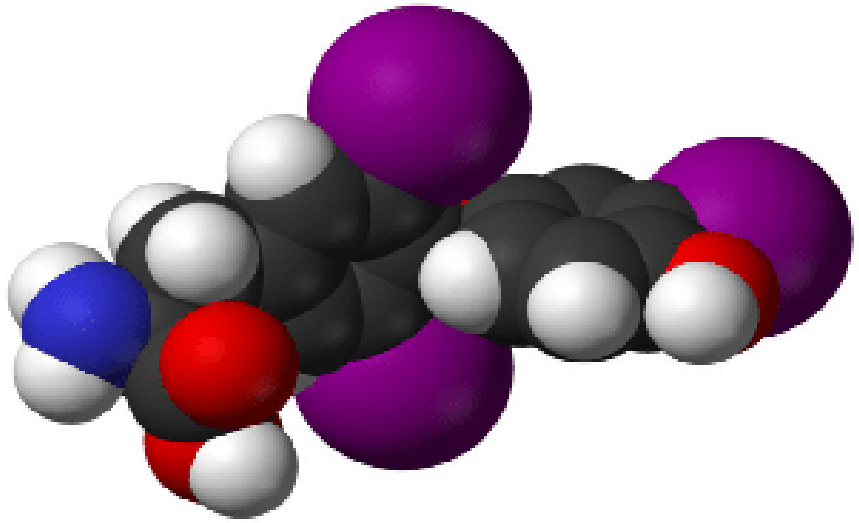
Hormones: chemical structure

And the implications



Thyroxine
(T_4)

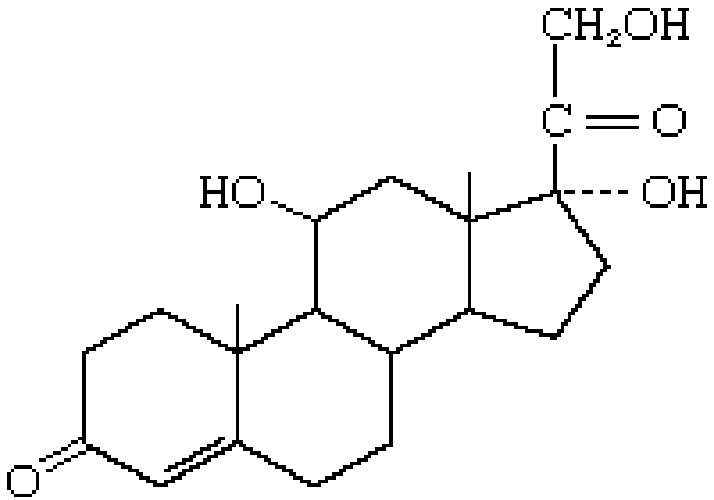
Triiodo-L-thyronine
(T3)



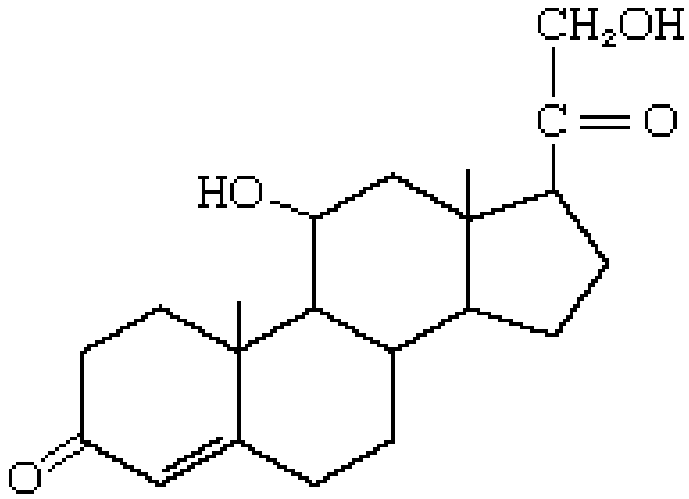
Iodine Oxigen Nitrogen

Carbon Hidrogen

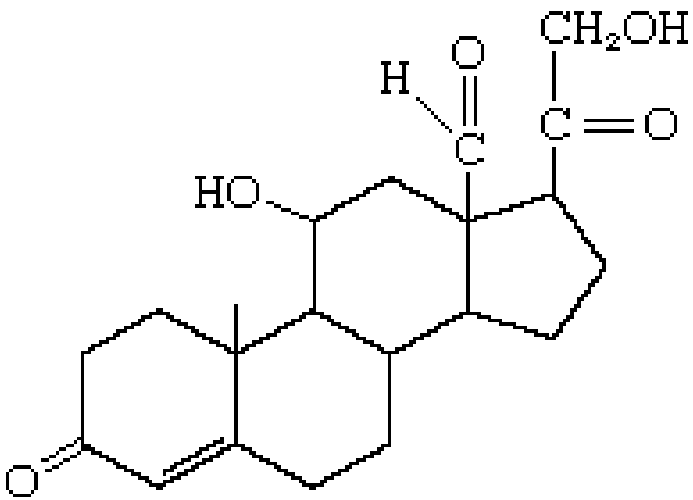
- small
- lipophilic



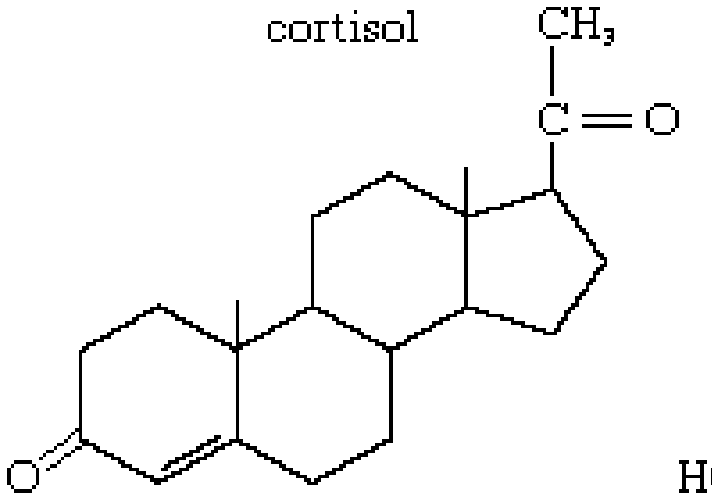
cortisol



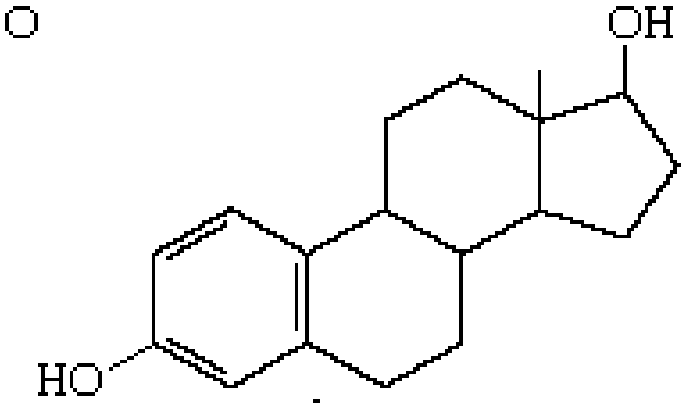
corticosterone



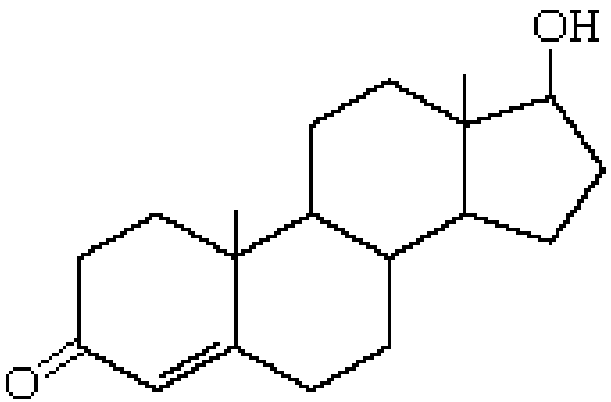
aldosterone



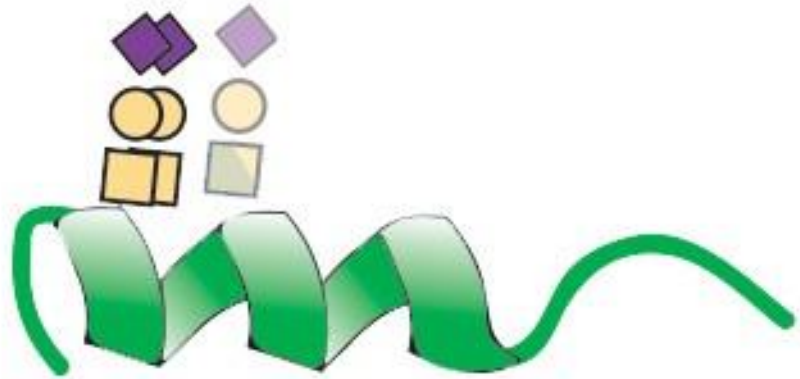
progesterone



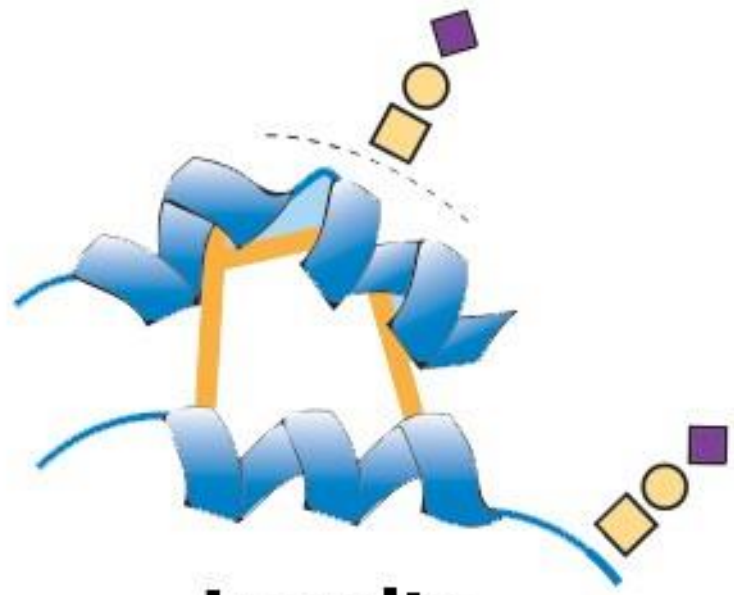
β-estradiol



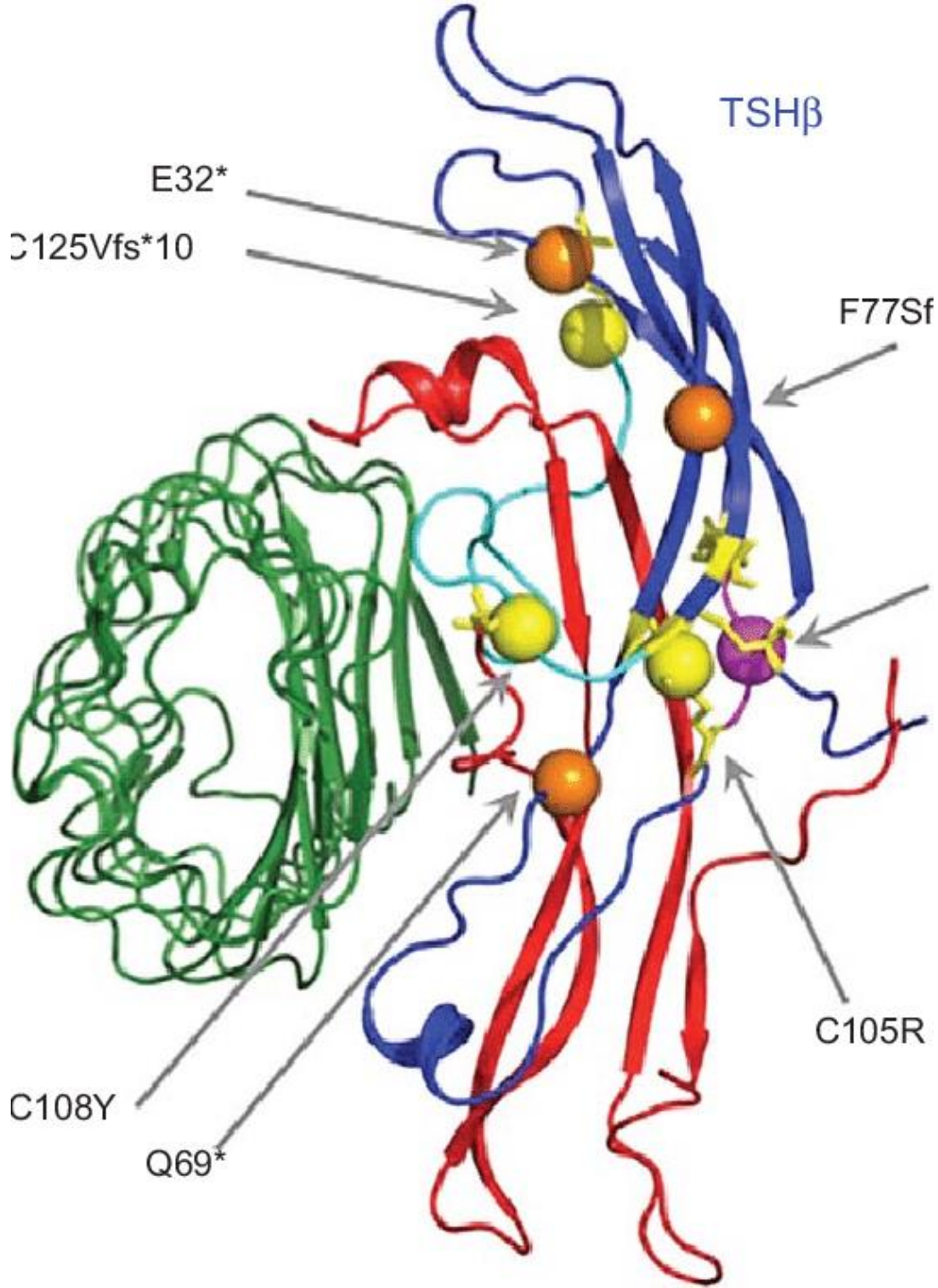
testosterone



Glucagon



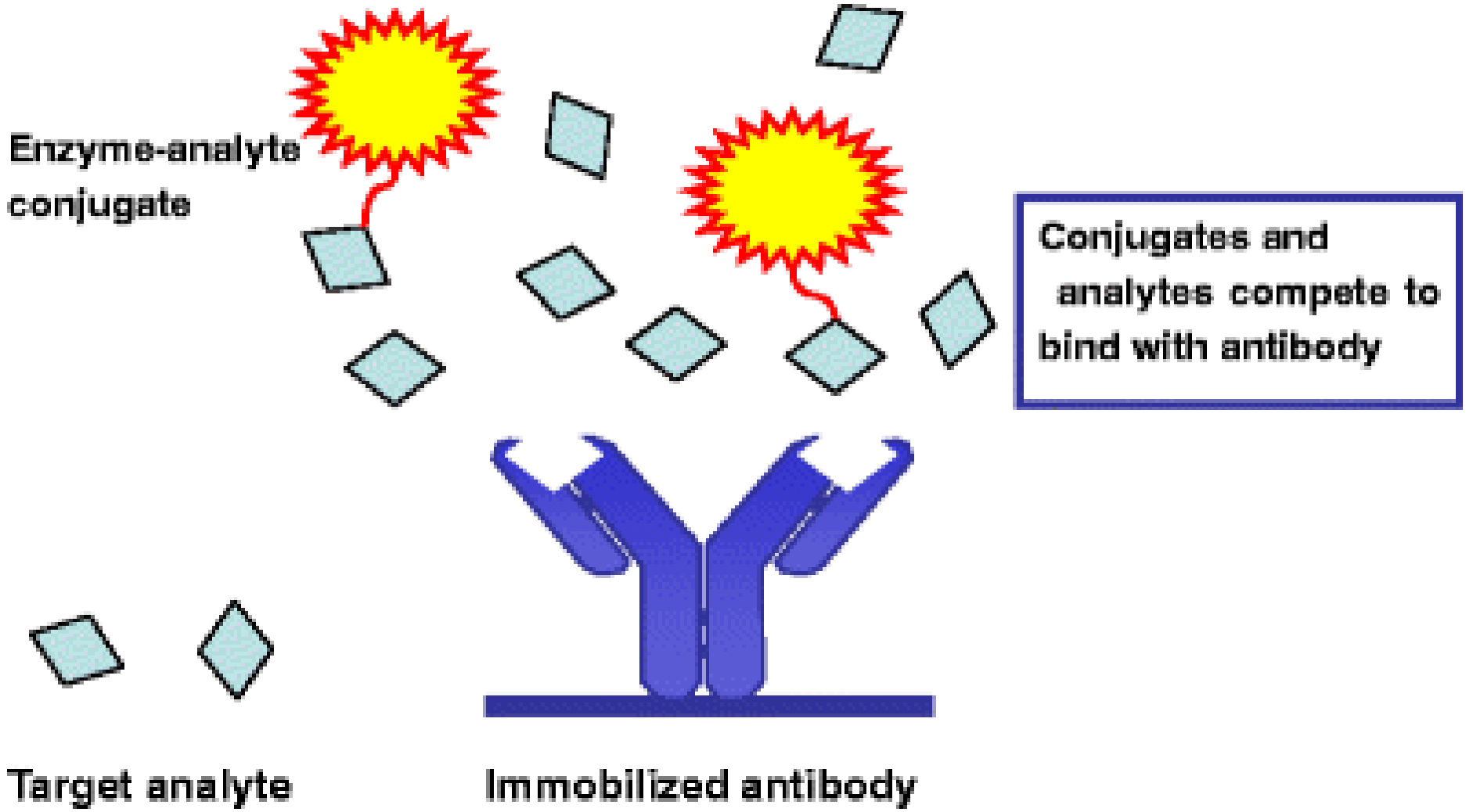
Insulin


TSHβ
E32*
C125Vfs*10
F77Sf
C105R
C108Y
Q69*

Immunoassay: the workhorse of the endocrine laboratory

Immunoassay and its pitfalls

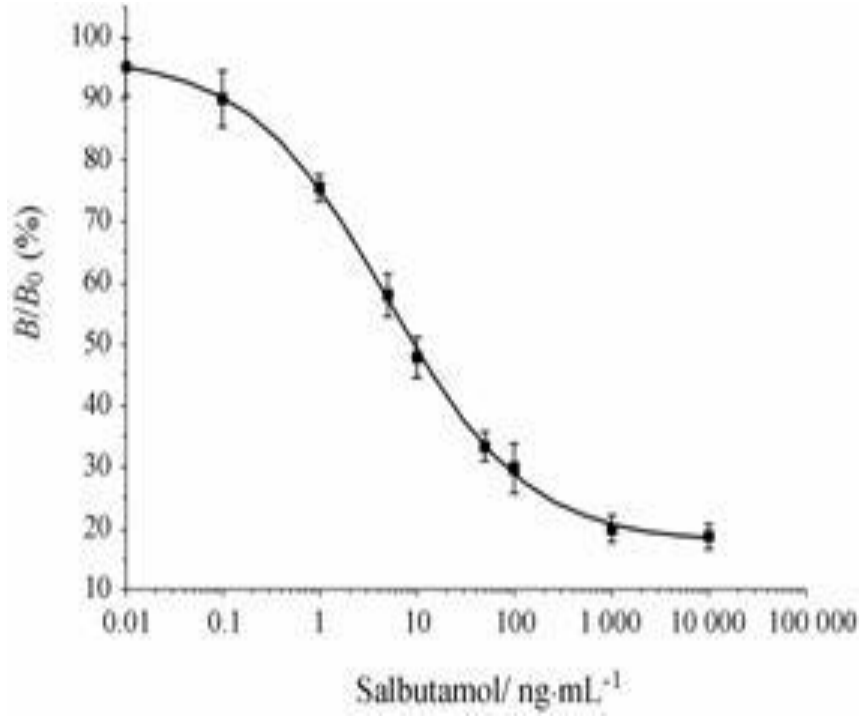
Direct Competitive ELISA



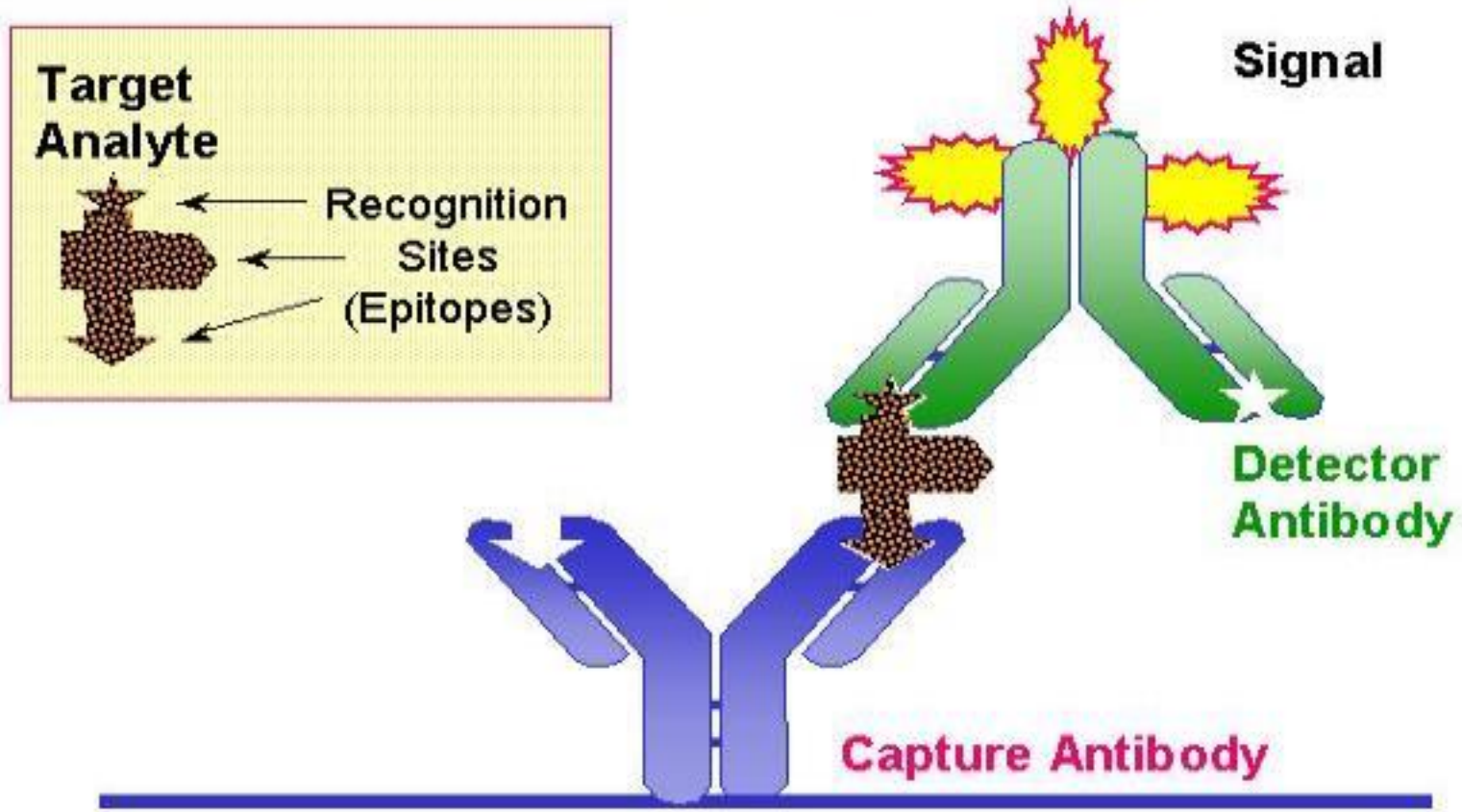
Competitive immunoassay

- Only one epitope
- One antibody
- Competion for antibody

- Less sensitive
- Less specific
- Small dynamic range



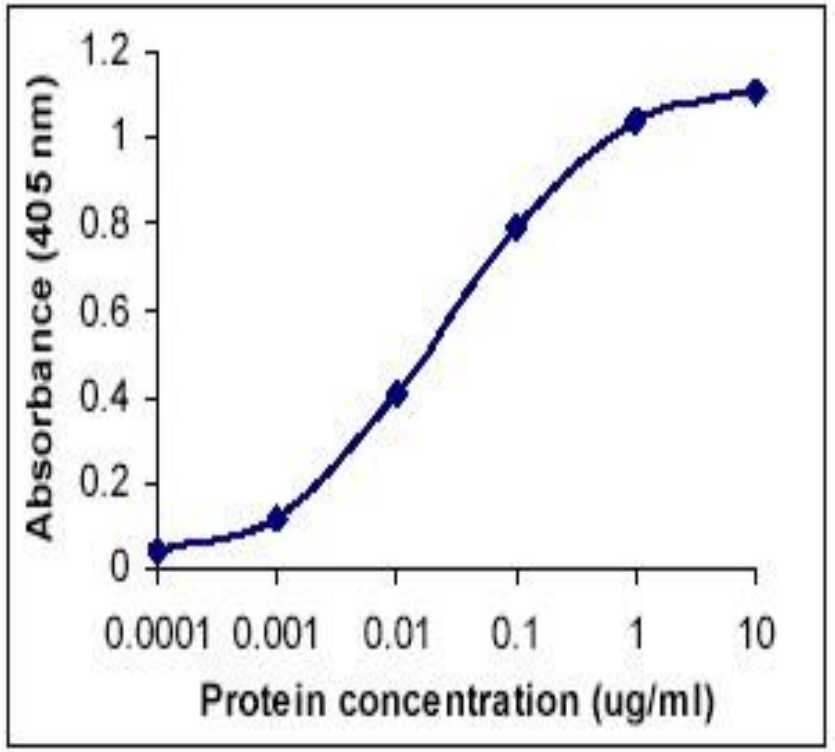
Double Antibody Sandwich Immunoassay



Sandwich assay

- Two epitopes
- Excess capture antibody
- Excess detection antibody

- Most sensitive immunoassays
- Large dynamic range
- High specificity



Immuno-assay

pro

- Relatively sensitive
- Easy
- Fully automated
- Direct measurement

contra

- Lack of accuracy
- Lack of sensitivity
- Lack of specificity
- Limited dynamic range
- Expensive (kit) reagents
- Black box

Preanalytical considerations

Serum tube

Plastic

Vacuum

Rubber

Separator gel

Surfactants

Clot activator

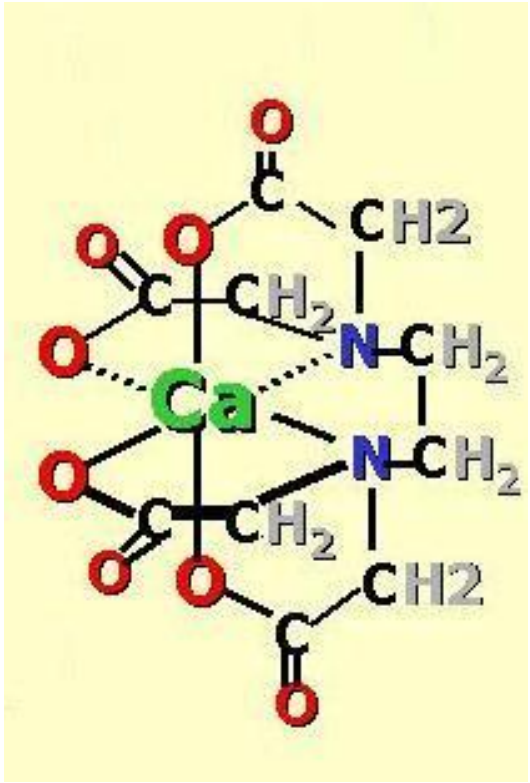
Plasma tube

Li heparin (green)

- fast centrifugation
- no clotting

EDTA (purple)

- ACTH
- renine
- aldosteron



Stability and storage

STAT analysis

- PTH
- Insulin
- C peptide
- ACTH
- …

Frozen till analysis

- Plasma Renin Activity
- Aldosterone
- Gastrin
- Glucagon
- …

hemolysis

Small effect on immunoassays

but:

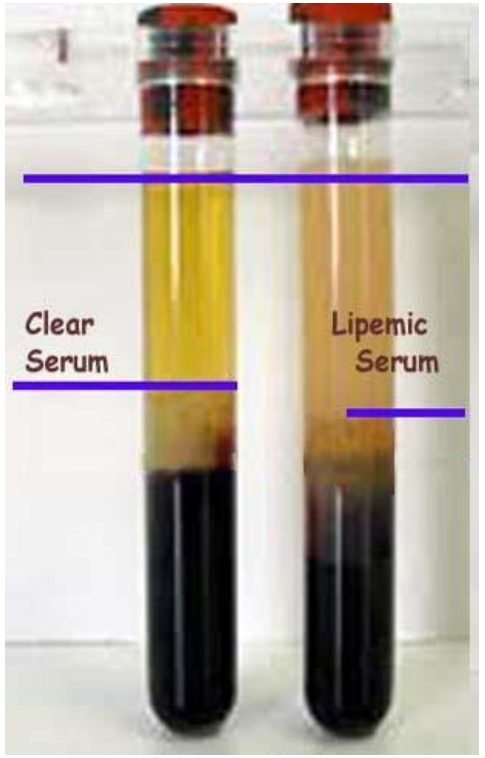
- proteolysis
  - insulin
  - gastrin
  - glucagon
  - PTH
- cellular release
  - NSE

lipemia

Small effect on immunoassays

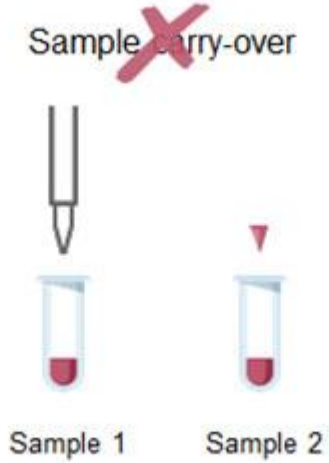
But:

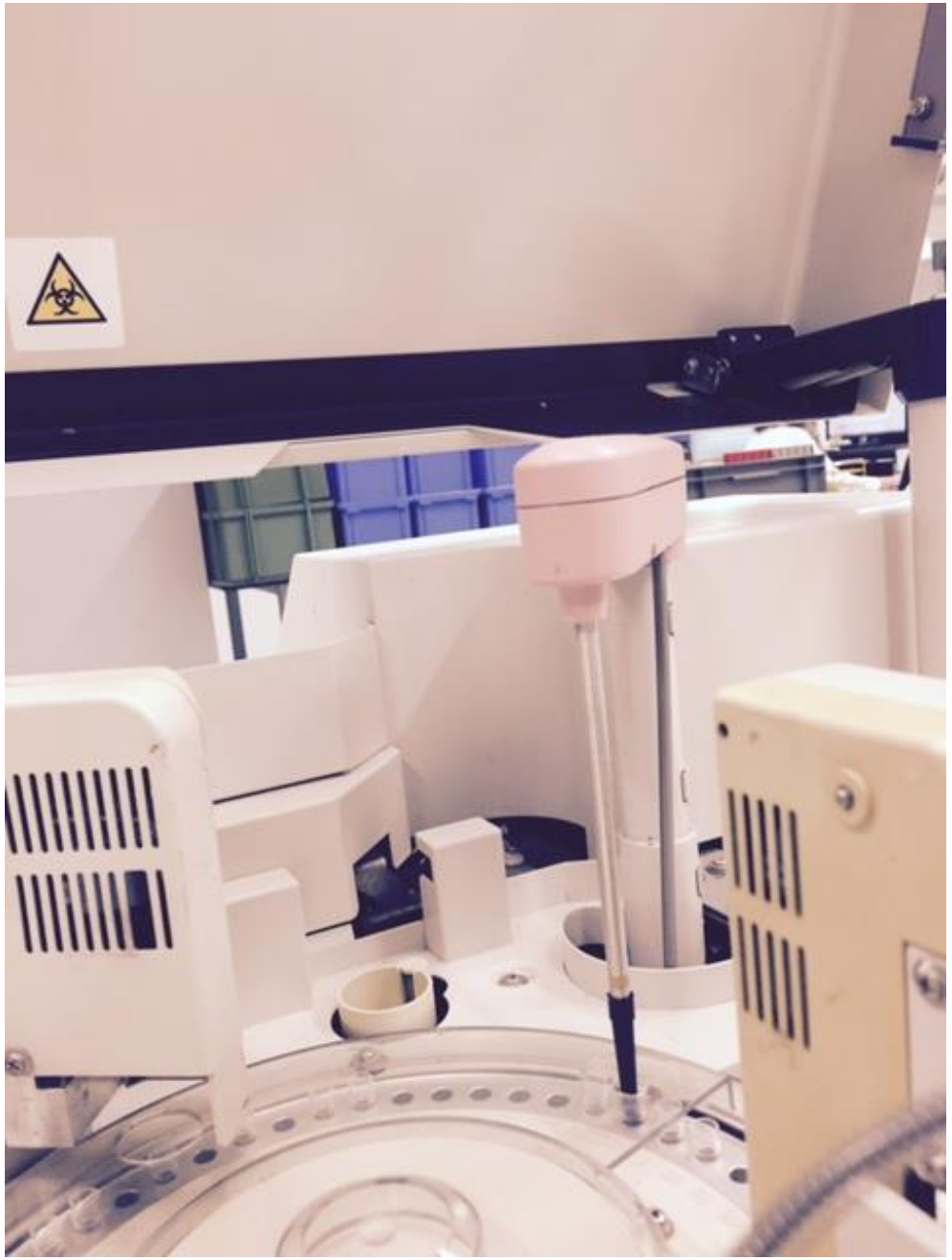
- turbidimetric
- nephelometric



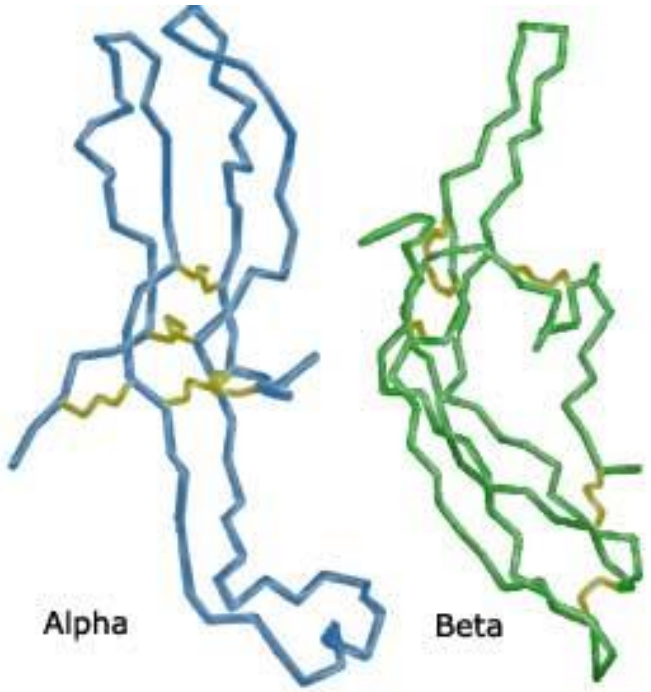
Analytical pitfalls

Carry-over



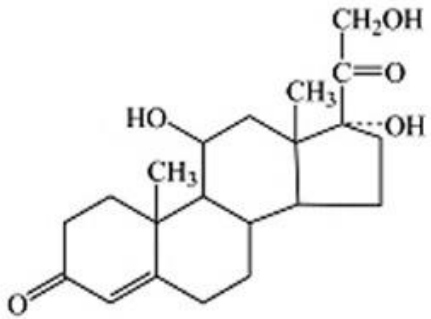


Cross-reactivity: hCG and LH

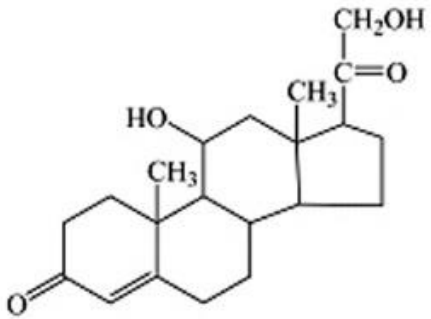


hCG subunits

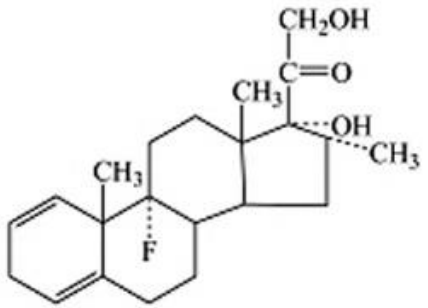
Cross-reactivity: steroids



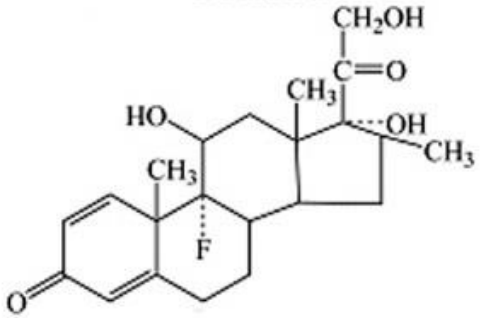
Cortisol



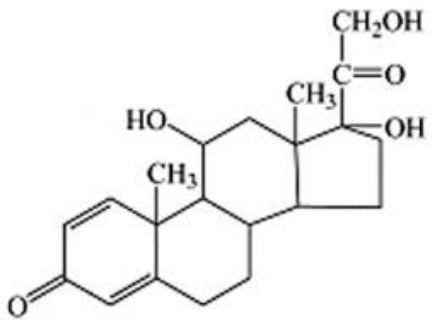
Corticosterone



Dexamethasone



Betamethasone



Prednisolone

Hormone binding proteins

Albumin, SHBG, TBG, CBG

For <u>total</u> hormone measurement, it is essential to displace all bound hormone from endogenous binding sites

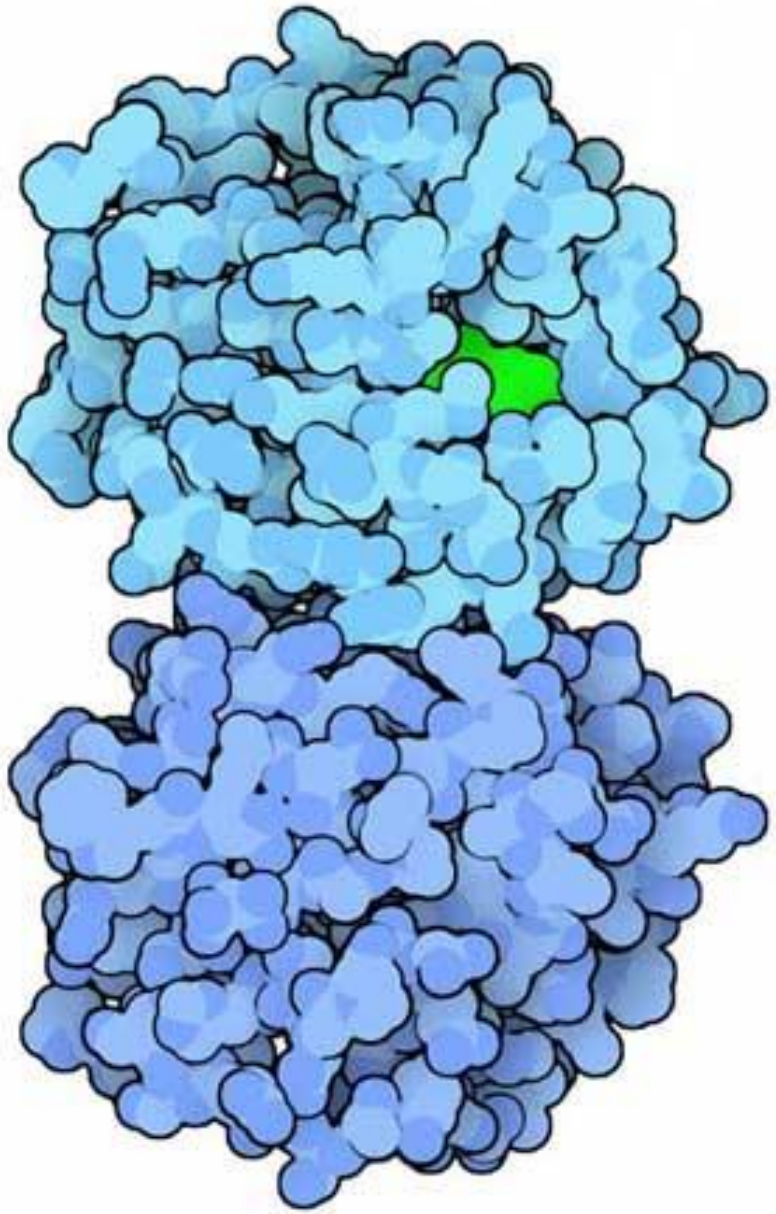
solvent extraction

anilino naphtalene sulfonic acid etc.

For <u>free</u> hormone measurement, displacement can alter the equilibrium

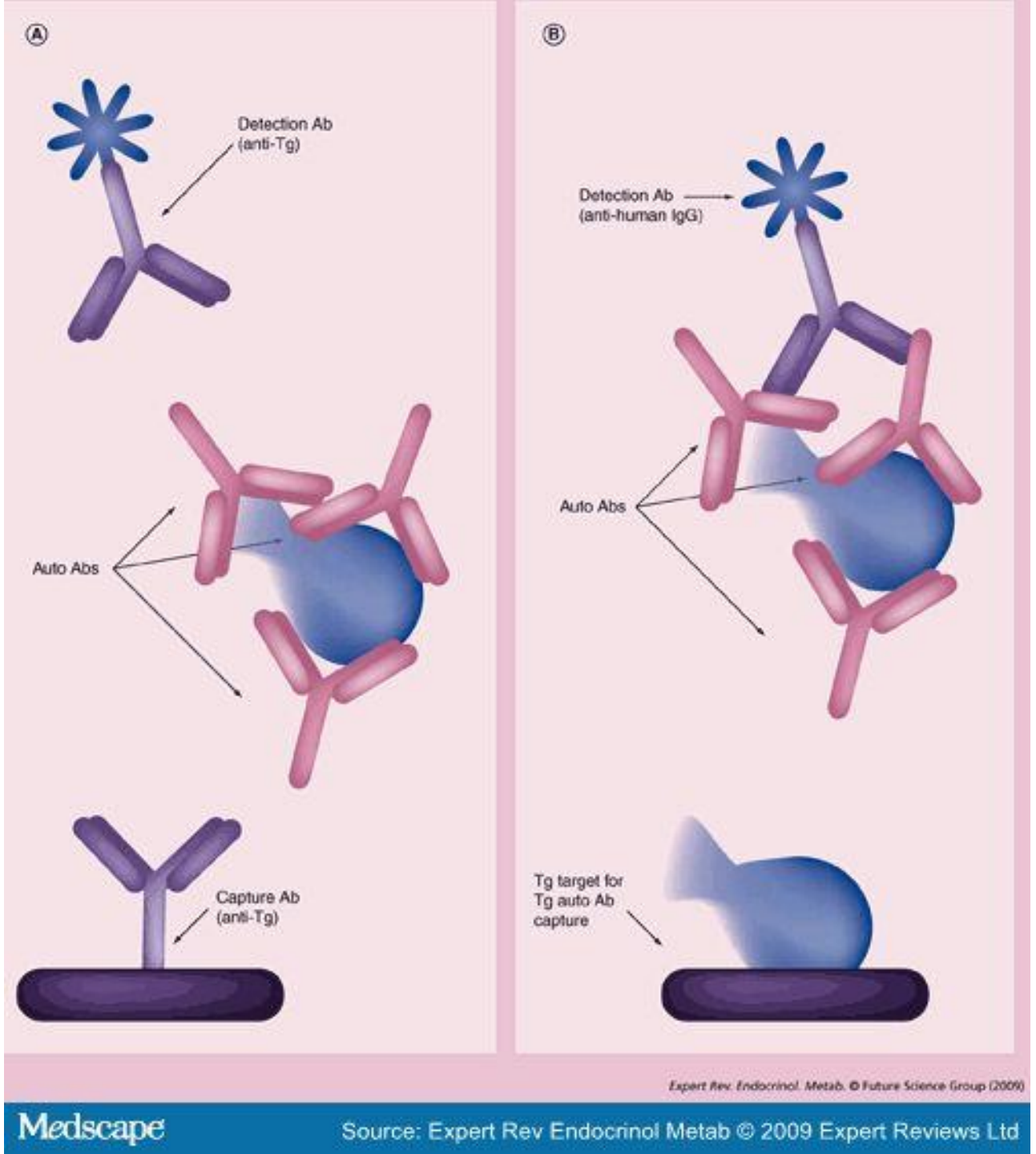
(always happens to a certain degree in free hormone assays + low concentration!)

NEFA in free thyroxine assays



Autoanalyte antibodies

Thyroglobulin

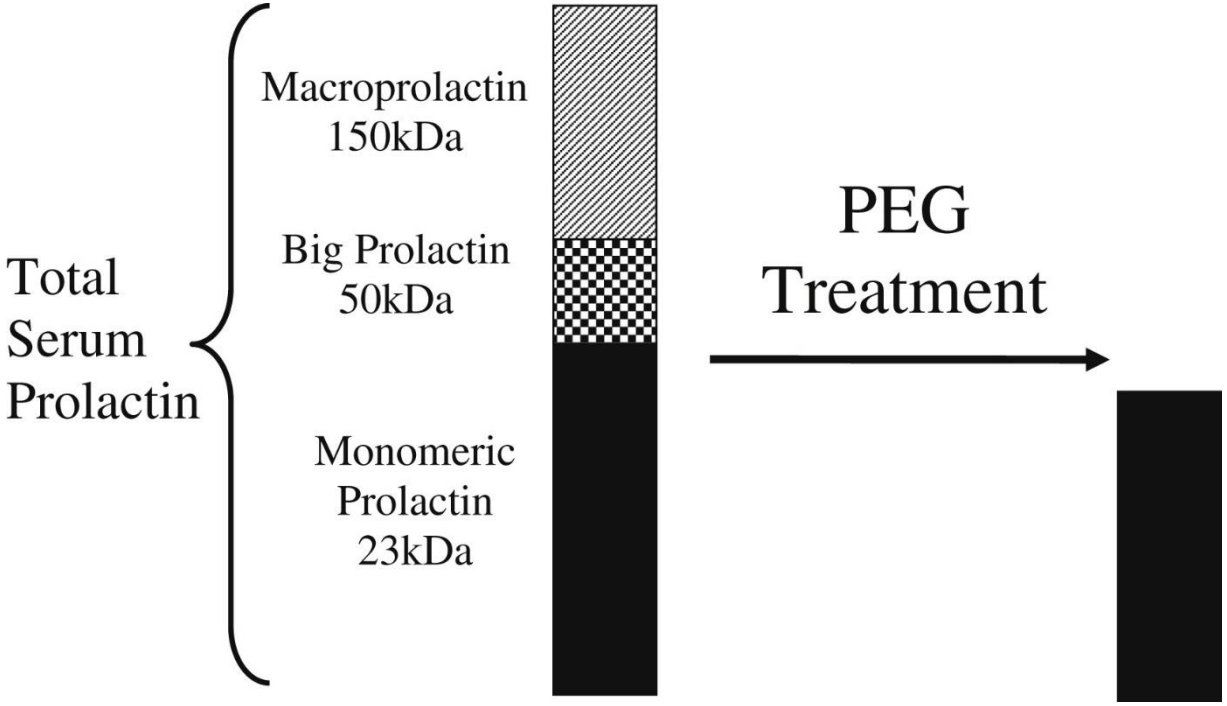


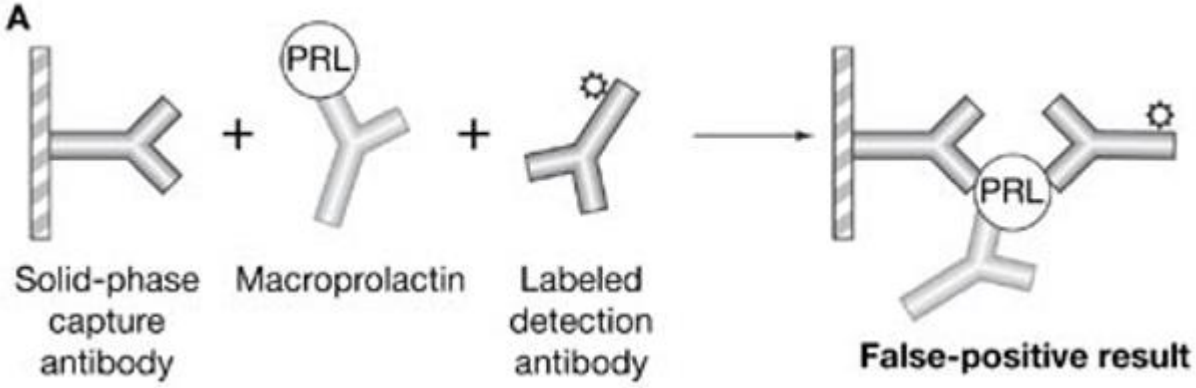
Macroprolactin

Biologically inactive

Cleared slowly

Causes false high prolactin





250 µL Serum + 250 µL PEG Reagent*

↓ *Vortex Mix*

Incubate 10 min at Room Temperature

↓ *Centrifuge 4,000RPM*

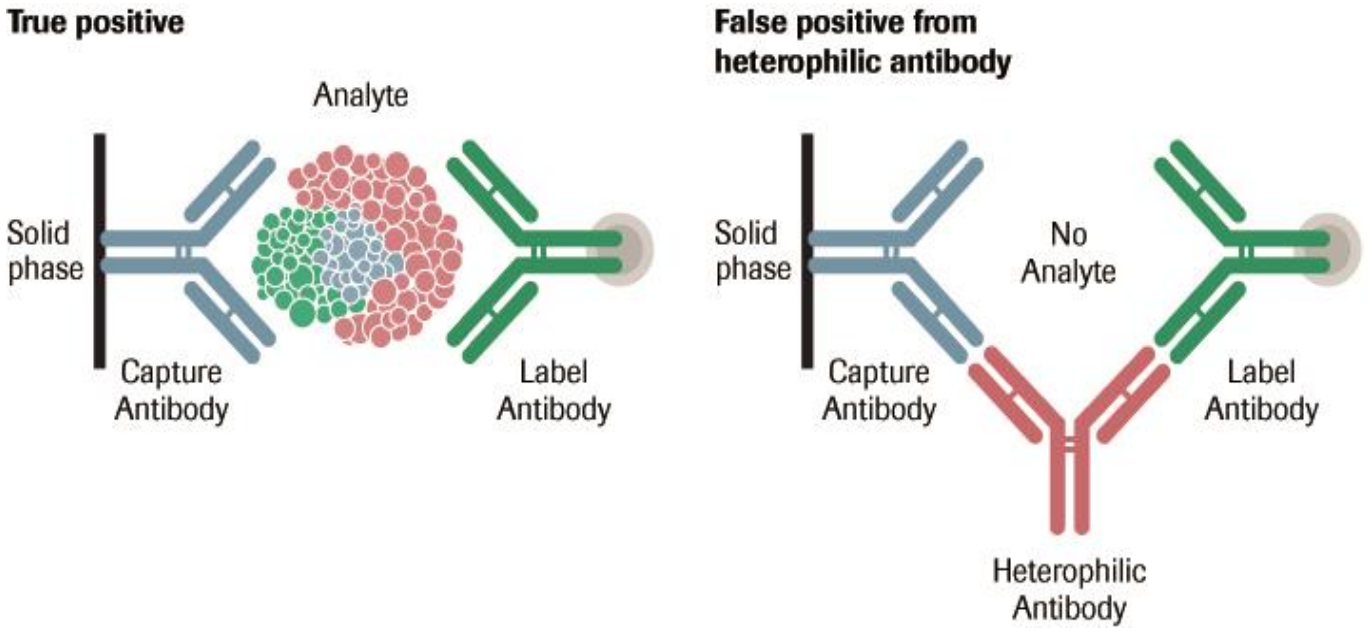
Measure Prolactin in Supernatant

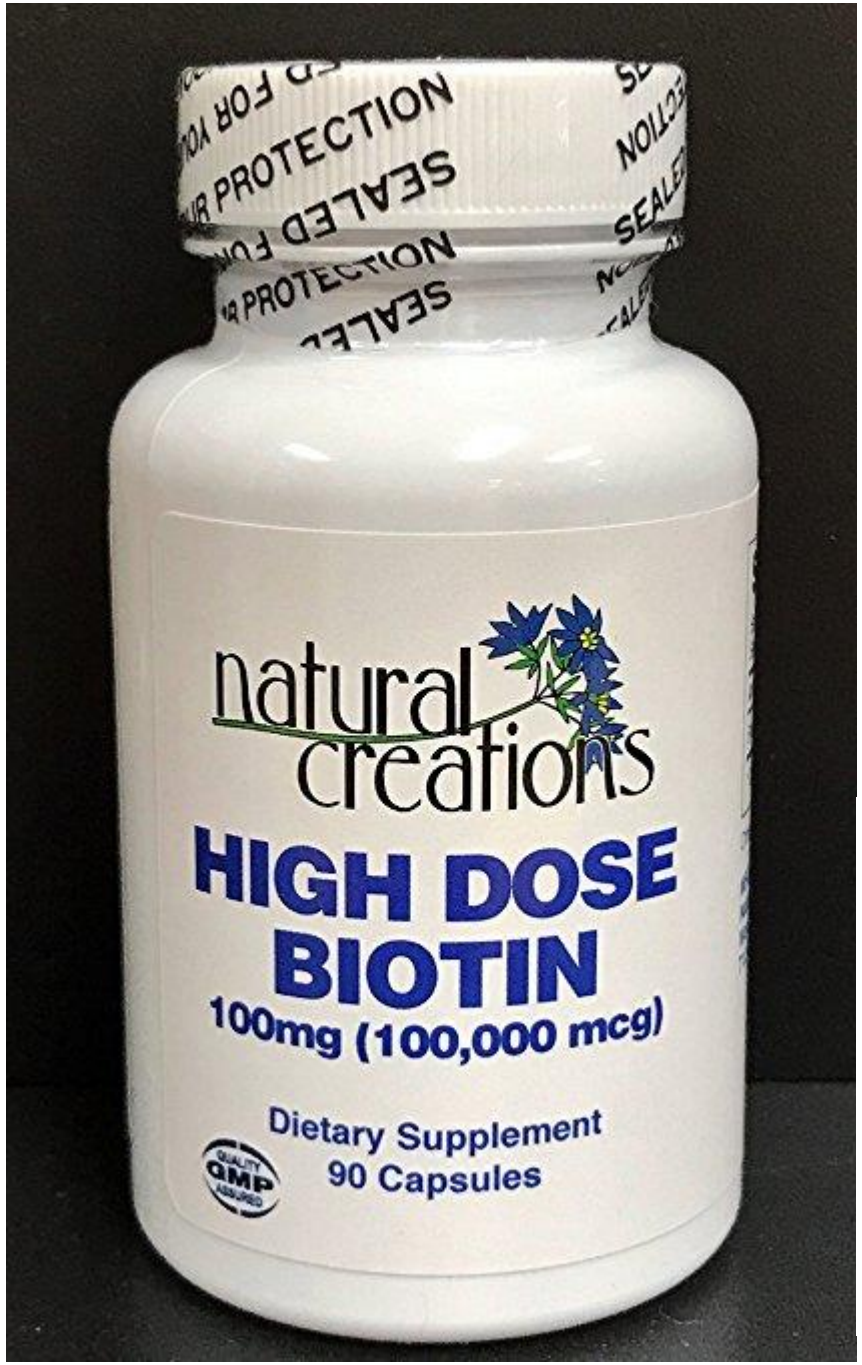
↓

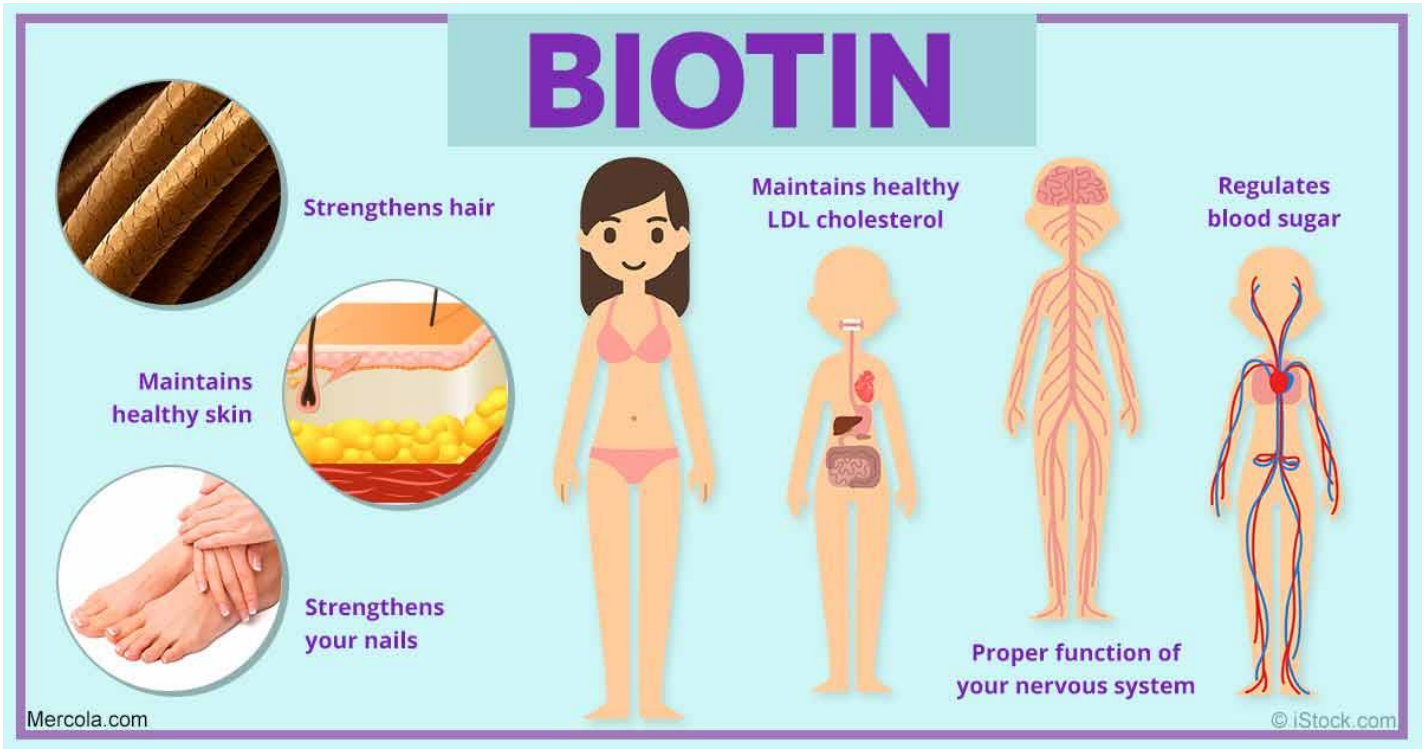
Multiply Result X2 to Correct for Dilution

Heterophile antibodies

HAMA







HEALTHLINE NEWS

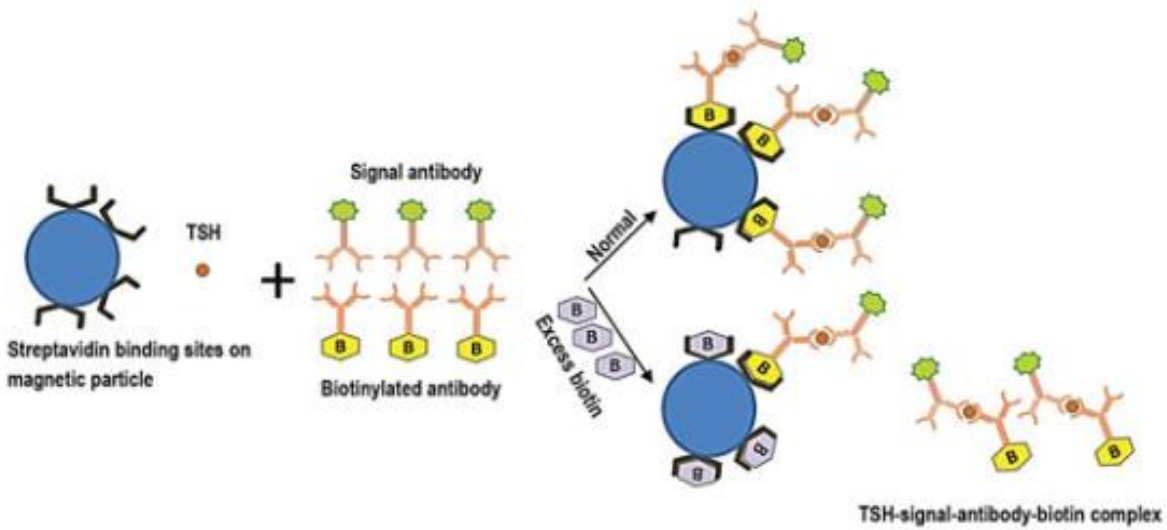
Study Looking at High-Dose Biotin as a Treatment for Multiple Sclerosis

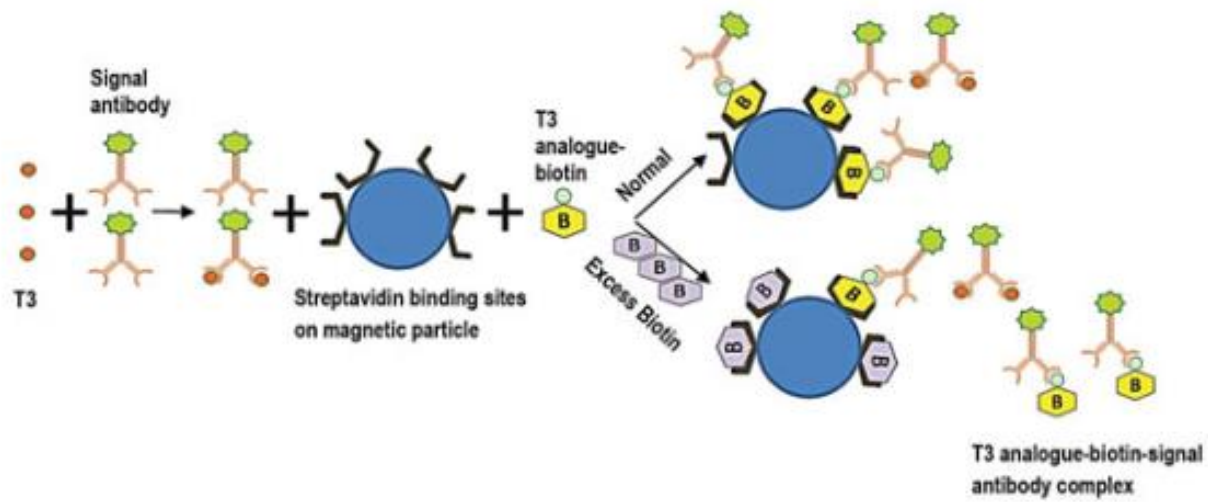
Written by Caroline Craven on 12 mei 2017

✓ Fact Checked

IMMUNOMETRIC

COMPETITIVE





DE GRUYTER

Clin Chem Lab Med 2017; 55(6): 777–779

Editorial

Aldo Clerico and Mario Plebani

Biotin interference on immunoassay methods: sporadic cases or hidden epidemic?

September 26, 2017

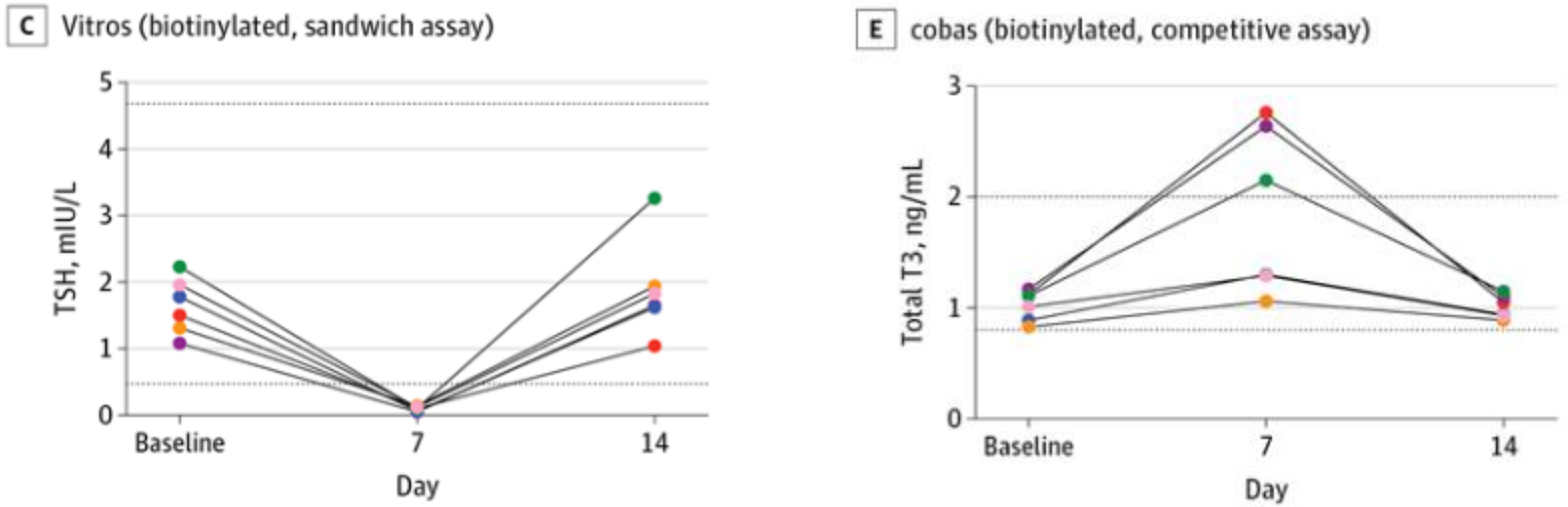
Association of Biotin Ingestion With Performance of Hormone and Nonhormone Assays in Healthy Adults

Danni Li, PhD[1]; Angela Radulescu, MD[2]; Rupendra T. Shrestha, MD[2]; et al

» Author Affiliations | Article Information

JAMA. 2017;318(12):1150-1160. doi:10.1001/jama.2017.13705

Affected systems: COBAS, Vista, Vitros, Centaur



Six patients taking 5 mg biotin for 7 days

High dose hook effect

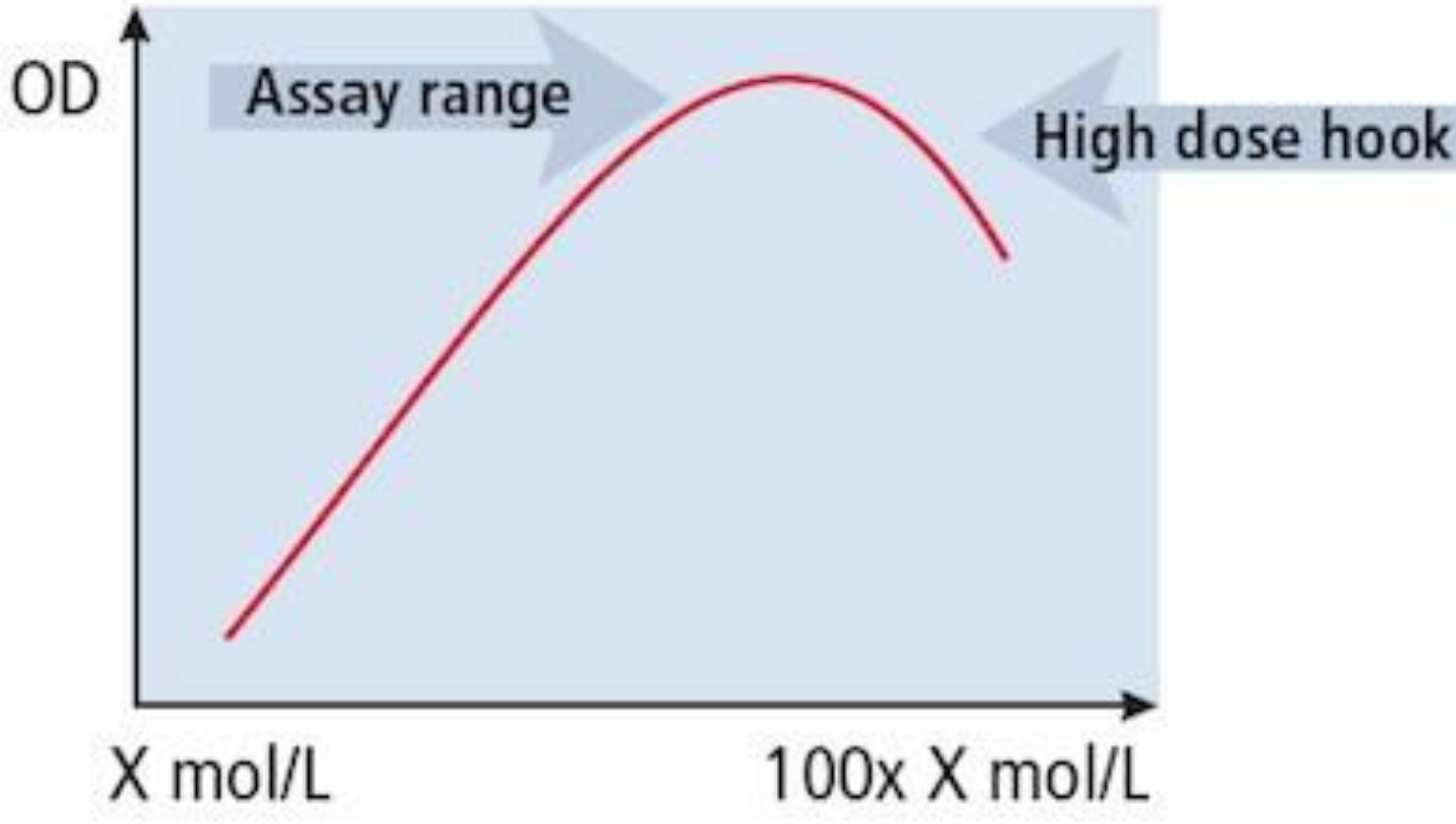
Tumor markers

hCG

Ferritin

Thyroglobulin

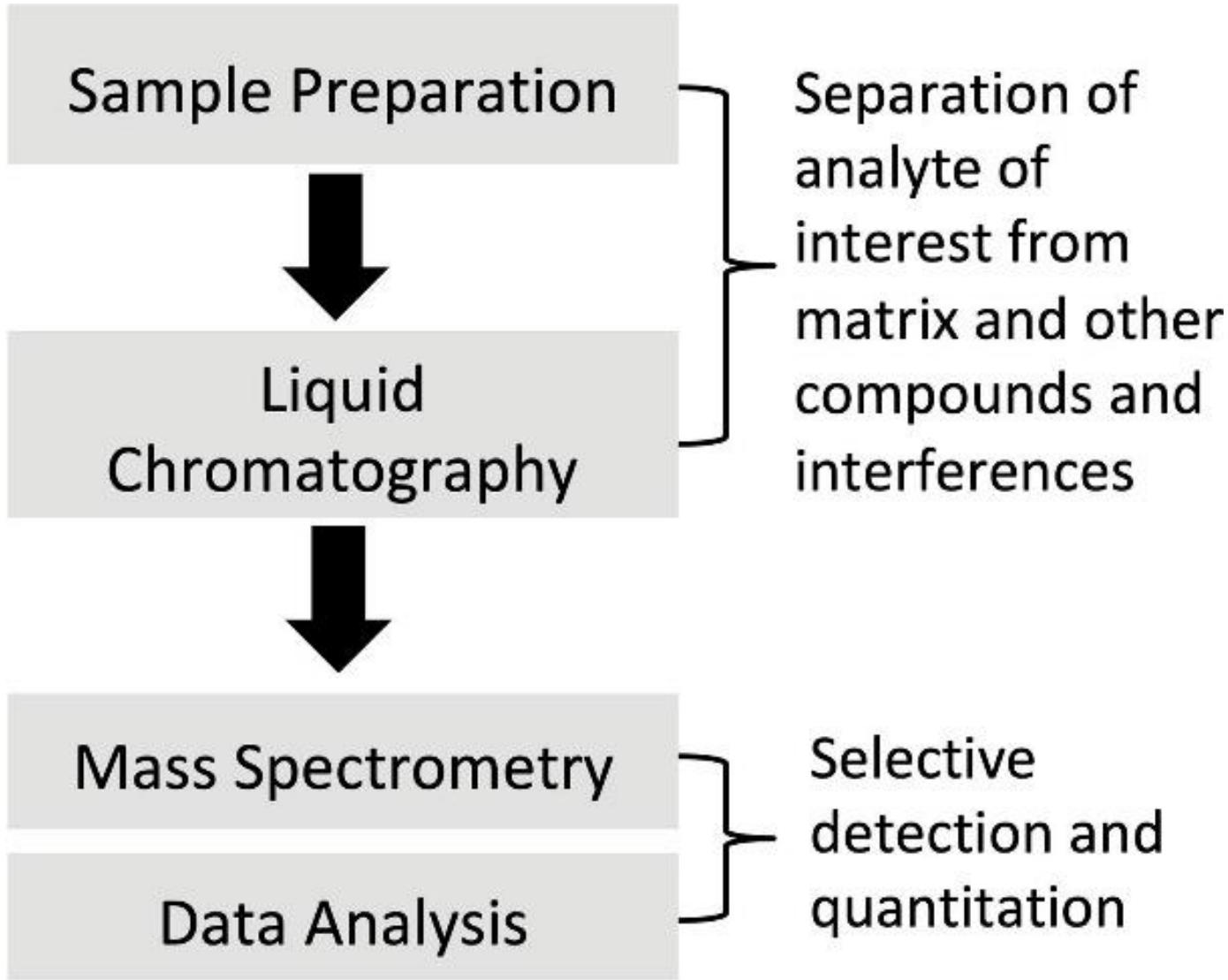
High analyte concentration



Repeat the analysis with an alternative immunoassay, preferably using assay antibodies from a different species
Treat the sample with an additional blocking agent (Heterophilic Blocking Tubes, Scantibodies)
Dilute the sample: non linearity indicates assay interference

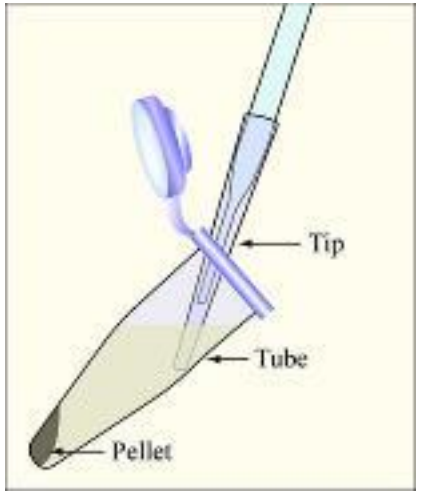
Mass Spectrometry

A reference method for hormones

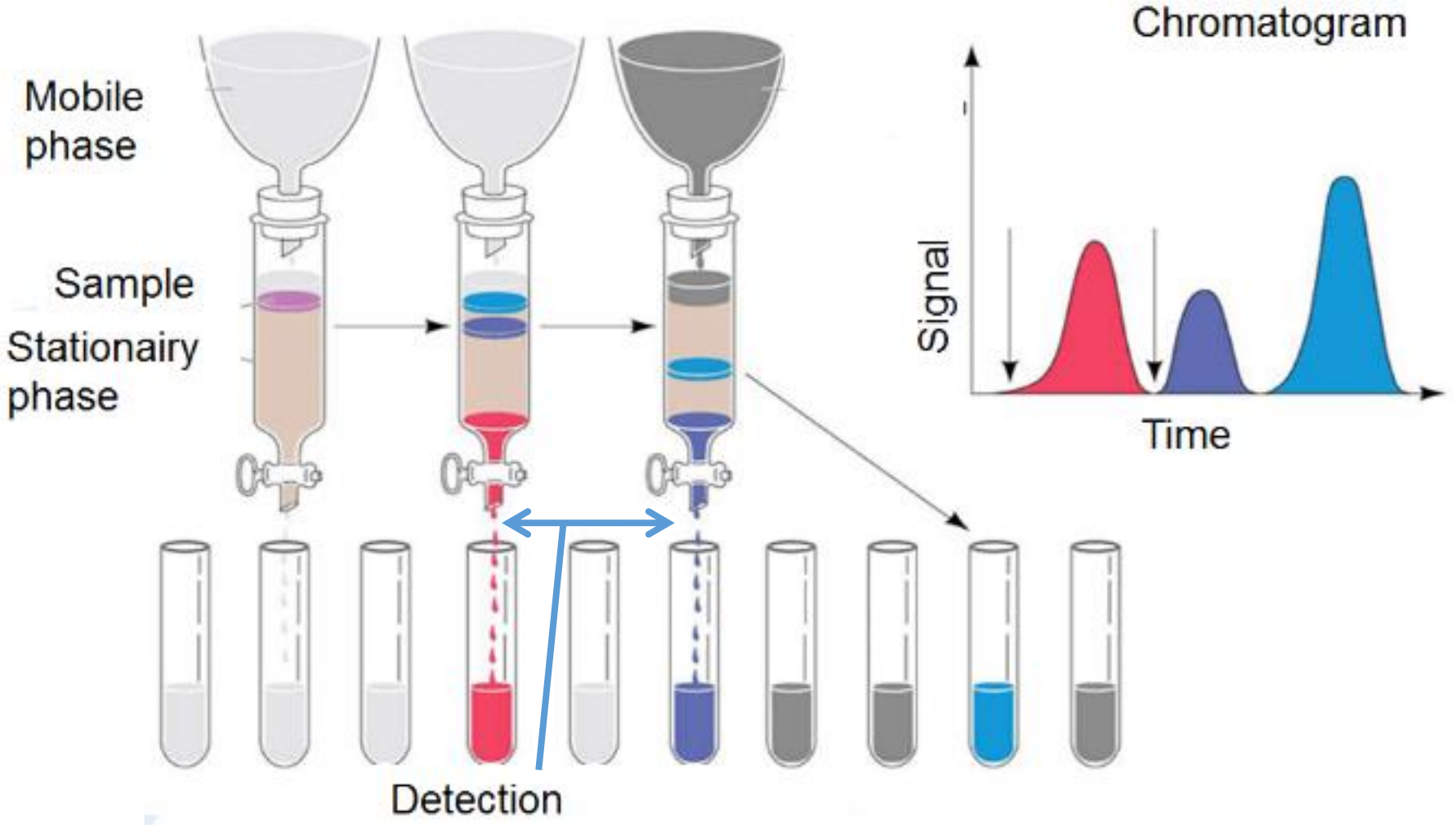

Sample Preparation
Liquid Chromatography
Mass Spectrometry
Data Analysis
Separation of analyte of interest from matrix and other compounds and interferences
Selective detection and quantitation

1) Sample preparation

- Protein precipitation (methanol, acetonitrile, $ZnSO_4$)
- Liquid liquid extraction
- Solid phase extraction



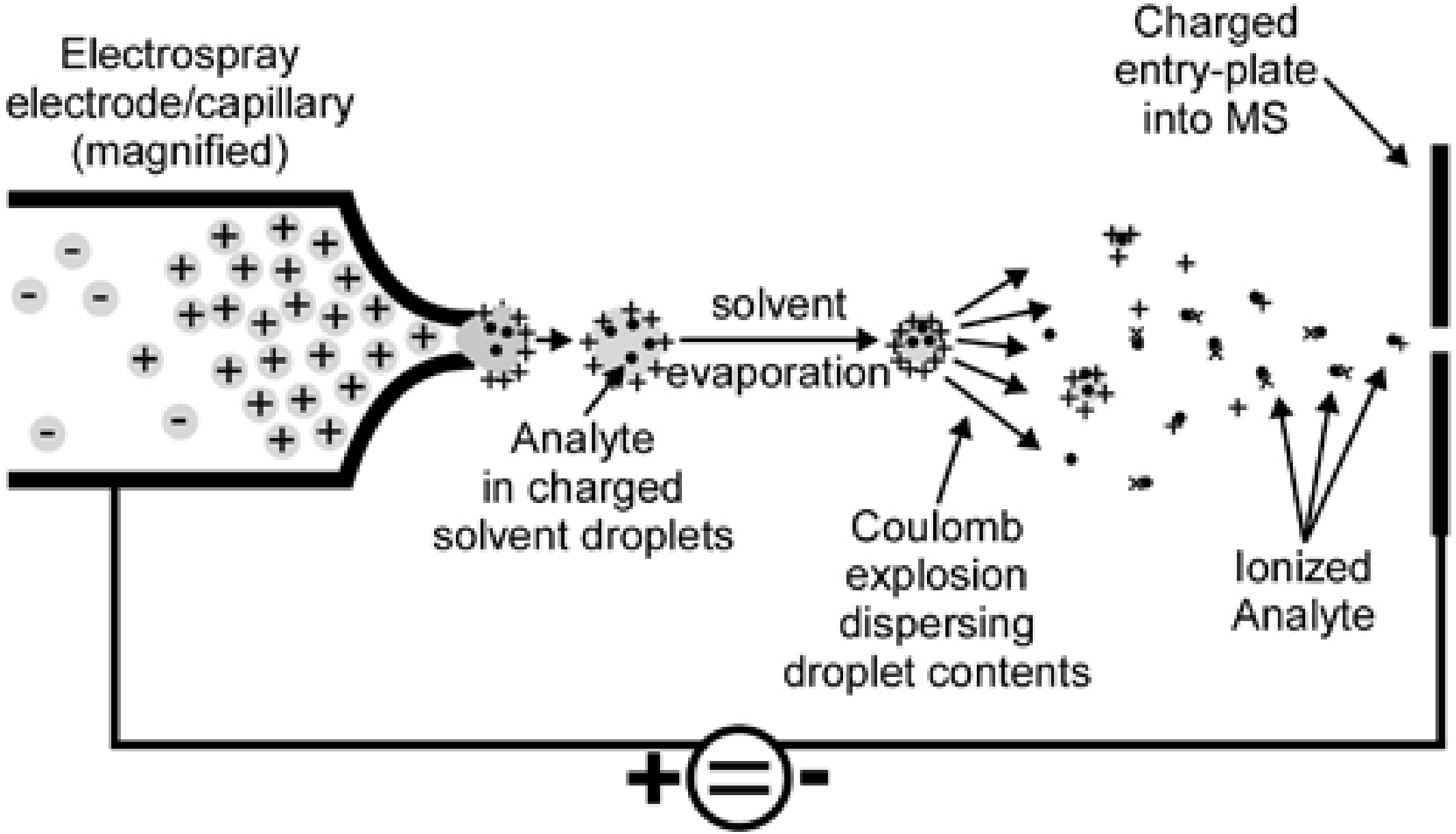
2) HPLC


Mobile phase
Sample
Stationairy phase
Detection
Chromatogram
Signal
Time

HPLC in practice

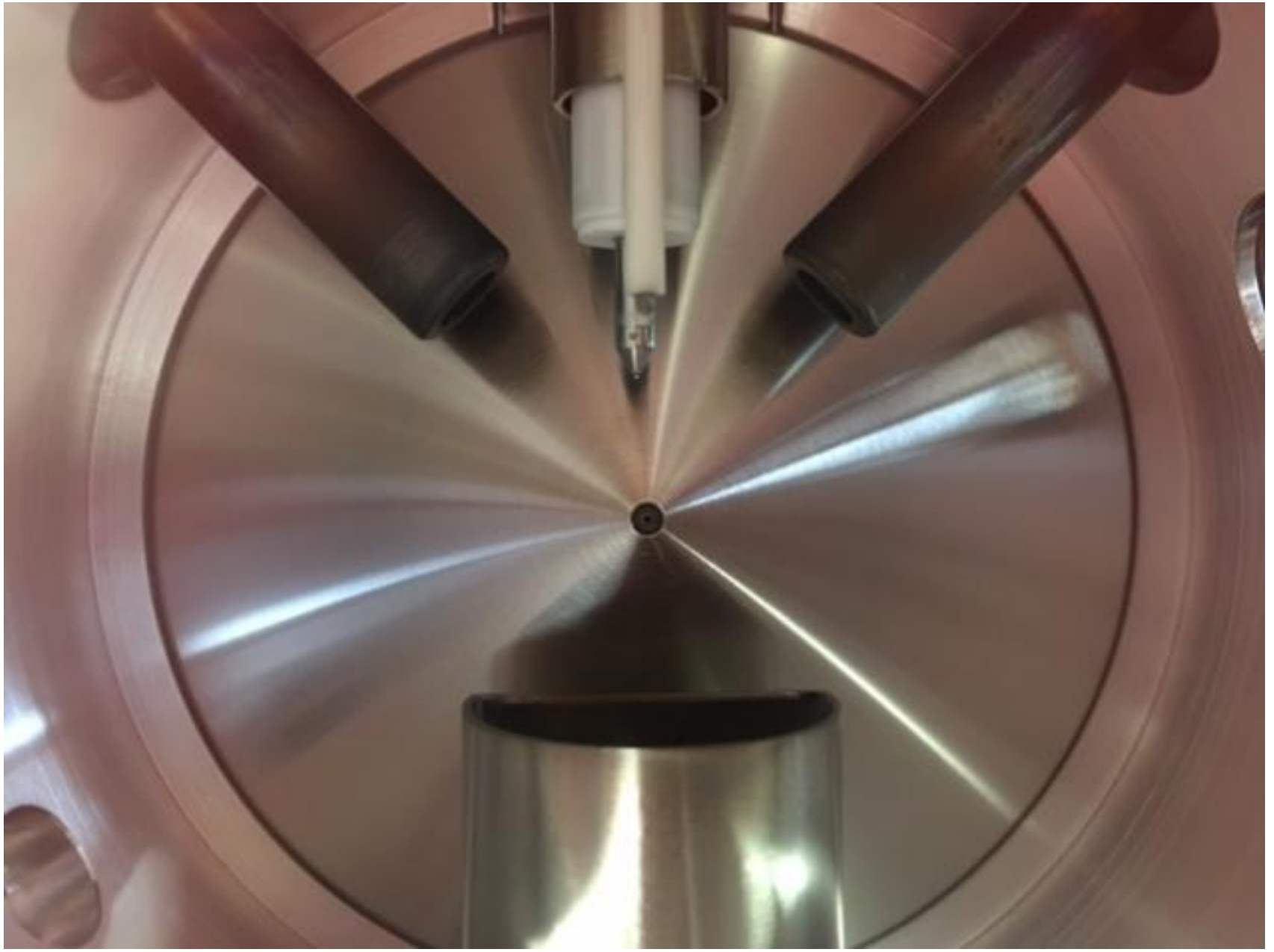
- Pumps
- Fluids
- Tubings
- Sample loader
- Complex valves
- Column

B: ESI-Source


Electrospray
electrode/capillary
(magnified)
Charged
entry-plate
into MS
solvent
evaporation
Analyte
in charged
solvent droplets
Coulomb
explosion
dispersing
droplet contents
Ionized
Analyte

Mass Spectrometry in practice

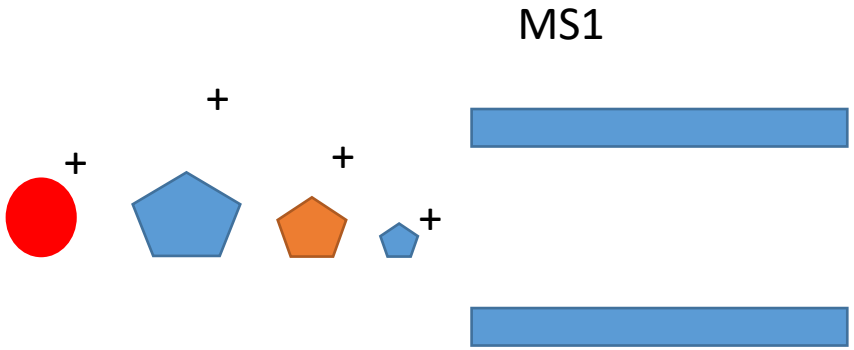
- Spray probe
- Heating probes
- Curtain plate
- Curtain plate aperture
- Orifice

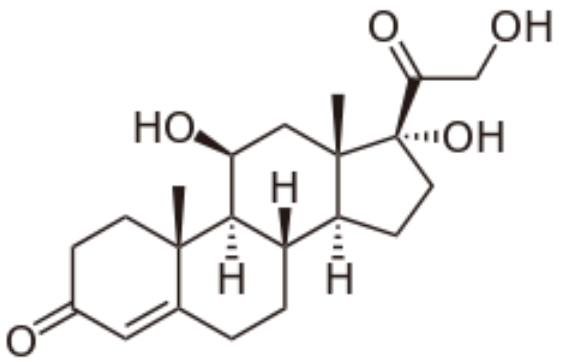


Mass Spectrometry in practice

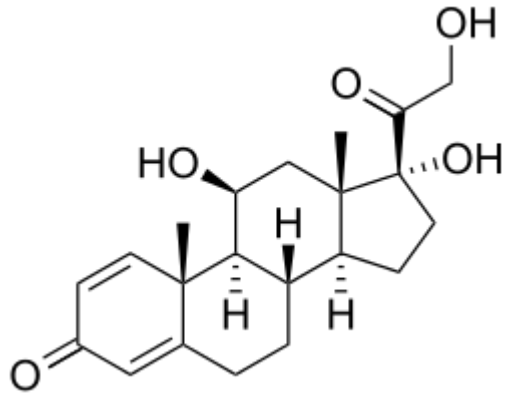
- Ion source
- Switching valves
- Tubings
- Vacuum pump
- Nitrogen generator

Massaspectrometer (Q)



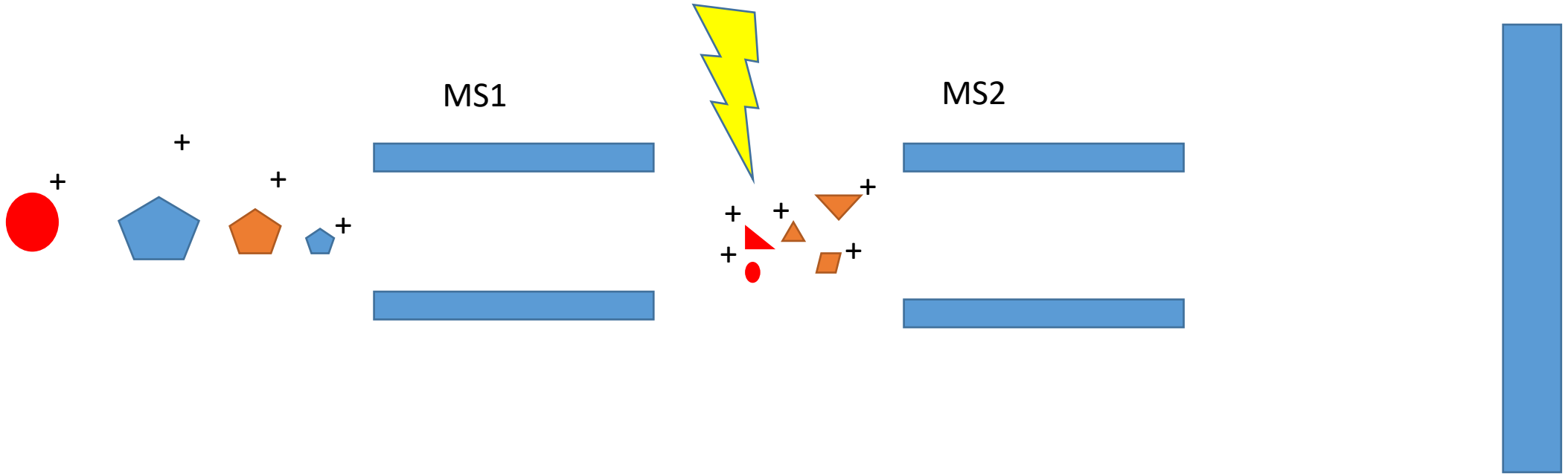


Cortisol 362,460 Da



Prednisolon 360,444 Da

Tandem Massaspectrometer (QQQ)

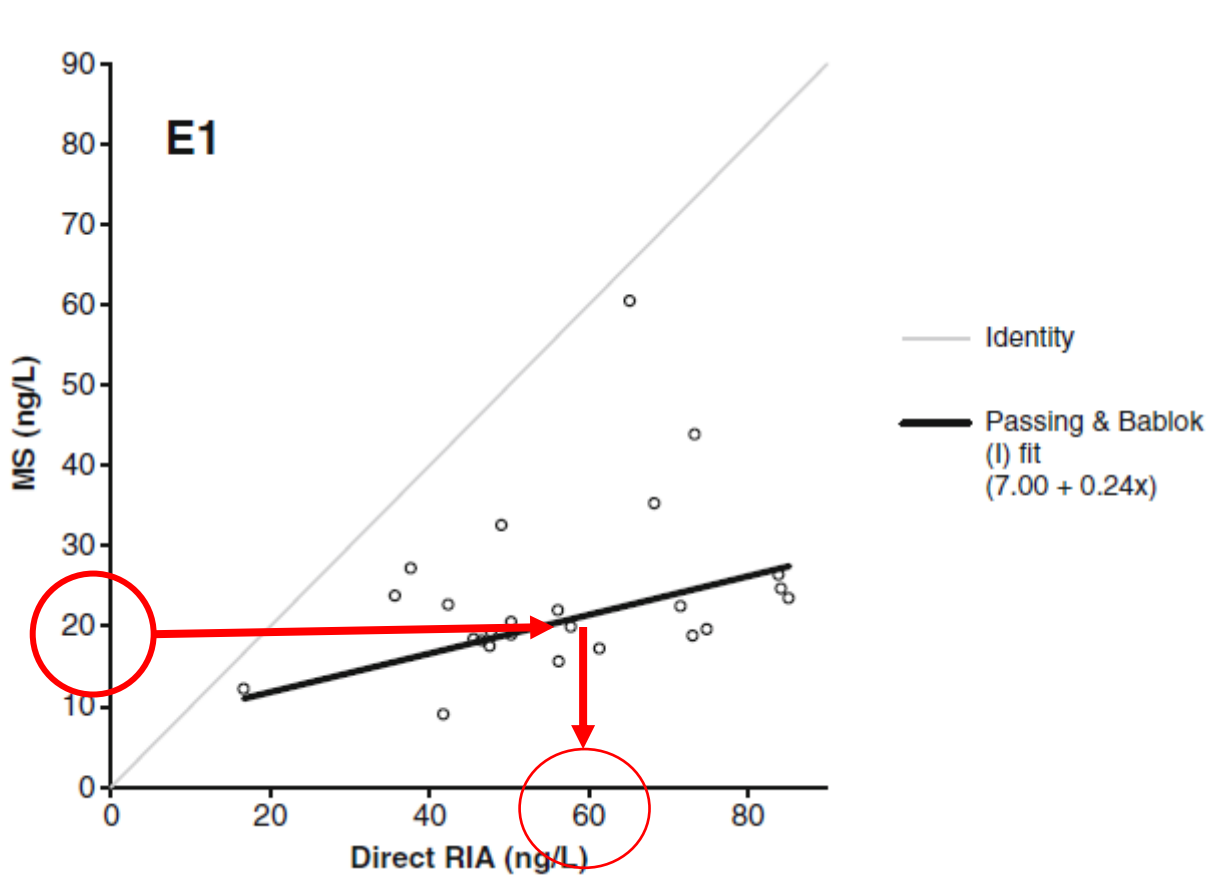


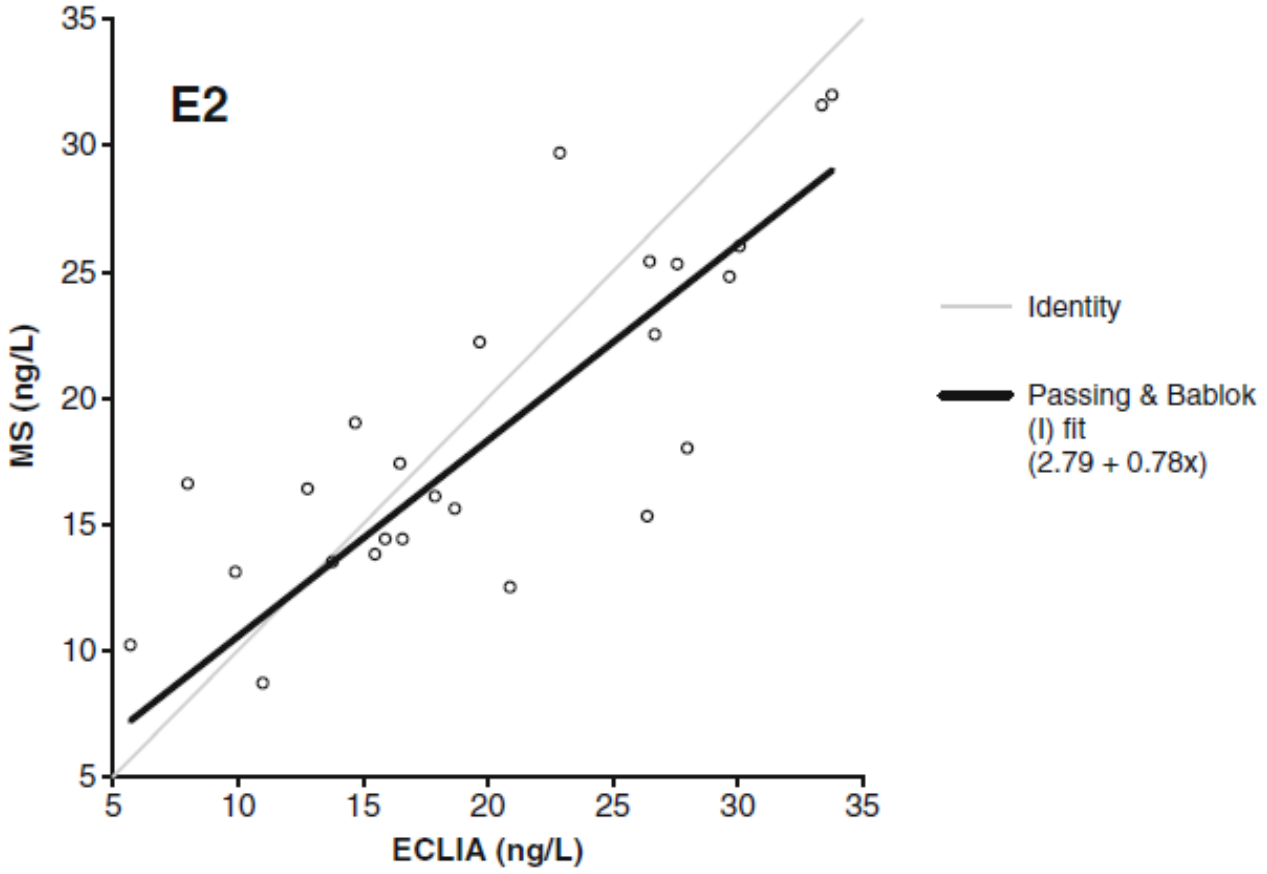
Future directions

LC-MS/MS in Endocrinology

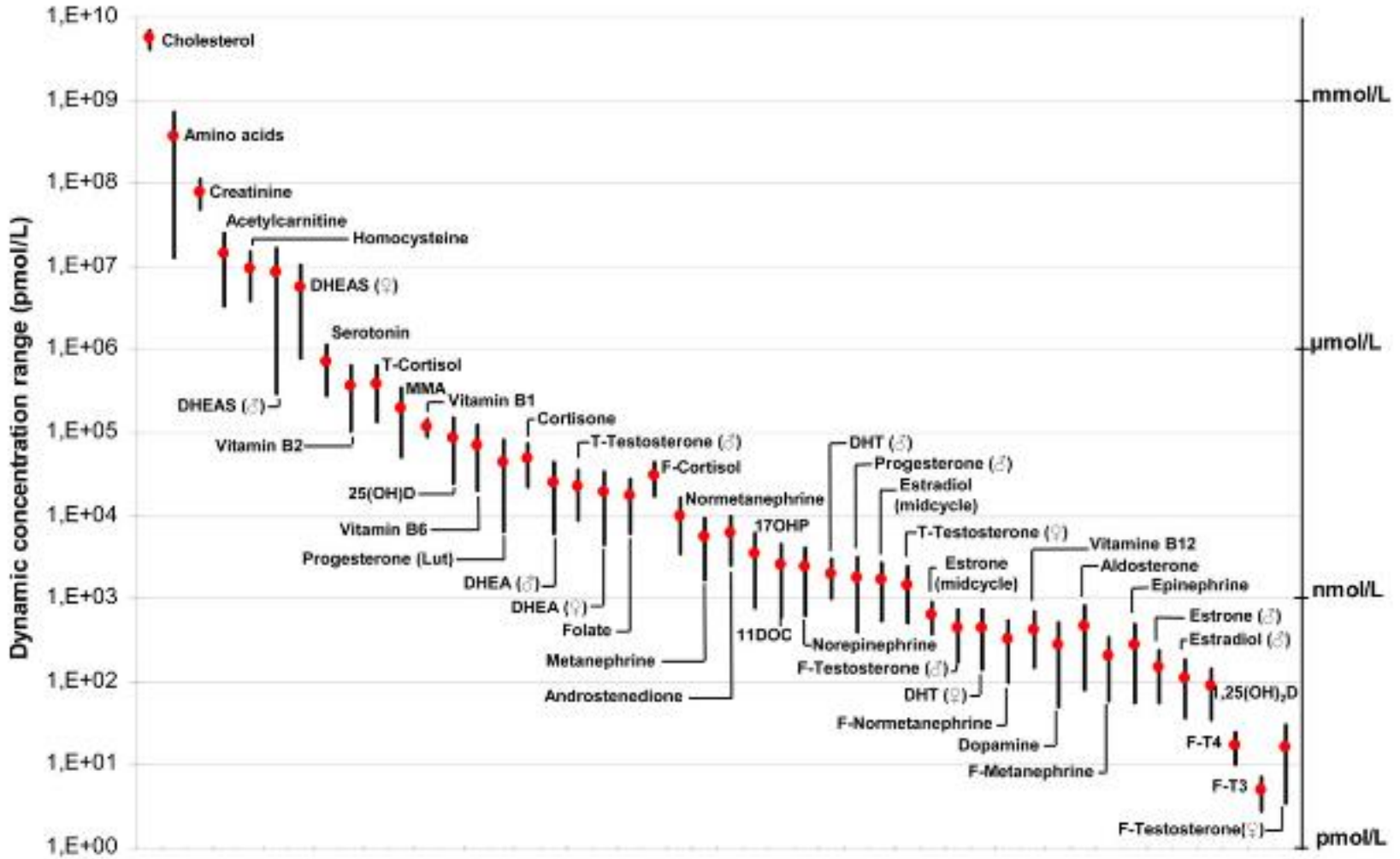
Analytical Superiority

Added value LC-MS/MS: Accuracy





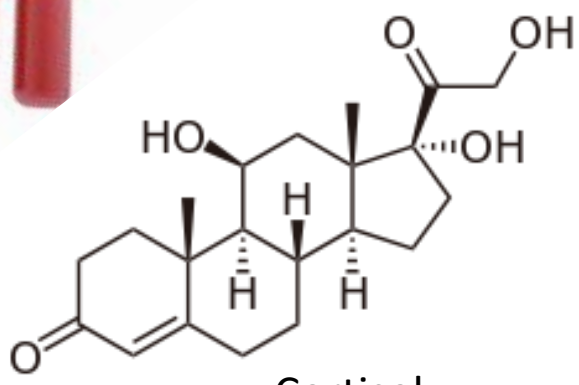
Added LC-MS/MS: Sensitivity



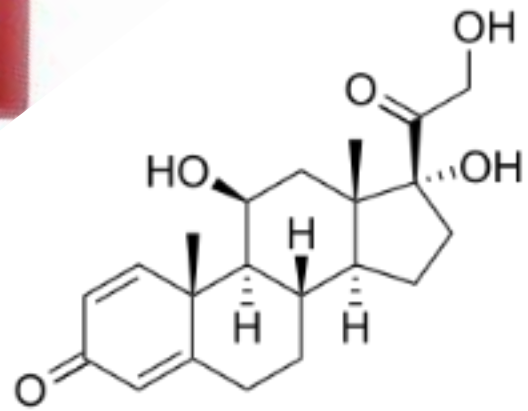
van den Ouweland JMW, Kema IP. J Chromatography B 2012

Added value LC-MS/MS: Specificity

Cross reactivity!

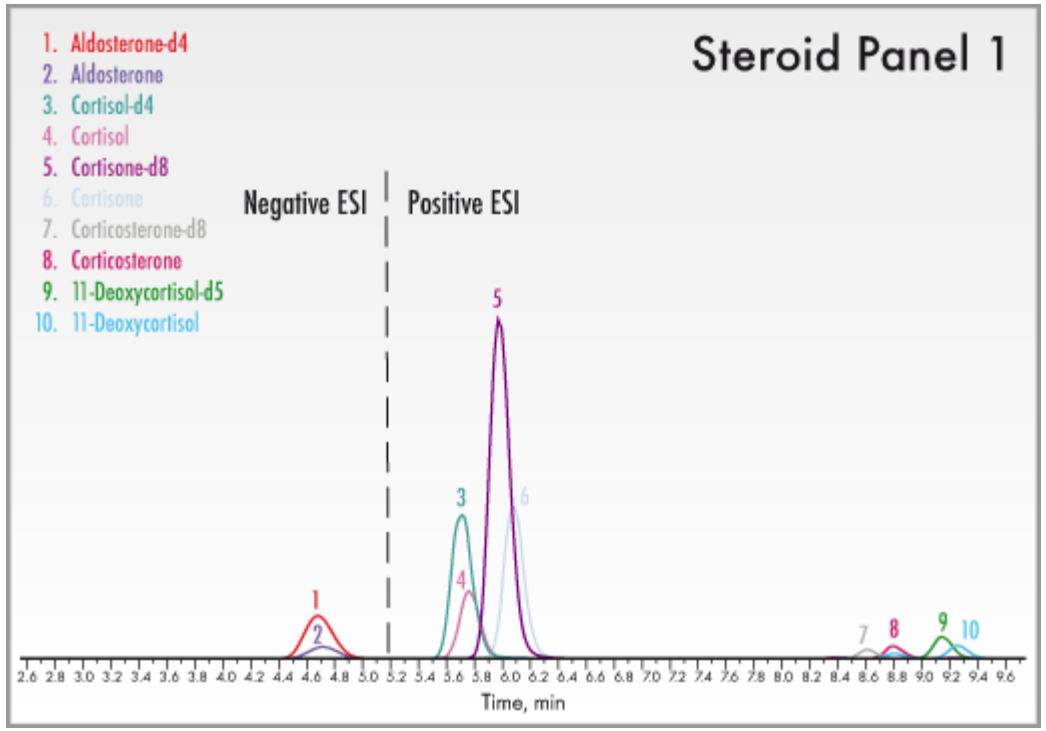


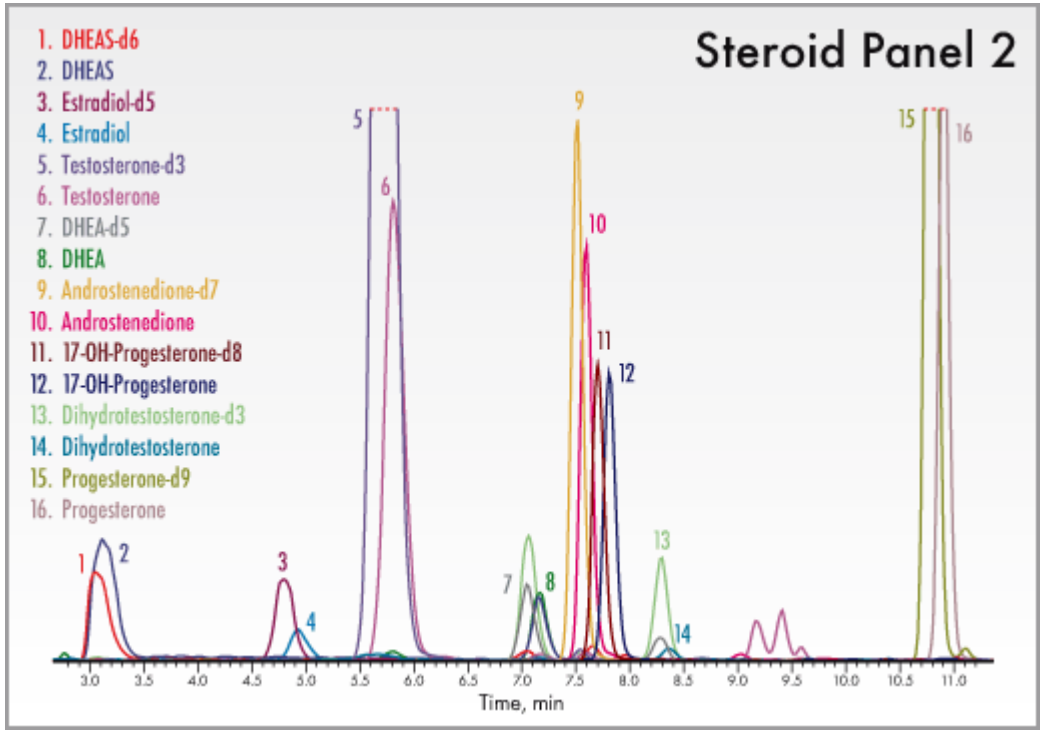
Cortisol



Prednisolon

Added value LC-MSMS: steroid profiling





Added value LC-MS/MS: pro and contra

Advantages

- Analytical superiority
- “Cheap” reagents
  - Organic solvents
  - Deuterated internal standards
  - Robust colums
  - Calibrator sets
  - Pipetting tips and plates
- High troughput possible
- Develop new assays

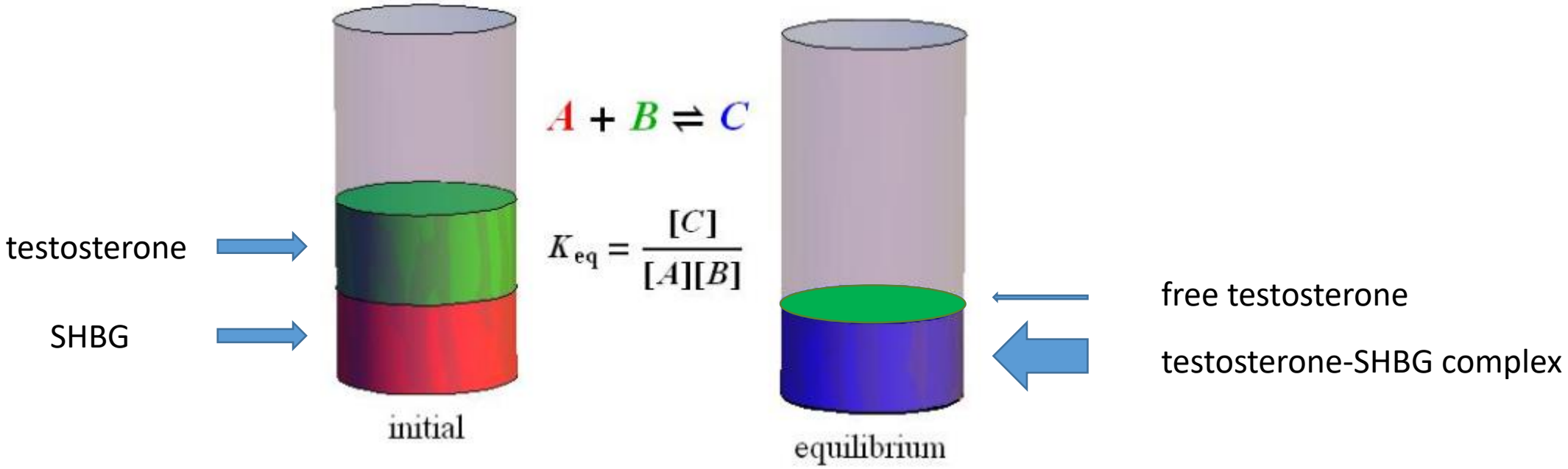
Disadvantages

- Expensive instruments
- Batch mode
- Skilled labor required
- LIS interface
- Regulartory uncertainty
- Ruggedness

Free hormone estimates

Never ending story….

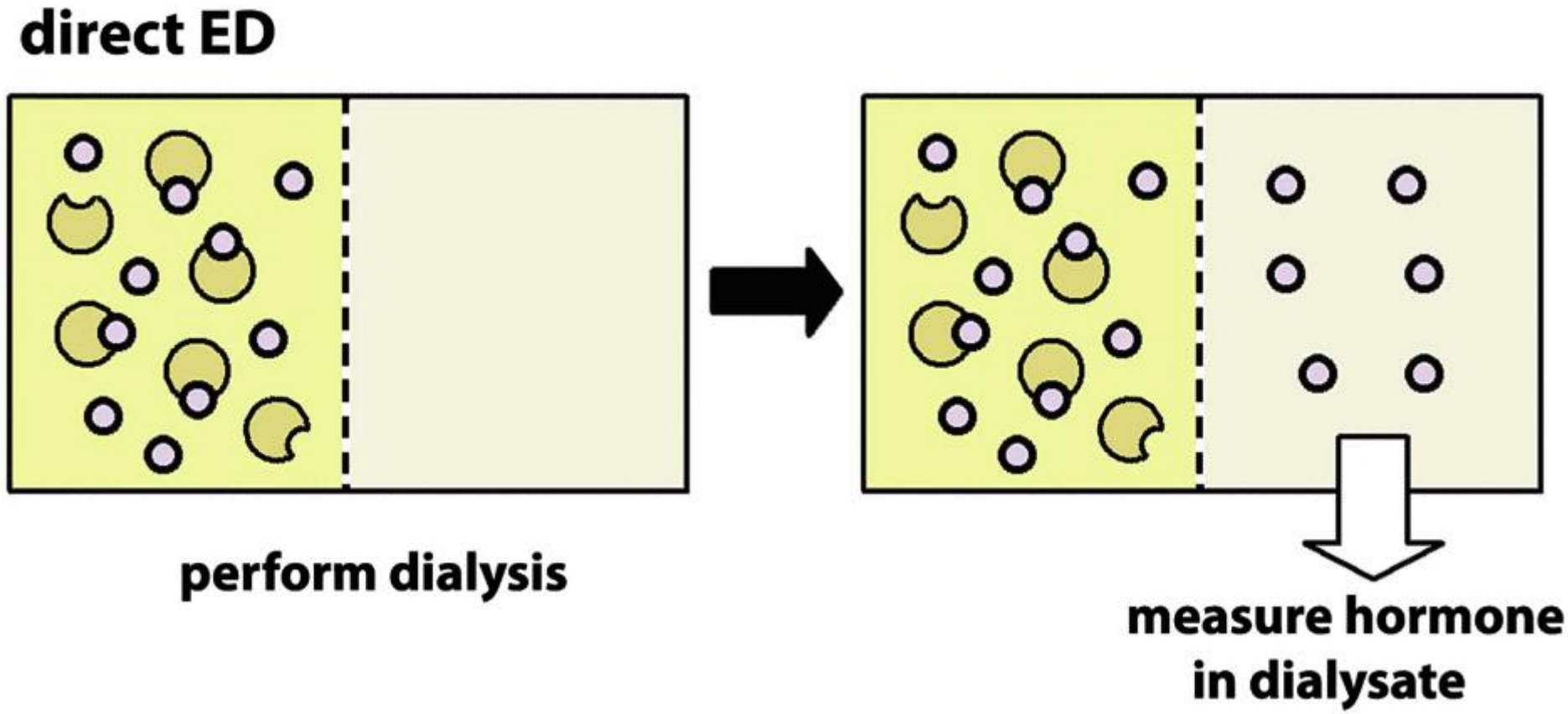
Law of mass action



Problems in free hormone assays

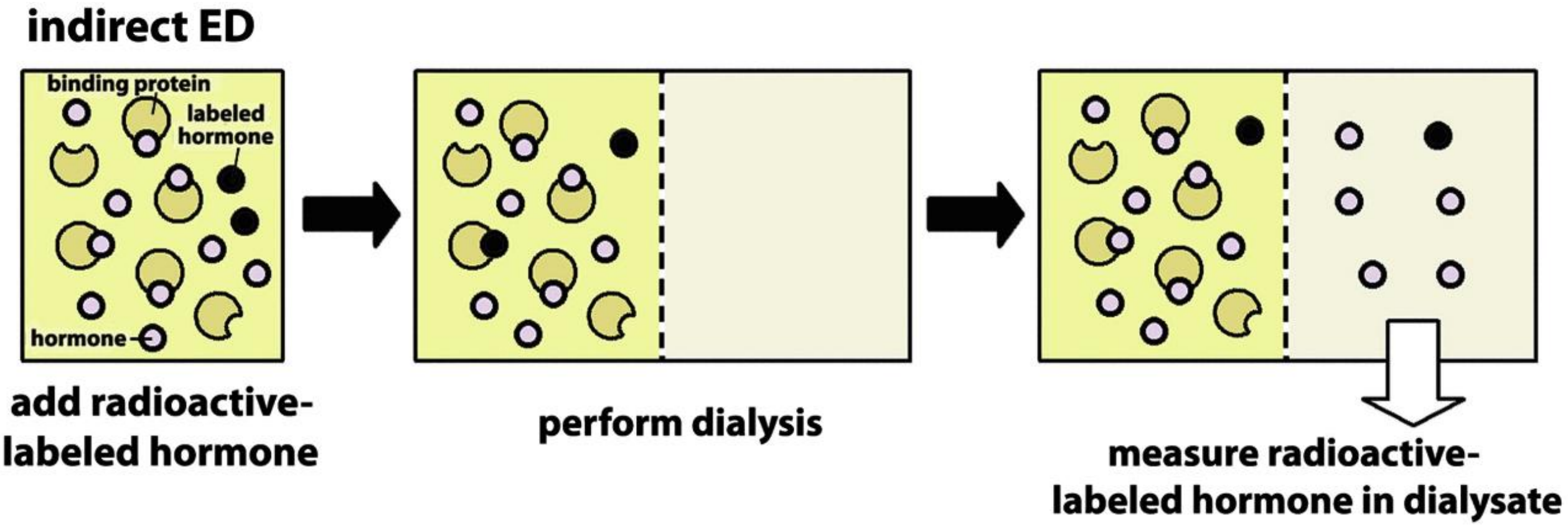
- Free hormone are found in ultra low concentrations
- Various factors disturb the equilibrium between the bound and free
  - Detergents
  - Preservatives
  - Albumin binding
  - Proteins
  - Diluents
  - Buffer components
  - Temperature
  - pH

Free T4 by equilibrium dialysis: the gold standard



Too low to quantify (exept some LC-MS/MS methods)

Free T4 by equilibrium dialysis



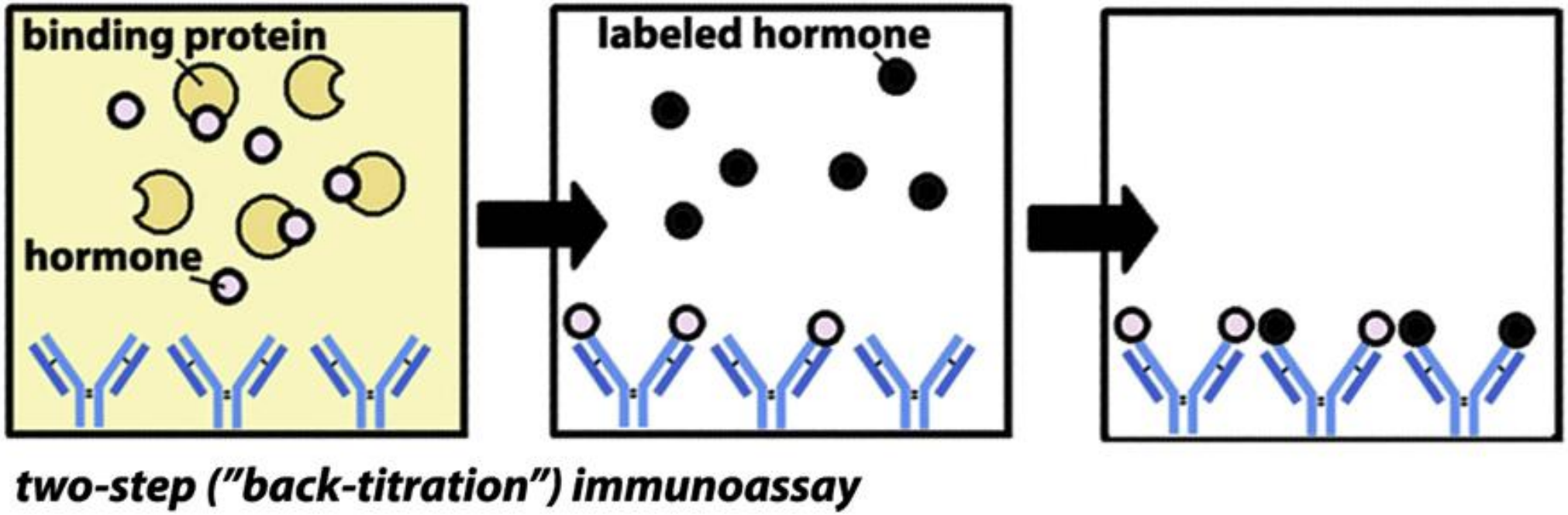
Calculate free fraction and multiply by total T4

Methods employing a specific, high affinity antibody

Thyroid hormone methods

- Two step labeled hormone/**Back-Titration** methods
- One step **labeled analog** methods
- **Labeled antibody** methods

Two step labeled hormone/Back-Titration methods

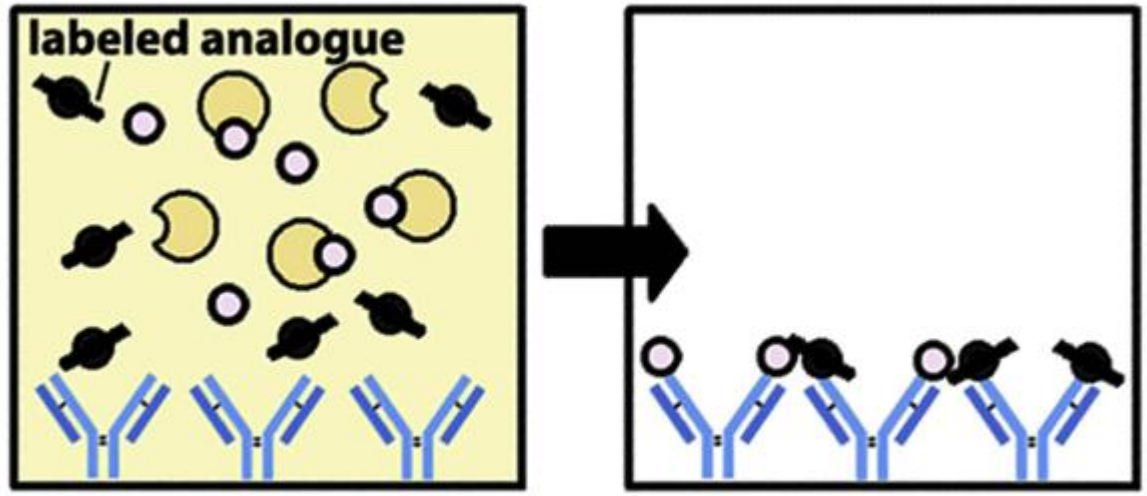


two-step ("back-titration") immunoassay

- Less labor intensive (compared to reference methods)
- Less affected by albumin and binding proteins

One-step, labeled Hormone-Analog methods

- Based on **hormone analog**, non-reactive with binding proteins

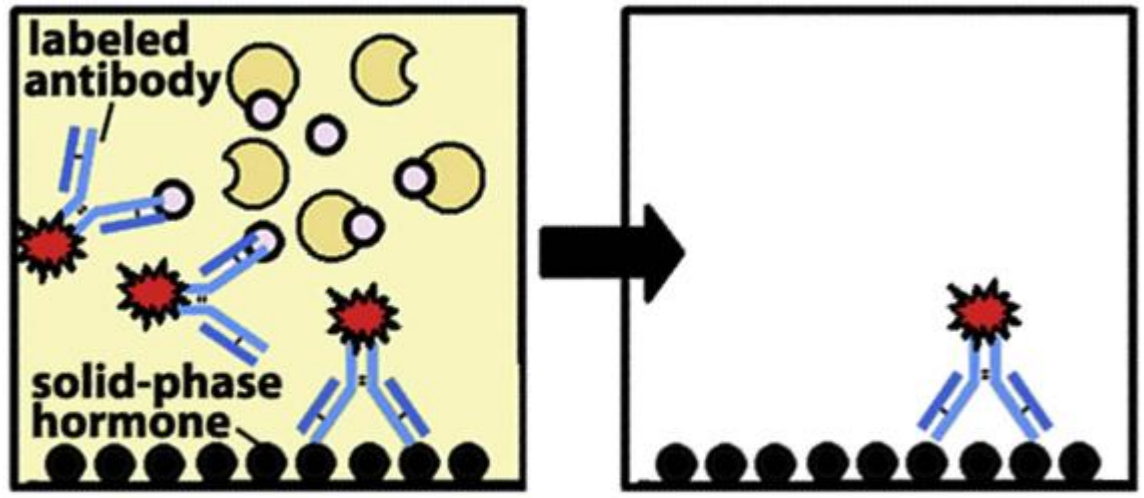


one-step ("analogue") immunoassay

- Poor diagnostic accuracy (analog binds to proteins)
- Prone to many interferences

One-step, labeled Antibody-Analog methods

- Based on **labeled antibody**, non-reactive with protein bound hormone



labeled antibody immunoassay

- Better diagnostic accuracy
- Prone to interferences (abnormal binding proteins, pregnany, DRUGS, NTI)

Testosterone REFERENCE methods

- Testosterone (total): LC-MS/MS
- Free testosterone: equilibrium dialysis
- Bioavailable testosterone: differential precipitation (ammonium sulphate)

Testosterone PRACTICAL methods

- Testosterone (total): competitive immuno-assay (robust in men)
- SHBG: immunometric (sandwich) assay (robust but standardisation??)
- Free testosterone: calculated
- Bioavailable testosterone: calculated

Vermeulen formula

Free & Bioavailable Testosterone calculator

These calculated parameters more accurately reflect the level of bioactive testosterone than does the sole measurement of total serum testosterone. Testosterone and dihydrotestosterone (DHT) circulate in plasma unbound (free approximately 2 - 3%) ,bound to specific plasma proteins (sex hormone-binding globulin SHBG) and weakly bound to nonspecific proteins such as albumin. The SHBG-bound fraction is biologically inactive because of the high binding affinity of SHBG for testosterone. Free testosterone measures the free fraction, bioavailable testosterone includes free plus weakly bound to albumin.

Albumin	4.3	g/dL
SHBG	45	nmol/L
Testosterone	300	ng/dL

Calculate

Explanation and examples

Free Testosterone	4.85 ng/dL = 1.62 %
Bioavailable Testosterone	114 ng/dL = 37.9 %

Disclaimer: Results from this calculator should NOT be solely relied upon in making (or refraining from making) any decision in any case/ circumstances without the prior consultation of experts or professional persons. No responsability whatsoever is assumed for its correctness or suitability for any given purpose.

WARNING! The calculated free and bioavailable testosterone are reliable in most clinical situations, but should not be relied upon in situations with potential massive interference by steroids binding to SHBG; e.g. in women during pregnancy, in men during treatment inducing high levels of DHT (e.g. transdermal DHT, oral testosterone) or mesterolon

This calculator was developed at the Hormonology department, University Hospital of Ghent, Belgium. If you have suggestions to improve this calculator, or for further questions or help contact us Dr. Tom Fiers or Prof. Dr. J.M. Kaufman

Coolens equation for calculation of free cortisol in serum

J Steroid Biochem. 1987 Feb;26(2):197-202.

Clinical use of unbound plasma cortisol as calculated from total cortisol and corticosteroid-binding globulin.

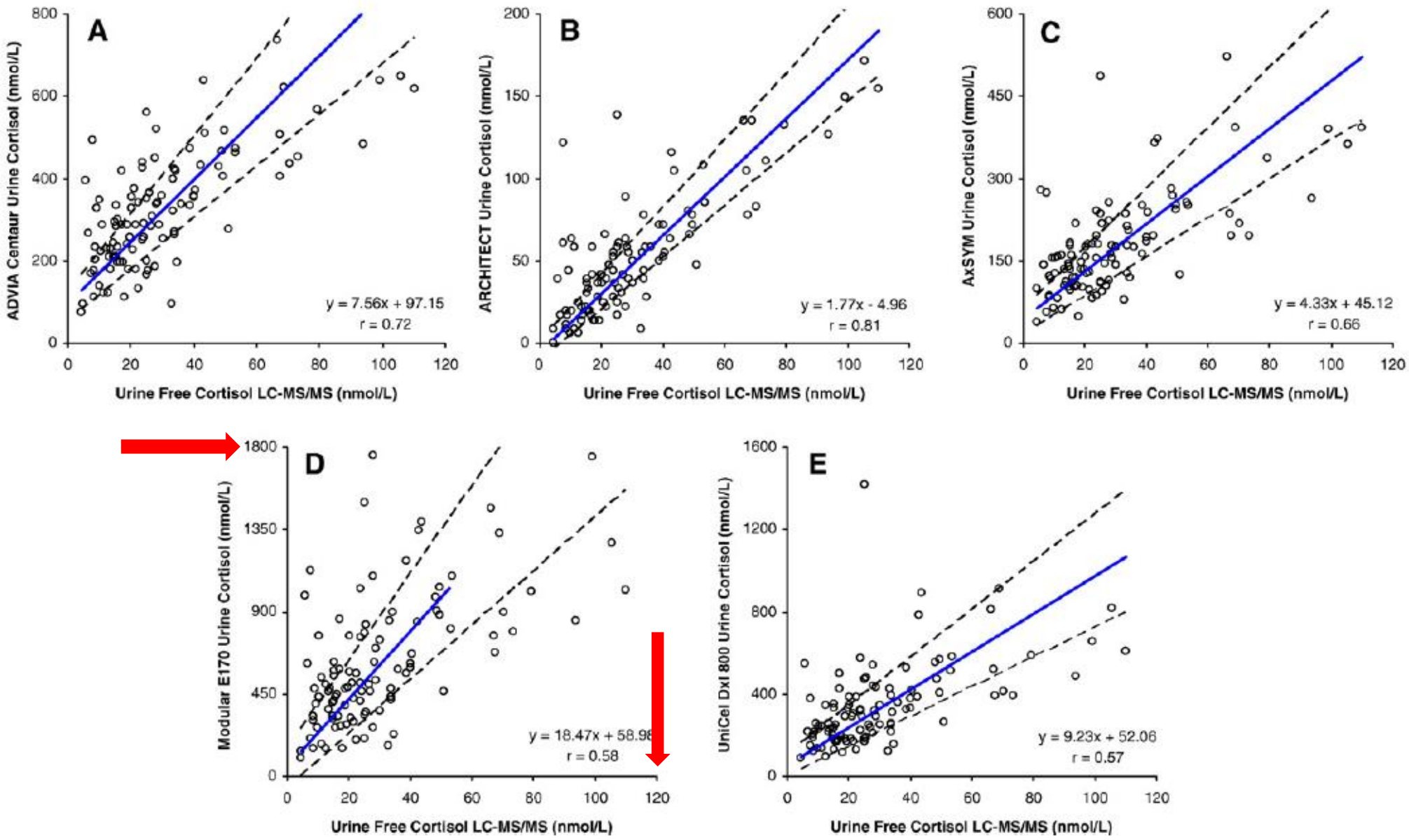
Coolens JL, Van Baelen H, Heyns W.

$$\text{Unbound cortisol } (\mu\text{mol/L}) = [(0{\cdot}0167 + 0{\cdot}182(\text{CBG} - \text{total cortisol}))^2 + (0{\cdot}0122 \times \text{total cortisol})]^{0{\cdot}5} - [0{\cdot}0167 + 0{\cdot}182(\text{CBG} - \text{total cortisol})]$$

Problems with calculated free cortisol:

- Septic shock
- Intensive care
- Cirrhosis
- Binding protein abnormalities
- …

Urinary free cortisol: direct measurement on automated analysers



Cortisol saliva

The fully automated lab

Around the analyzer street

UZ LEUVEN

LABORATORIUMGENEESKUND

AANVRAAGFORMULIER

STANDAARD

HEMATOLOGIE

71 ❑ Hemoglobine
72 ❑ RBC telling + hematocriet
84 ❑ WBC telling
80 ❑ Trombocyten telling
78 ❑ Reticulocyten telling
86 ❑ WBC differentiatie
4213 ❑ Haptoglobine
4239 ❑ Hemoglobine vrij (plasma)

BLOEDSTOLLINGSTESTS

7301 ❑ Protrombinetijd (PT)
7305 ❑ APTT
7306 ❑ Fibrinogeen
7309 ❑ D - dimeren

IMMUNOHEMATOLOGIE

8201 ❑ Bloedgroep ABO en RhD

TUMORMERKERS

4706 ❑ α-foetoproteïne
4702 ❑ CEA
4629 ❑ Neuron Specific Enolase

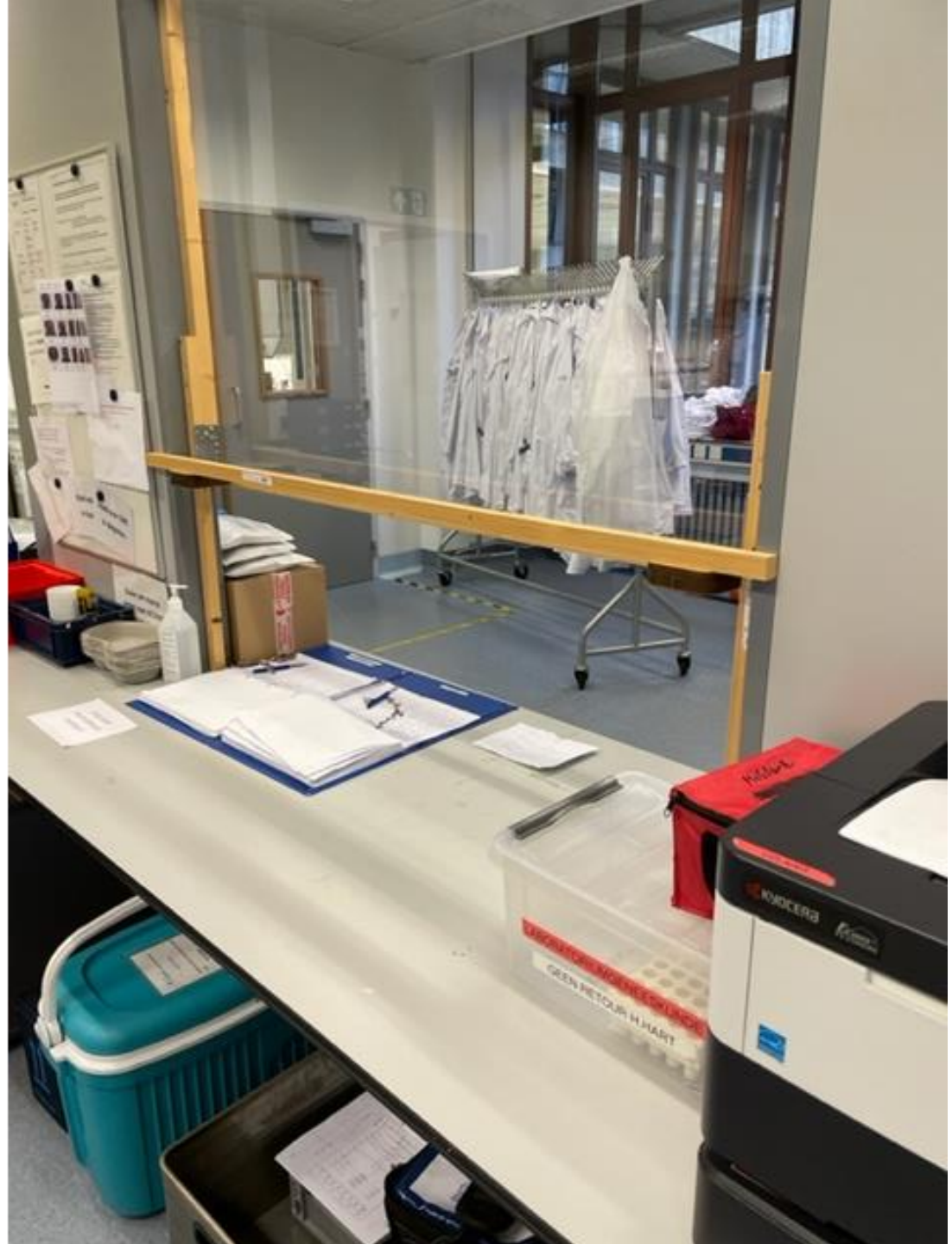
4710 ❑ PSA follow-up therapie
of 4716 ❑ PSA screening man 40-50 j
bij familiaal antecedent
of 4714 ❑ PSA (buiten RIZIV)

4701 ❑ hCG totaal

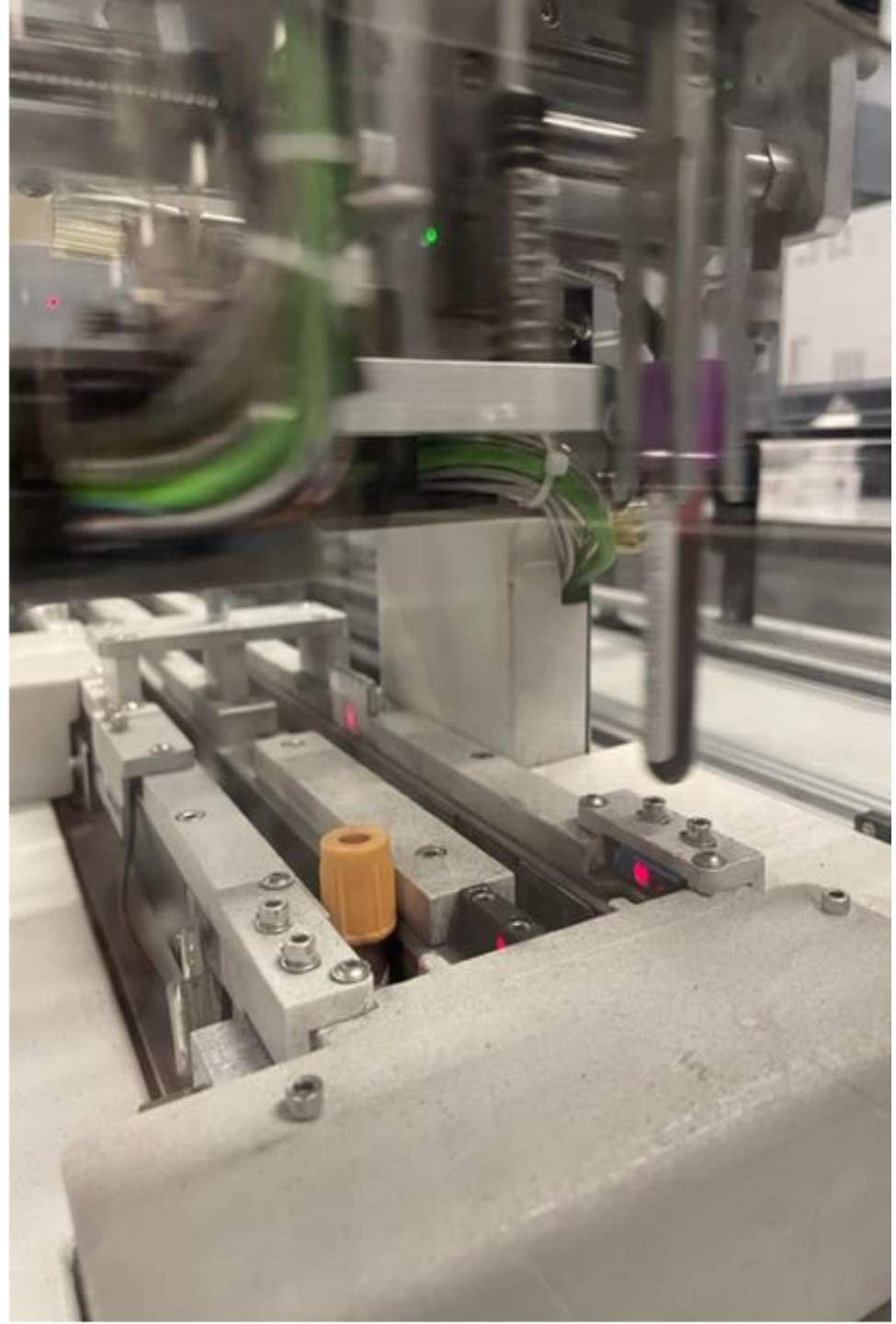
CHEMIE

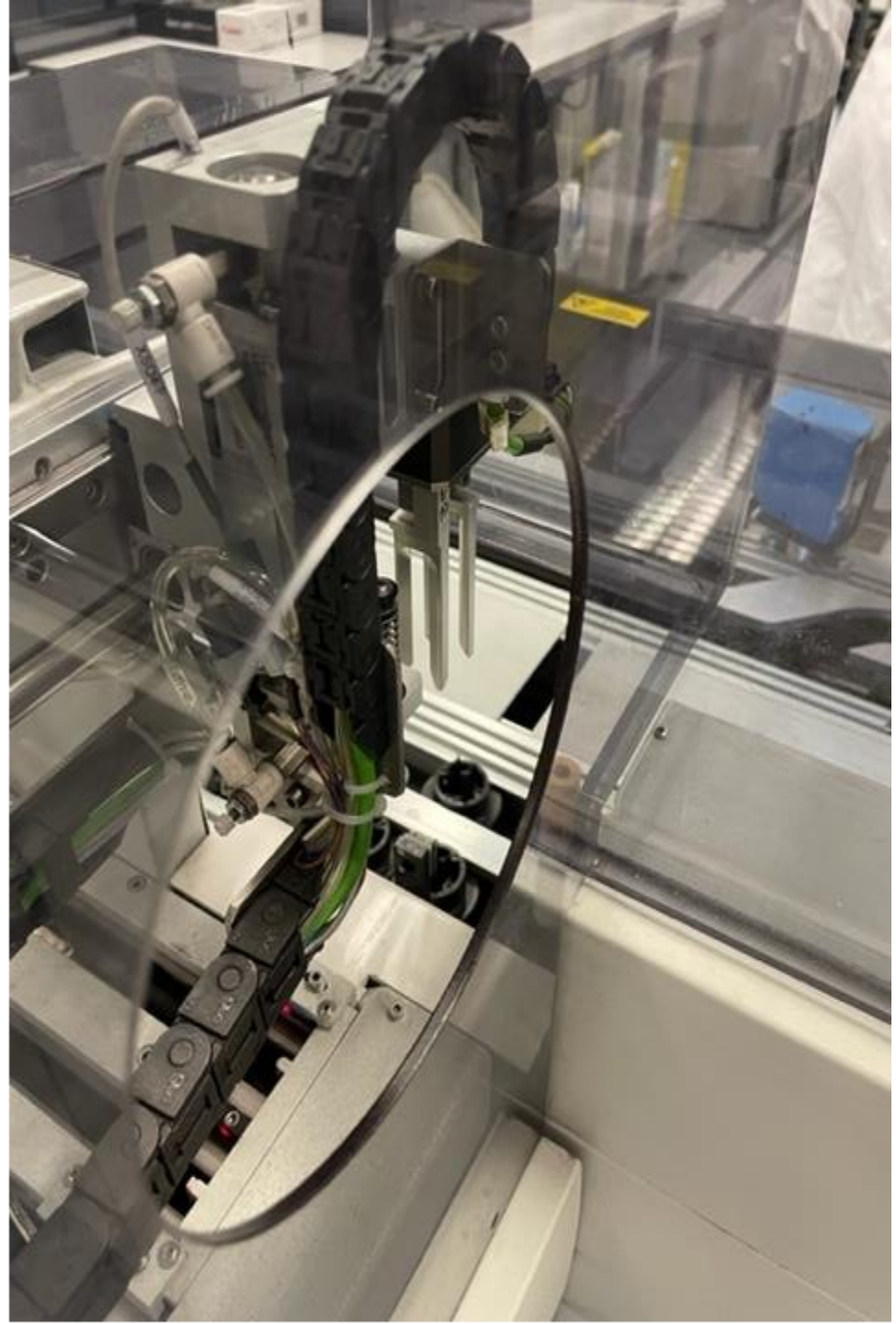
4201 ❑ Natrium
4202 ❑ Kalium
4203 ❑ Chloride
4204 ❑ Bicarbonaat
4206 ❑ Ureum
4207 ❑ Creatinine
4215 ❑ Urinezuur
4211 ❑ Eiwit totaal
4212 ❑ Albumine
4208 ❑ Calcium totaal
4209 ❑ Fosfaat
4205 ❑ Osmolaliteit
292 ❑ Lactaat ☛
218 ❑ Glucose
246 ❑ HbA1c
4216 ❑ Alkalische fosfatase
4217 ❑ AST (GOT)
4218 ❑ ALT (GPT)
4219 ❑ Gamma - GT
4220 ❑ Bilirubine totaal
4221 ❑ Bilirubine totaal en direct
132 ❑ Ammonia ☛
4222 ❑ CK totaal
4236 ❑ Troponine T
4223 ❑ CK MB
4226 ❑ Lipase (pancreatitis)
4224 ❑ LDH
4225 ❑ Amylase (parotitis)

4235 ❑ CRP
4227 ❑ Cholesterol totaal
4228 ❑ Triglyceriden
4229 ❑ HDL + berek.LDL-ch
+ berek. Non-HDL-
cholesterol
4230 ❑ LDL cholesterol
4690 ❑ Apolipoproteïnen A
4662 ❑ Lipoproteïne Lp(a)
4242 ❑ Kininase II (ACE)
4237 ❑ NT-proBNP
4231 ❑ IJzer
4233 ❑ Transferrine + % s
4234 ❑ Ferritine
4627 ❑ Vitamine B12
4628 ❑ Folaat (serum)
82 ❑ Folaat (RBC)
5483 ❑ Eiwitelektroforese
5644 ❑ Eiwitelektroforese
met IF indien M p
4210 ❑ Magnesium
263 ❑ Koper
4661 ❑ Ceruloplasmine
265 ❑ Zink
264 ❑ Lood
4291 ❑ Lithium
261 ❑ Aluminium ☛
4393 ❑ Vitamine A ☛
4394 ❑ Vitamine E ☛
[illegible] ❑ Vitamine B1 ☛

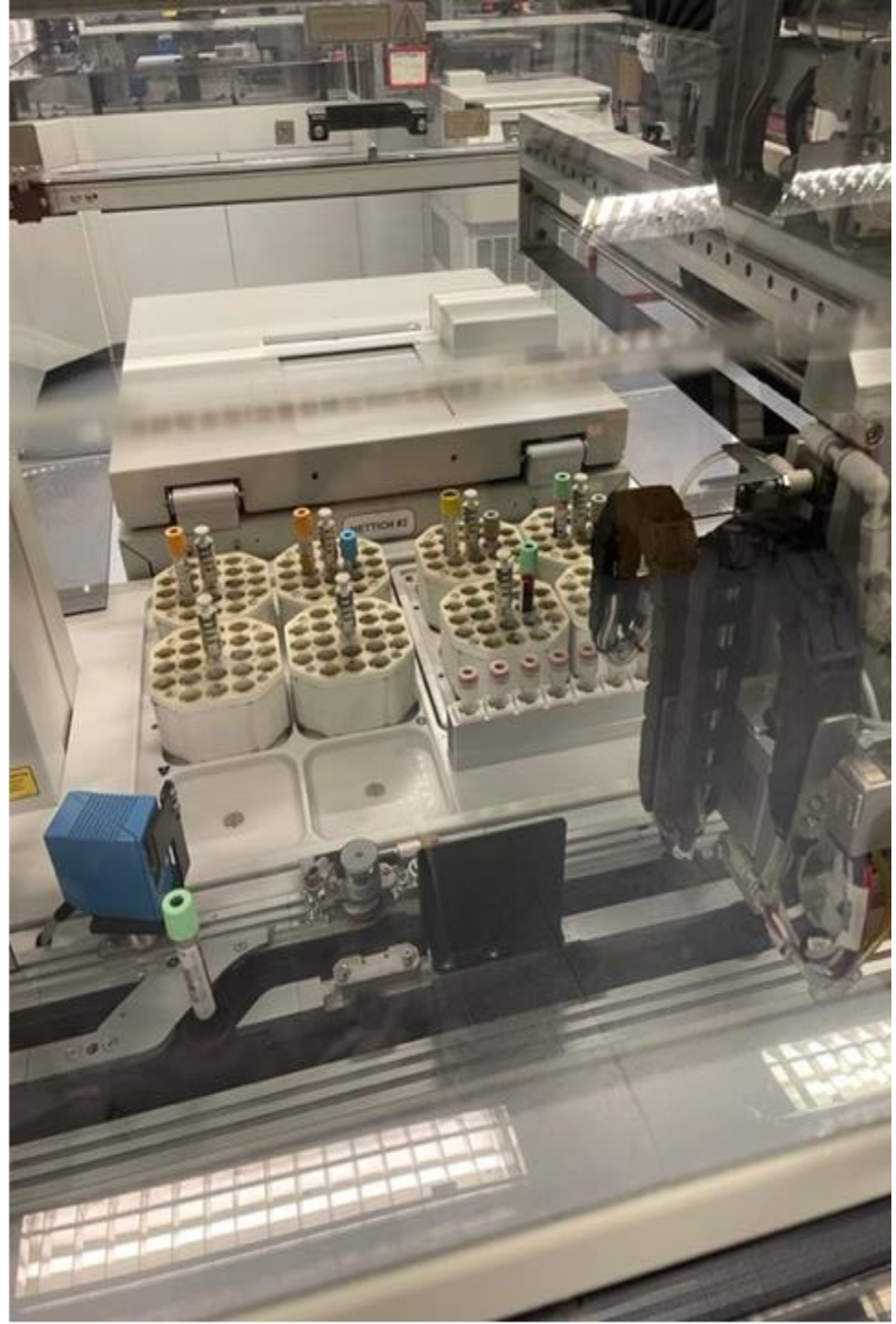

KYOCERA

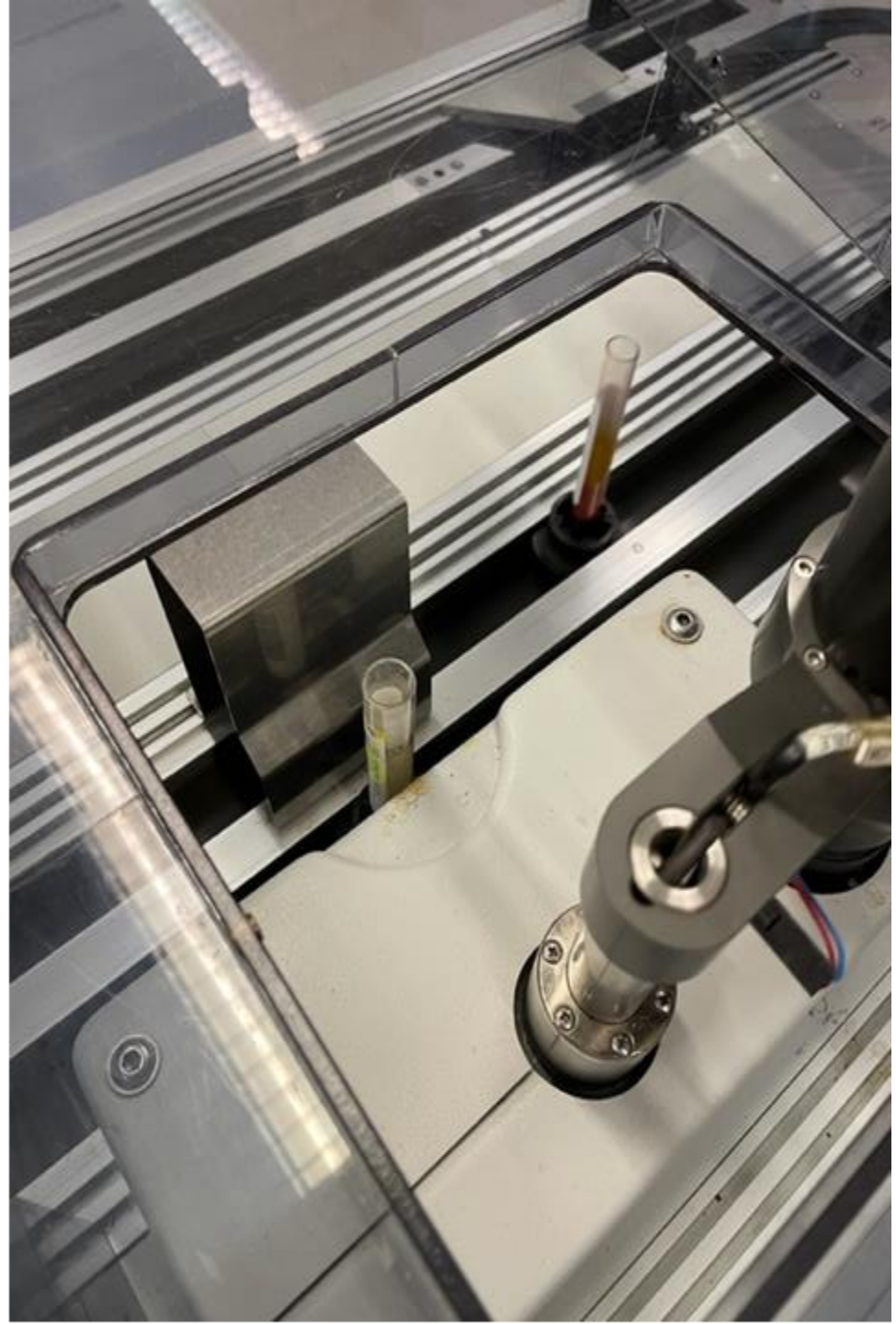

TANIA
MAD



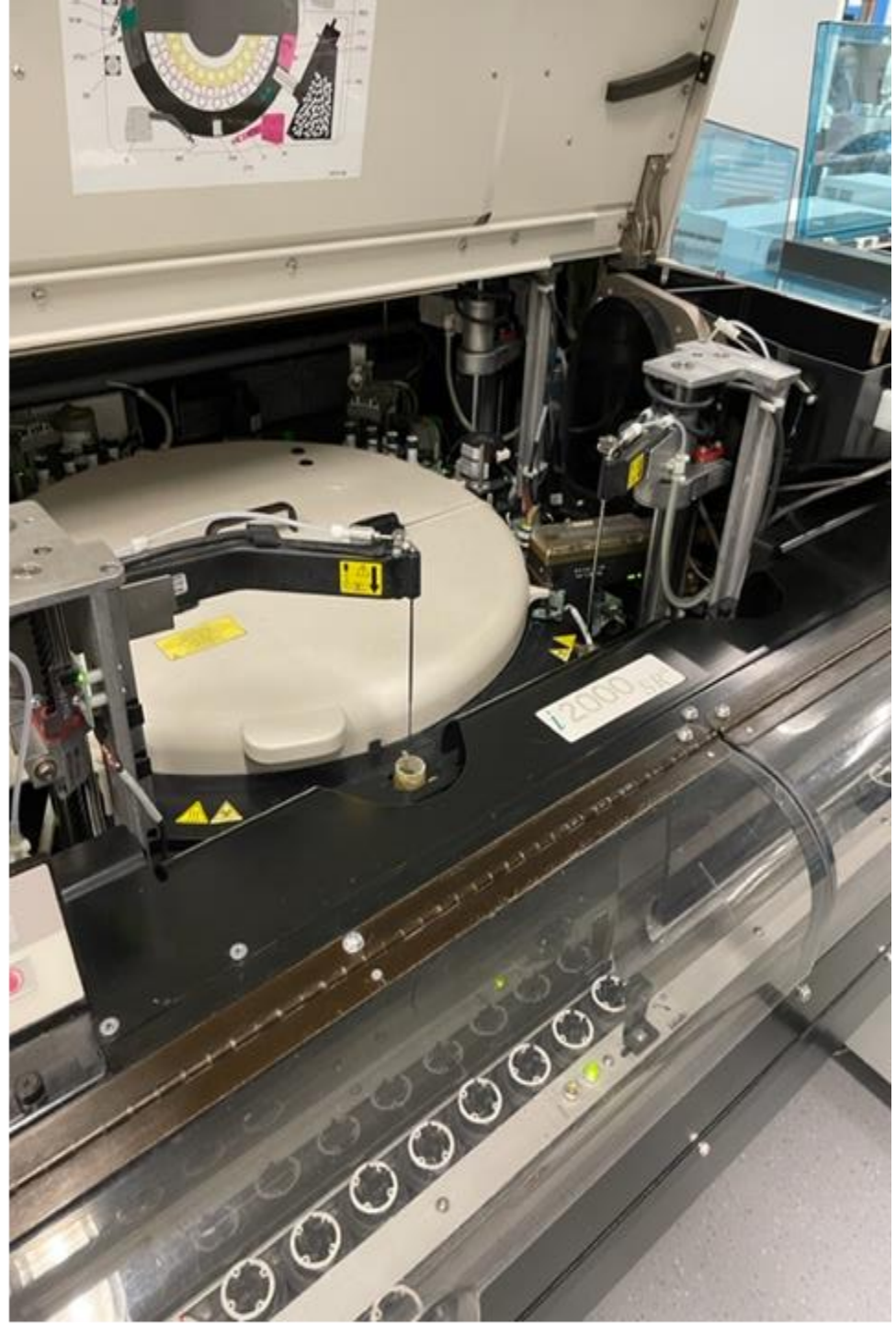



Centrifuge 1






peco

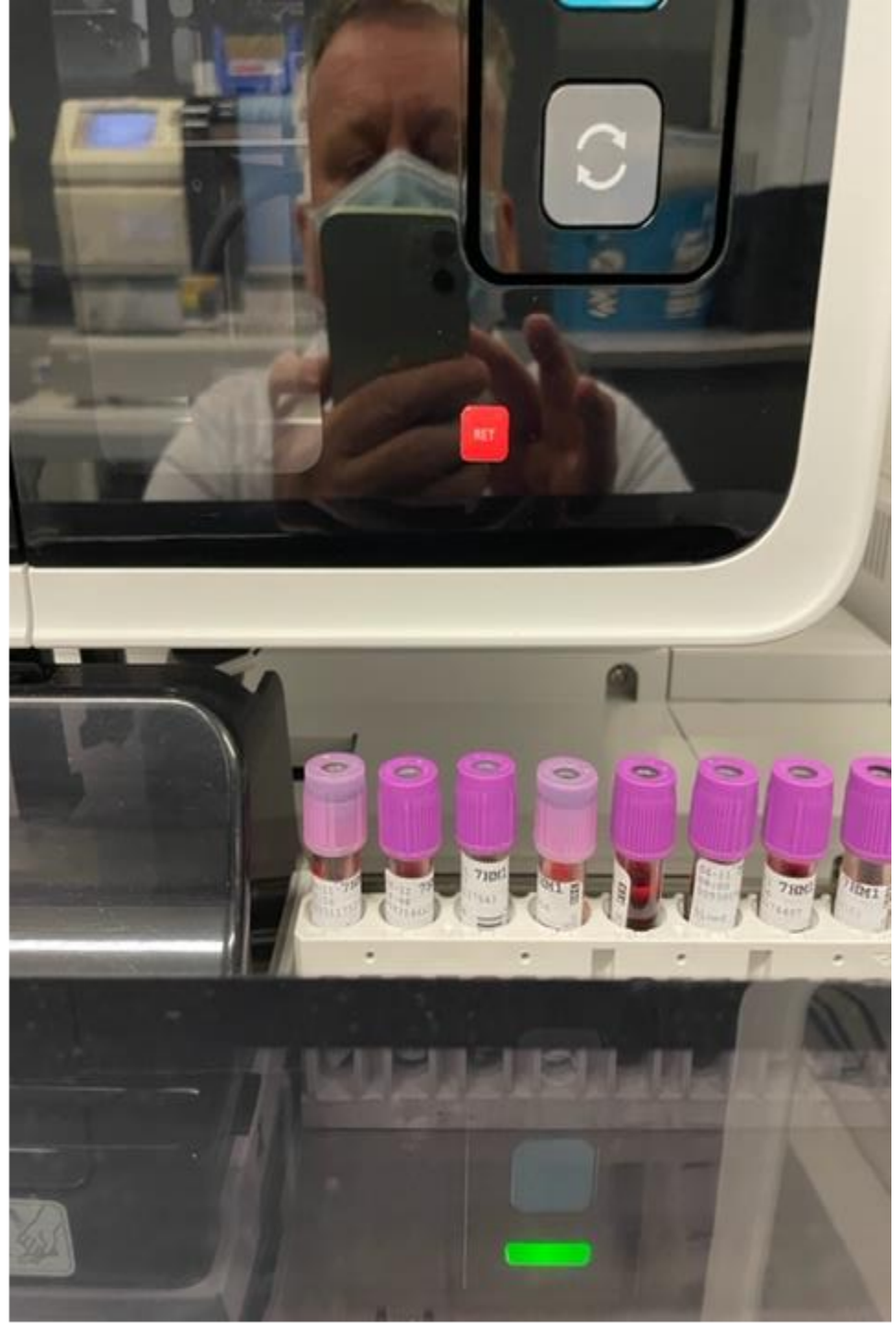



NAME
ASSAY
STATUS
System time:
Architect HAVAB IGM, L
HAVAbIgM2
Running
Architect HAVAB IGG, L
HAVABIgG2
Running
Architect ANTI-HBS, L
Anti-HBs 3
Running
Architect ANTI-HBS, H1
Anti-HBs 3
Running
Architect ANTI-HBe, L
Anti-HBe
Running
SARS-COV2 IgG ANTI-N, L
COV-2 IgG
Running
Architect SYPHILIS T, L
SyphilisTP
Running
SARS-COV2 IgG II ANT, L
COV-2IgGII
Running
SARS-COV2 IgG II ANT, M
COV-2IgGII
Running
Architect HIV COMBO, H1
_HIV Ag/Ab
Running
ARCHITECT HTLV I/I, L
HTLV-I/II
Running
Architect ANTI-HBe, L
Anti-HBe
Running
Architect ANTI-HBe, H
Anti-HBe
Running
Print
Details...
Delete

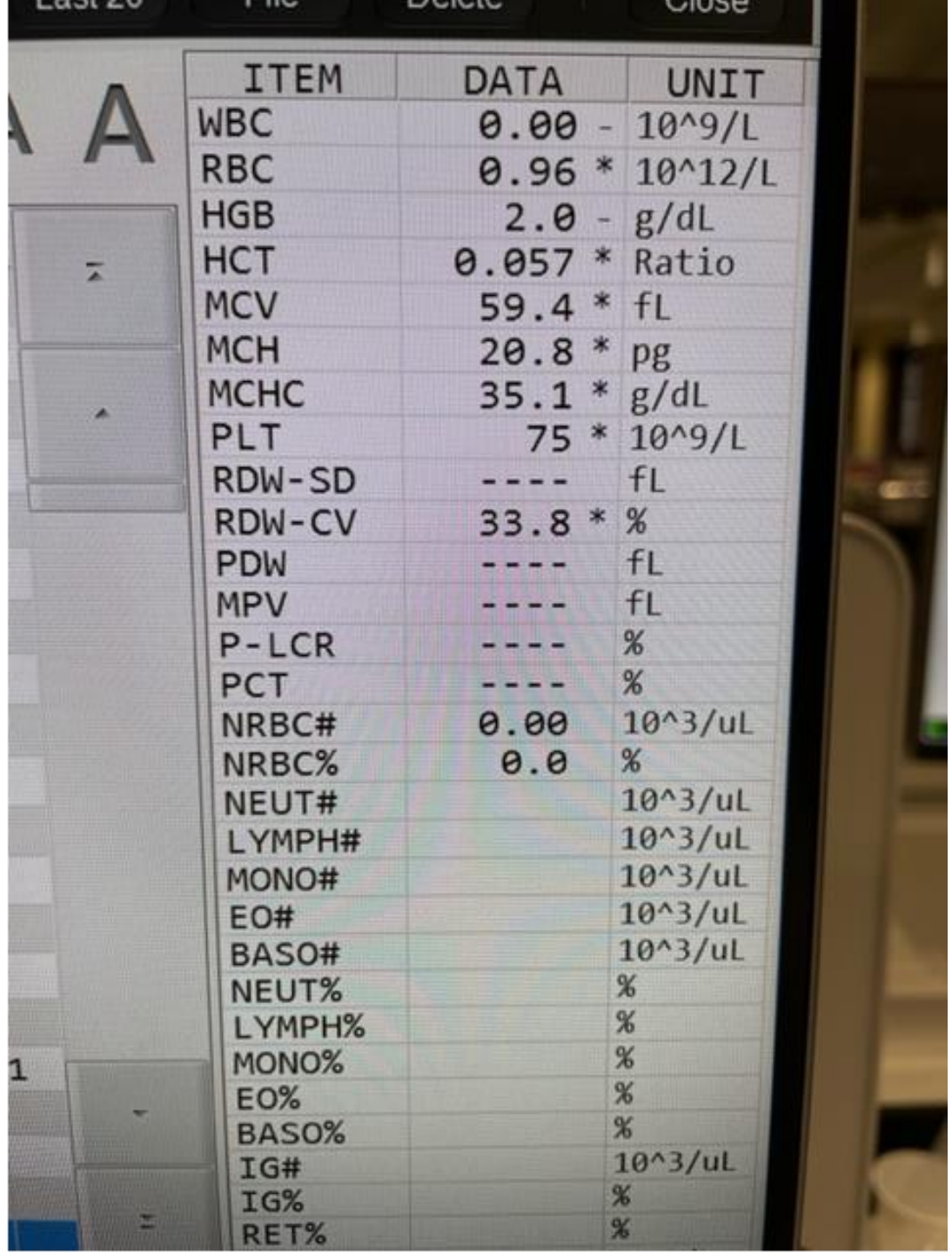

cobas 8000


ACL TOP
OFF-LINE


A




DI
Slide moet op DI-60.
NA
Slide moet niet op DI-60.
Onderaan slide (beginletter van code)
R
RBC_INFO: Nazicht malaria
D
Differentatie nodig
N
Nazicht NRBC
P
PLT_INFO: Nazicht aggregaten
I
Order microscopie door aanvrager
C
Aferese-collectie

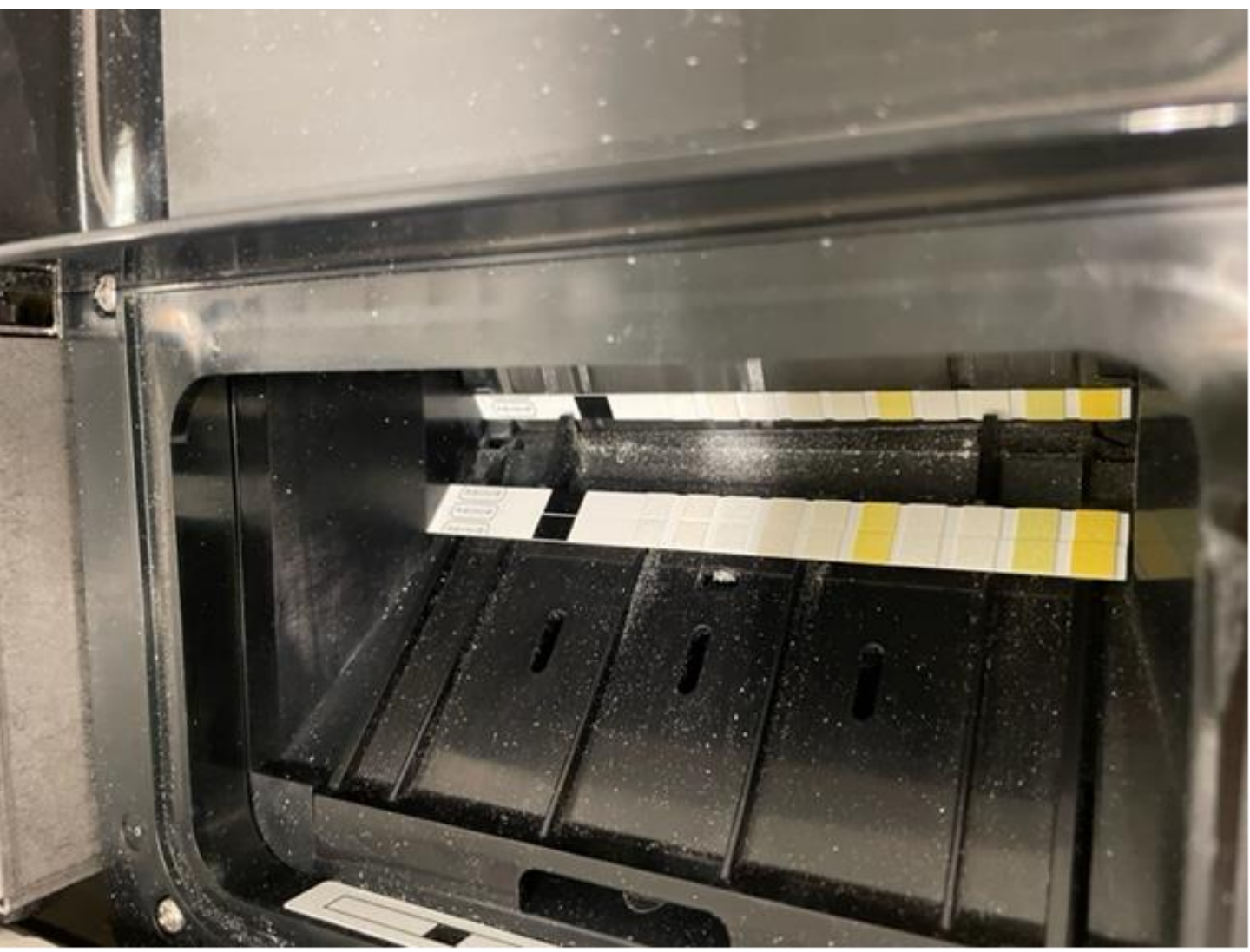


ITEM	DATA	UNIT
WBC	0.00 -	10^9/L
RBC	0.96 *	10^12/L
HGB	2.0 -	g/dL
HCT	0.057 *	Ratio
MCV	59.4 *	fL
MCH	20.8 *	pg
MCHC	35.1 *	g/dL
PLT	75 *	10^9/L
RDW-SD	----	fL
RDW-CV	33.8 *	%
PDW	----	fL
MPV	----	fL
P-LCR	----	%
PCT	----	%
NRBC#	0.00	10^3/uL
NRBC%	0.0	%
NEUT#		10^3/uL
LYMPH#		10^3/uL
MONO#		10^3/uL
EO#		10^3/uL
BASO#		10^3/uL
NEUT%		%
LYMPH%		%
MONO%		%
EO%		%
BASO%		%
IG#		10^3/uL
IG%		%
RET%		%

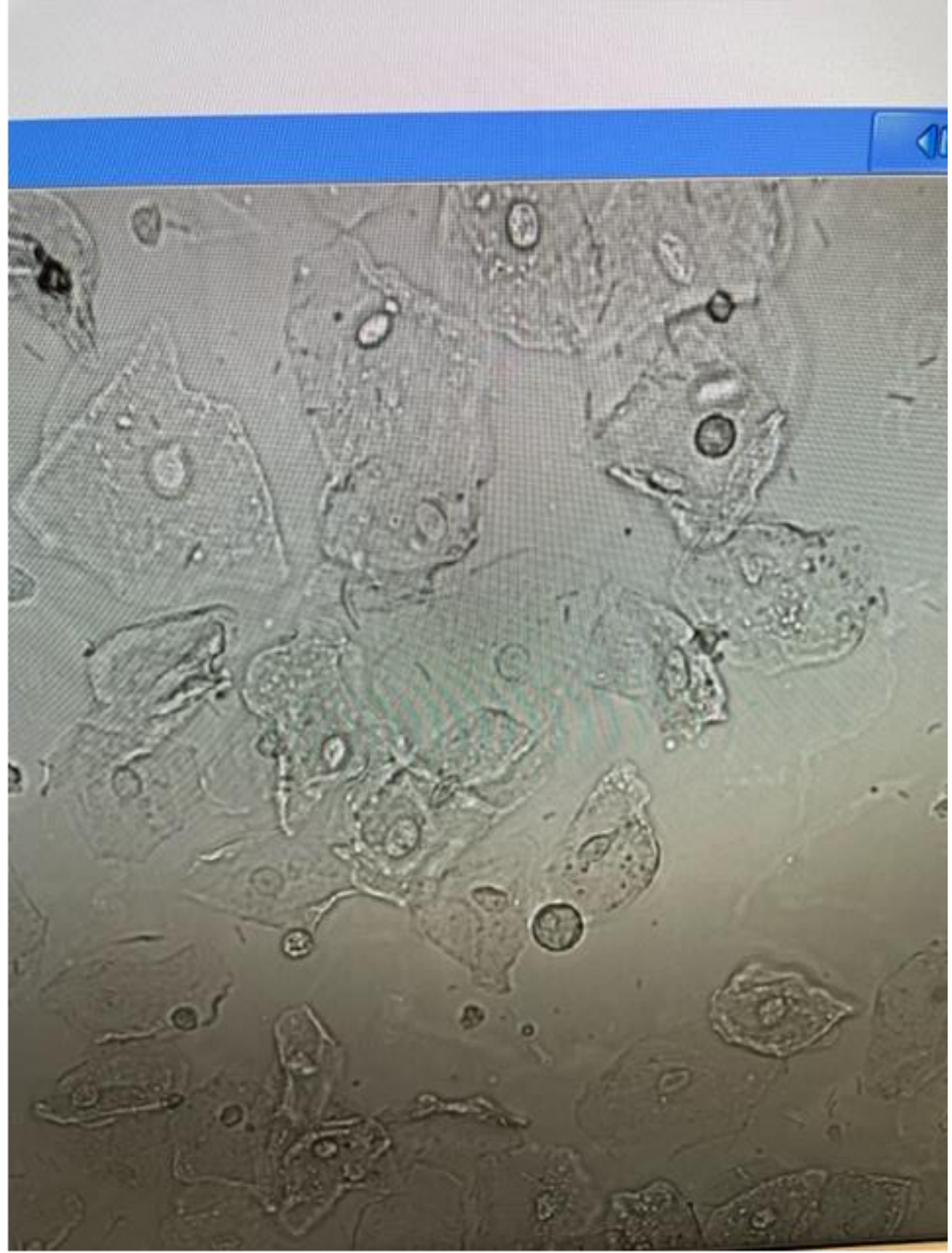

TYP 372
% TIME AREA
NAME 0.00 0.00 0.00
TYP 0.76 0.23 9.99
A1A 0.70 0.31 9.22
A1B 0.63 0.39 8.23
F 2.85 0.48 37.23
LA1C* 5.59 0.59 56.94
SA1C 81.33 0.89 1194.59
A0 TOTAL AREA 1316.19
IFCC 37.6mmol/mol
HbA1C 5.59%
HbA1 7.06 % HbF 0.63 %
0% 15%
0.0
1.0

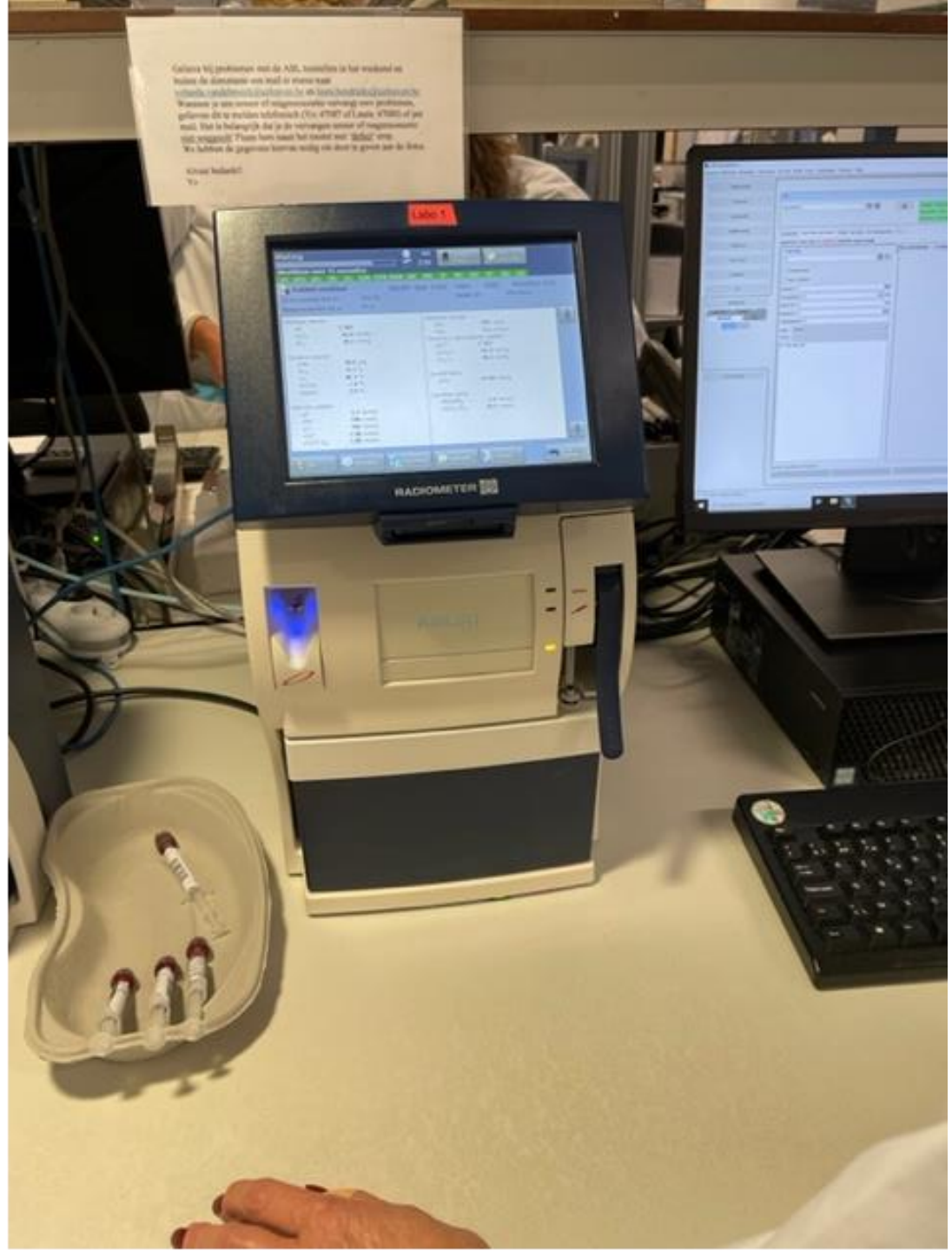

MANUELLE TESTEN URINEWP - LATEN STAAN AUB
URINEWP
0:00
Actim

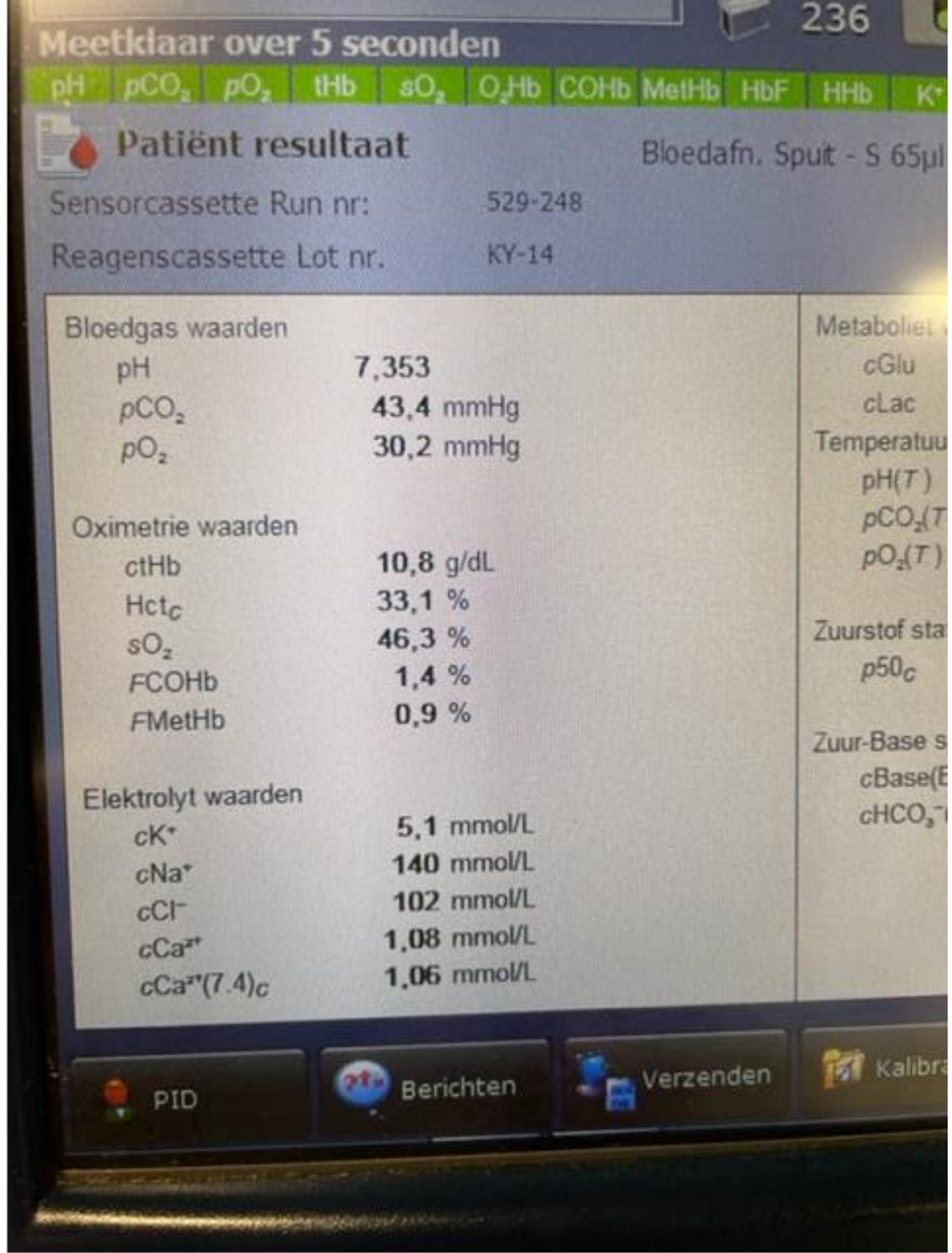

AUTION MAX




sediMAX
A.MENARINI
diagnostics

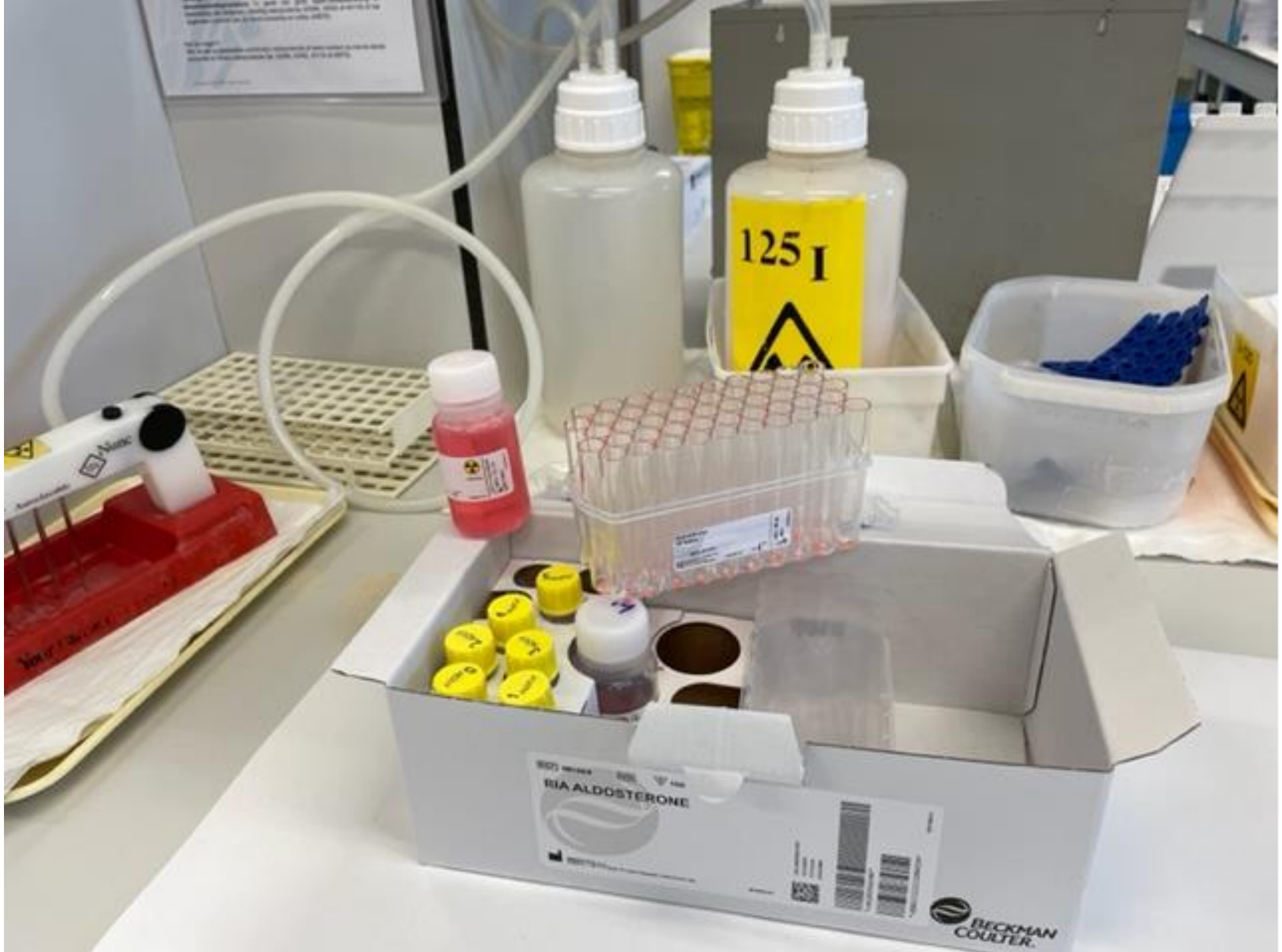


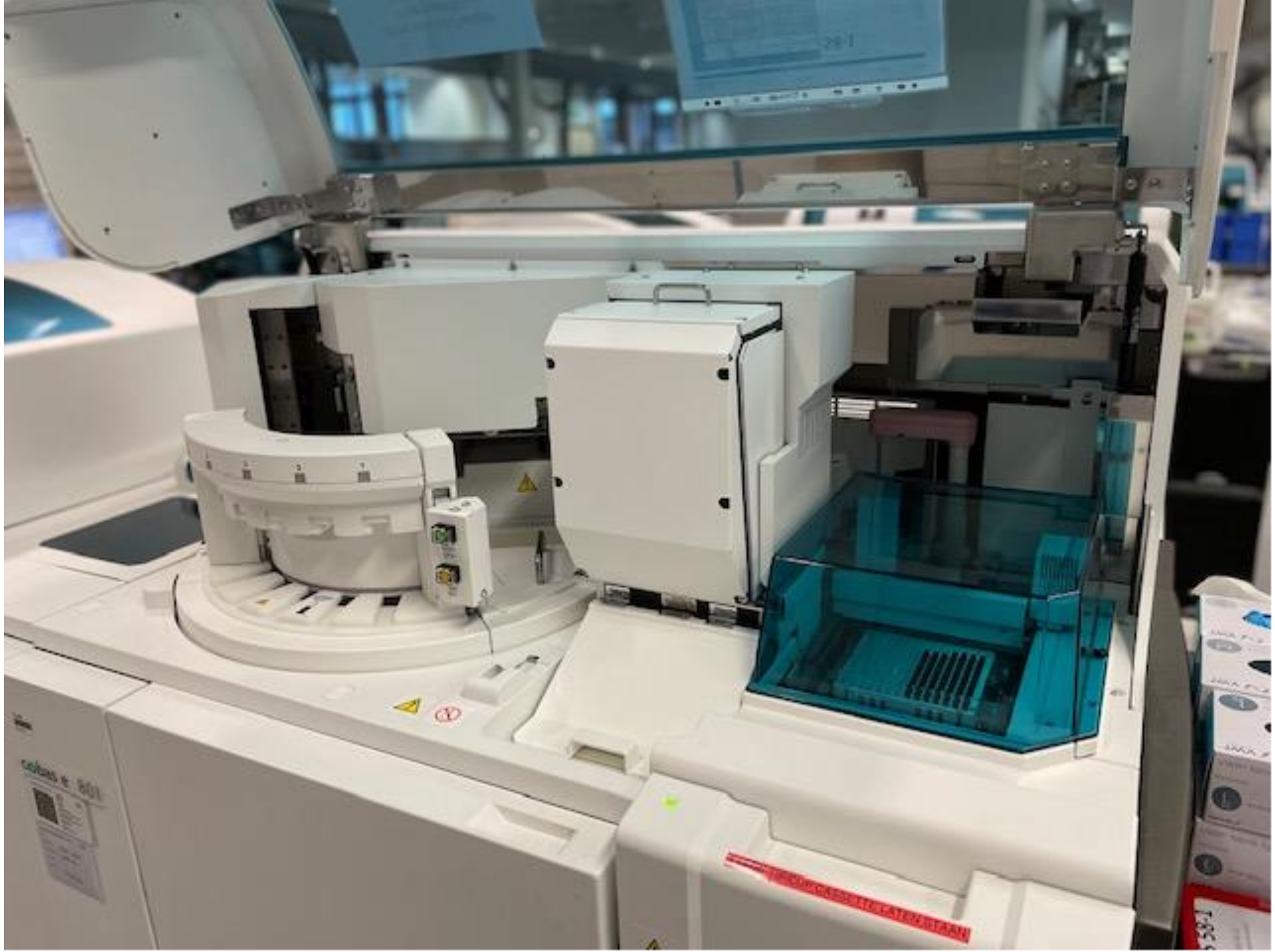

Labo 1
RADIOMETER


236
Meetklaar over 5 seconden
pH pCO2 pO2 tHb sO2 O2Hb COHb MetHb HbF HHb K+
Patiënt resultaat
Bloedafn. Sput - S 65µl
Sensorcassette Run nr: 529-248
Reagenscassette Lot nr. KY-14
Bloedgas waarden
pH 7,353
pCO2 43,4 mmHg
pO2 30,2 mmHg
Oximetrie waarden
ctHb 10,8 g/dL
HctC 33,1 %
sO2 46,3 %
FCOHb 1,4 %
FMetHb 0,9 %
Elektrolyt waarden
cK+ 5,1 mmol/L
cNa+ 140 mmol/L
cCl- 102 mmol/L
cCa2+ 1,08 mmol/L
cCa2+(7.4)C 1,06 mmol/L
Metaboliet
cGlu
cLac
Temperatuu
pH(T)
pCO2(T
pO2(T)
Zuurstof sta
p50C
Zuur-Base s
cBase(E
cHCO3-
PID
Berichten
Verzenden
Kalibra

Back to the immuno-assay…

RIA


125 I
RIA ALDOSTERONE
BECKMAN COULTER

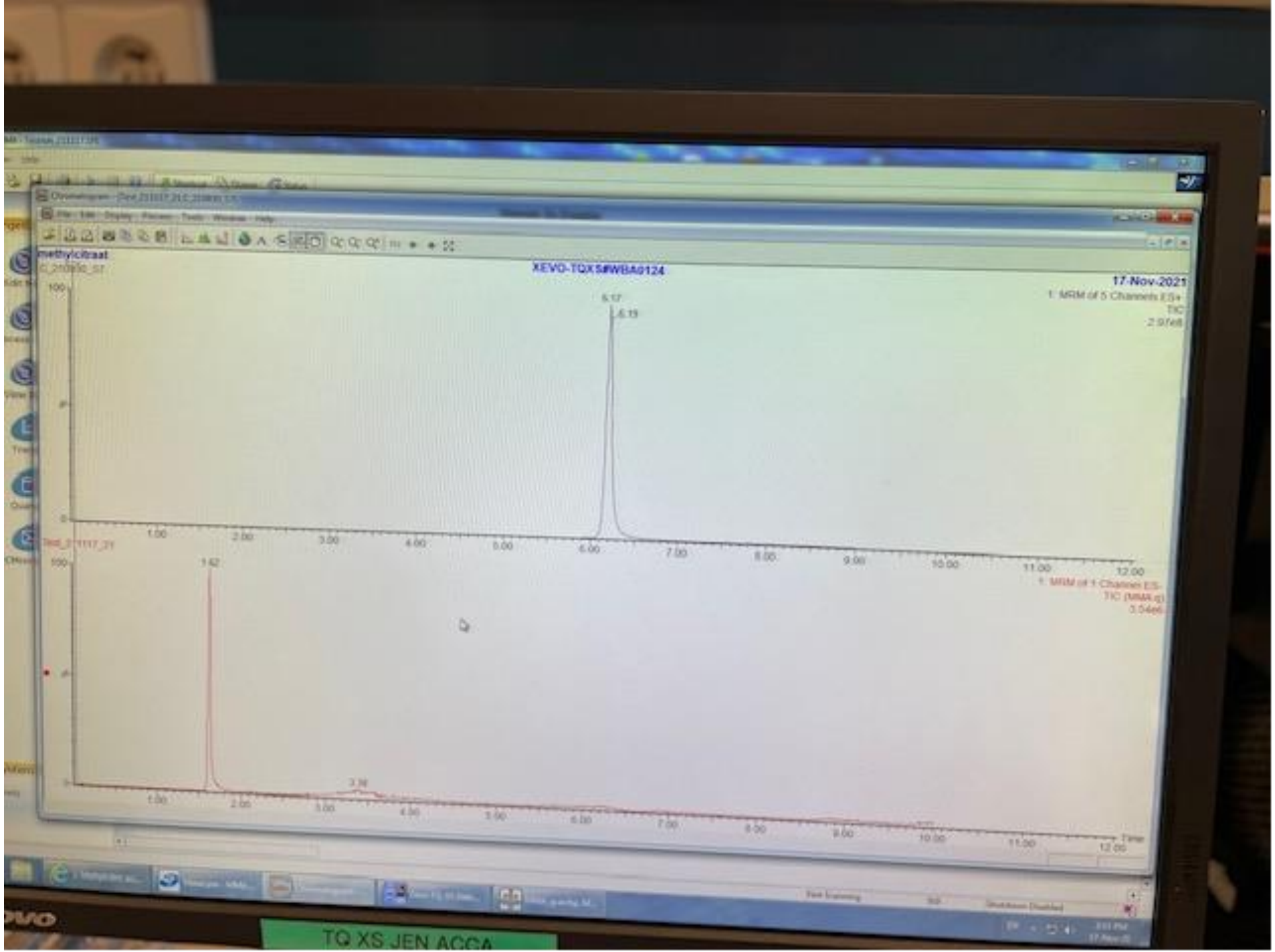


LC-MS/MS

Small hormones

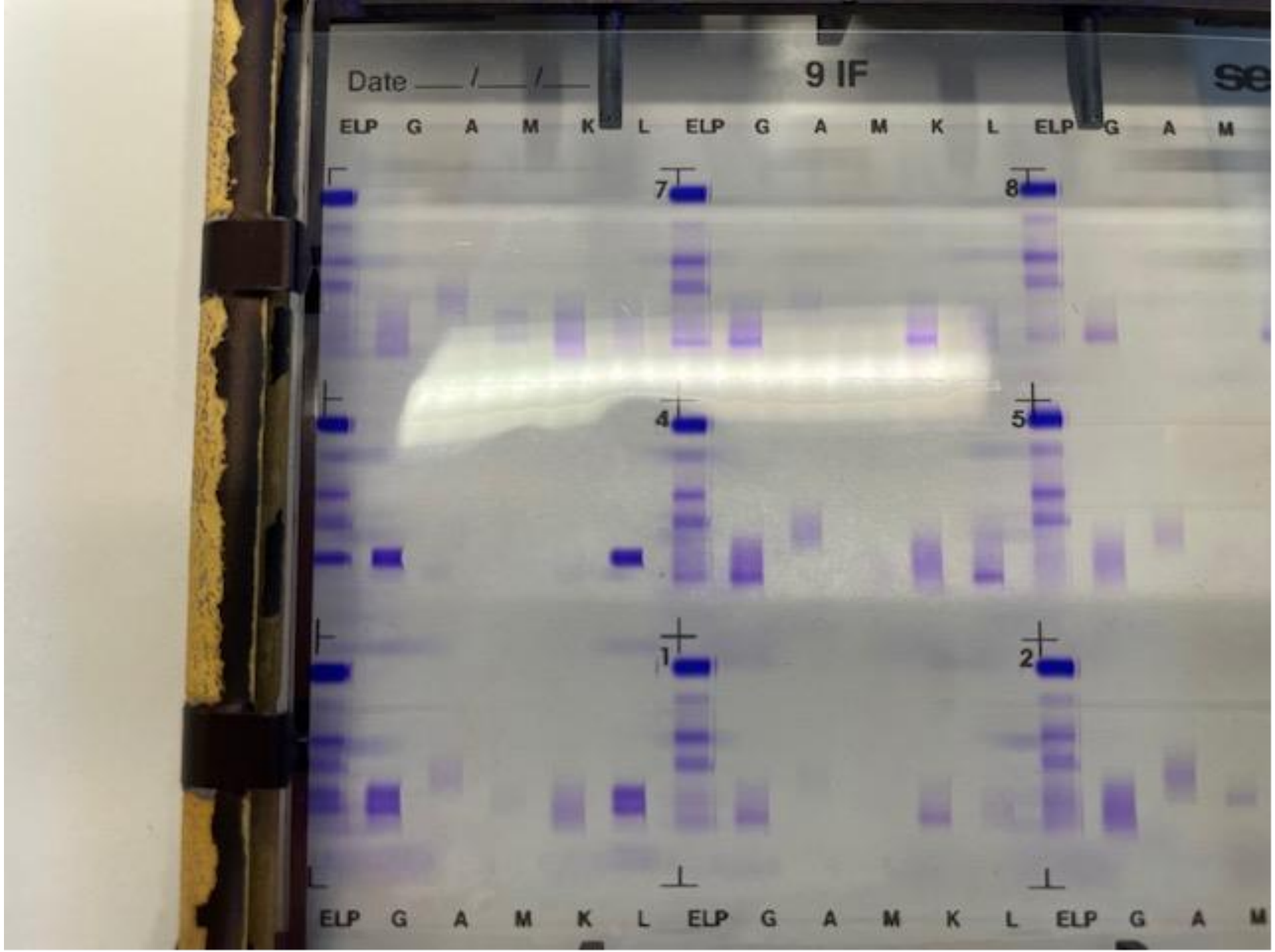

Biomek NXP
Laboratory Automation Workstation


AB SCIEX QTRAP 5500


methylcitraat
XEVO-TQXS#WBA0124
17-Nov-2021
1: MRM of 5 Channels ES+
TIC
2.97e8
6.17
6.19
1: MRM of 1 Channel ES-
TIC (MRM 1)
3.04e6
1.62
3.38
Time
TQ XS JEN ACCA

Special lab tests

Same technology/platform, different clinical applications


Date ___/___/___
9 IF
ELP G A M K L ELP G A M K L ELP G A M
7
8
4
5
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