High Performance Liquid Chromatography vs Immunoassay for HbA1c testing

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HPLC

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Objectives

By the end of this session, you will be able to:

1. **Describe different needs of IA (immunoassay)**
2. **Understand IA and HPLC differences**
3. **Discuss why HPLC is a superior methodology for HbA1c**

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Agenda

- **Theory behind the needs of a good immunoassay**
  - **Types of assays and why so many**
- **Theory of HPLC**
- **HbA1c testing in the lab - methodologies**

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6 in the World - MEXICO 16.4 MILLION

Top 10: Countries/territories of number of people with diabetes (20-79 years), 2011 and 2030

	COUNTRY /TERRITORY	2011 MILLIONS
1	China	90.0
2	India	61.3
3	United States of America	23.7
4	Russian Federation	12.6
5	Brazil	12.4
6	Japan	10.7
7	Mexico	10.3
8	Bangladesh	8.4
9	Egypt	7.3
10	Indonesia	7.3

	COUNTRY /TERRITORY	2030 MILLIONS
1	China	129.7
2	India	101.2
3	United States of America	29.6
4	Brazil	19.6
5	Bangladesh	16.8
6	Mexico	16.4
7	Russian Federation	14.1
8	Egypt	12.4
9	Indonesia	11.8
10	Pakistan	11.4

What is an immunoassay (IA) ?

- A test that uses Antigen (Ag) and Antibody (Ab) complexes that can generate a measurable result thru some signal. The unknown analyte in an IA that is being measured can be an Ag or Ab.

- This is different than a basic colorimetric test which just uses the analyte being measured and some chemical to generate a color change (ie. Creatinine – Jaffe reaction uses Picric Acid)

Optimal Strategy of Immunoassay Design

- **Allosteric effects - minimize**
- **Sample volume - optimize**
- **Incubation time - optimize**
- **One epitope**
- **No non - specific binding**

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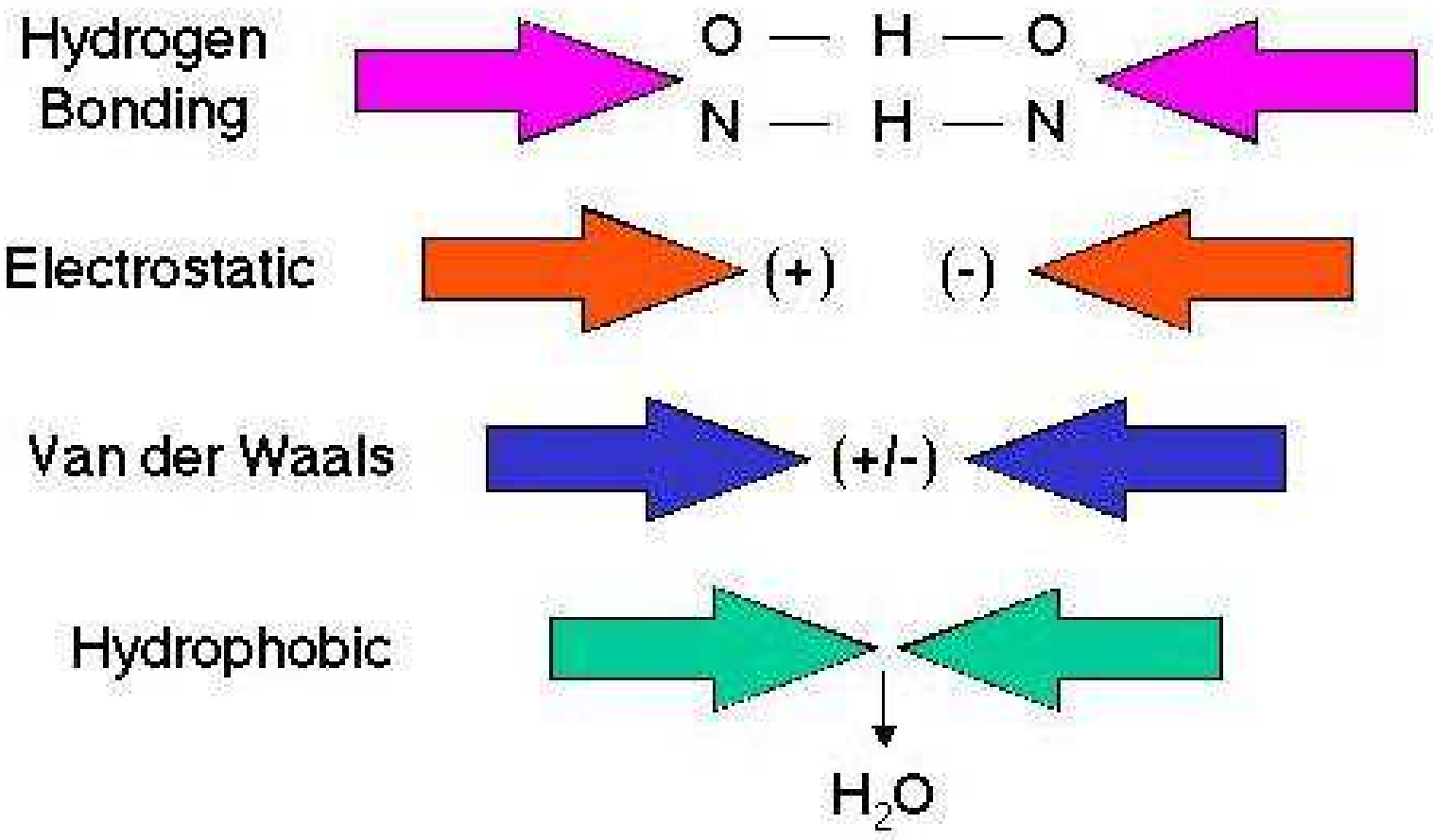
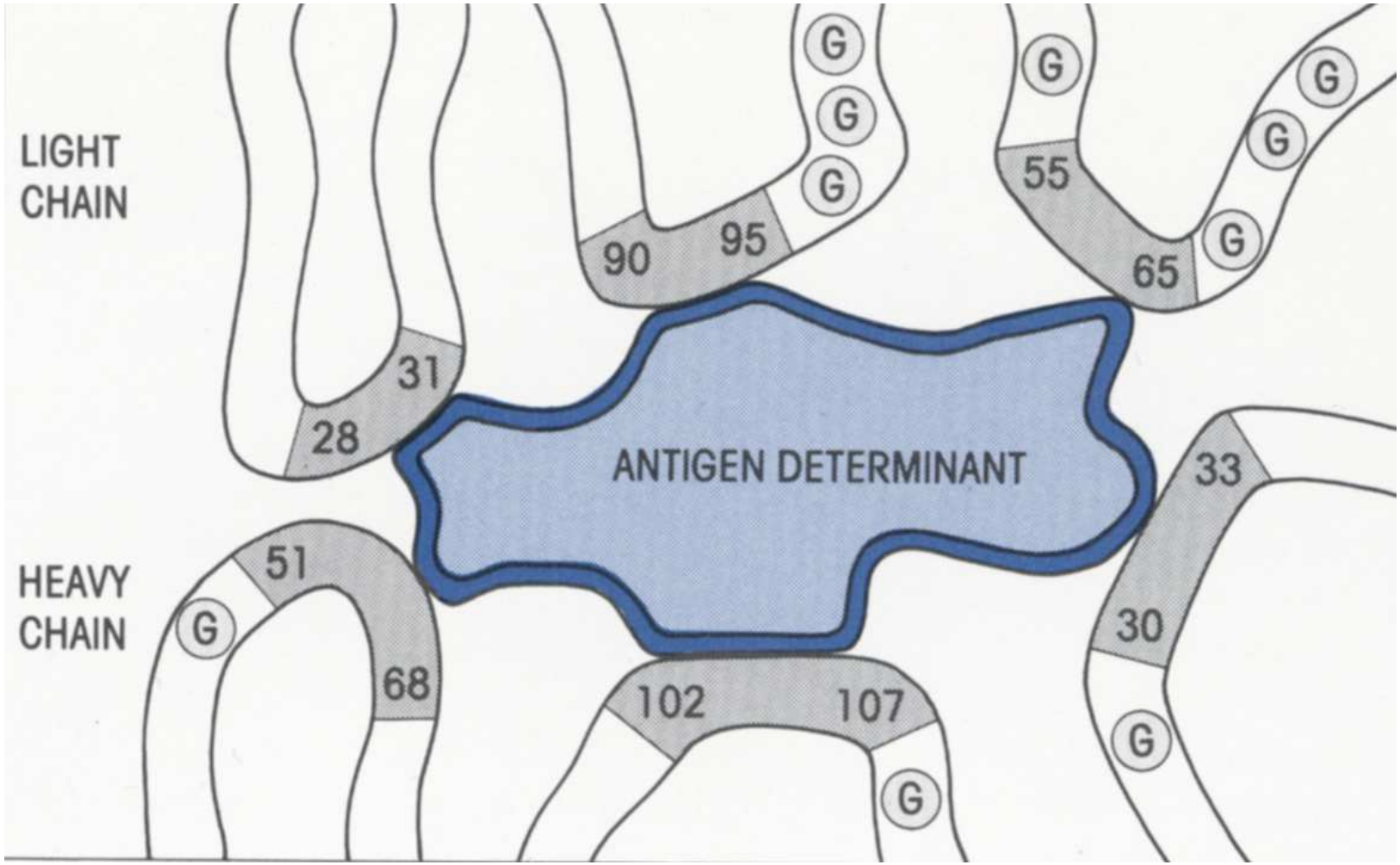
Bond strength / Binding energy



Epitope (multiple) Antigen determinant



Types of Assays

Another **example of confounding nomenclature**

- **Homogeneous vs. Heterogeneous**
- **Immunometric vs. ‘competitive’**
- **Reagent excess vs. reagent limited**
- **Rate or endpoint**

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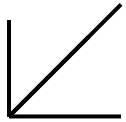
Types of immunoassays

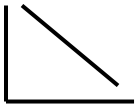
Another **example of confounding nomenclature**

- **Homogeneous** simply means that bound(**B**) and free(**F**) Ab are discriminated without separation. There is a change in signal due to the insolubility of the **Ag-Ab** complex detection.

- **Immunometric** is typically when Ab is in excess and Abs are against different epitopes of Ag. (reagent excess)



- **Competitive** is typically when the Ab is limited so as to have a certain # of Ab sites for the unknown Ag and the conjugated Ag to **'fight'** or '**compete'** for. The dose response curve is inversely proportional to concentration.



- **Equivalence** This is when a visual precipitate of Ag - Ab complexes forms. Examples of this are RID, Immunoelectrophoresis, Nephelometry and Turbidimetry.

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Why the diversity?

- **Permutations of:**
  - **Ab**
  - **Molecular Size**
  - **Calibration Methods**
  - **Separation Systems**
  - **Signal Generation**

Homogeneous Assay

- Simplicity
- NO separation of B/F - typically not as sensitive as a heterogeneous assay
- Good for drug monitoring - Why? Because therapeutic drugs circulate at high enough levels as not to be overly concerned with sensitivity
- Suitable for uncomplicated automation
- Examples
  - **Agglutination**
  - **Nephelometry**
  - **Turbidimetry**
  - **Fluorescent Polarization**

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Heterogeneous Assay

Who says size isn't important?

- Hapten + Peptide - MacroMolecule (Alice in Wonderland or pantalla)
- Immunometric typically refers to reagent excess or Ab excess
- Separation between B/F

- Basically 3 Types of heterogeneous immunoassays
  - A. Competitive Assay with Solid Phase Separation (small molecular weight SMW)
  - B. Immunometric Assay (for detecting Antigen) (LMW)
  - C. Immunometric Assay (for detecting Antibody)

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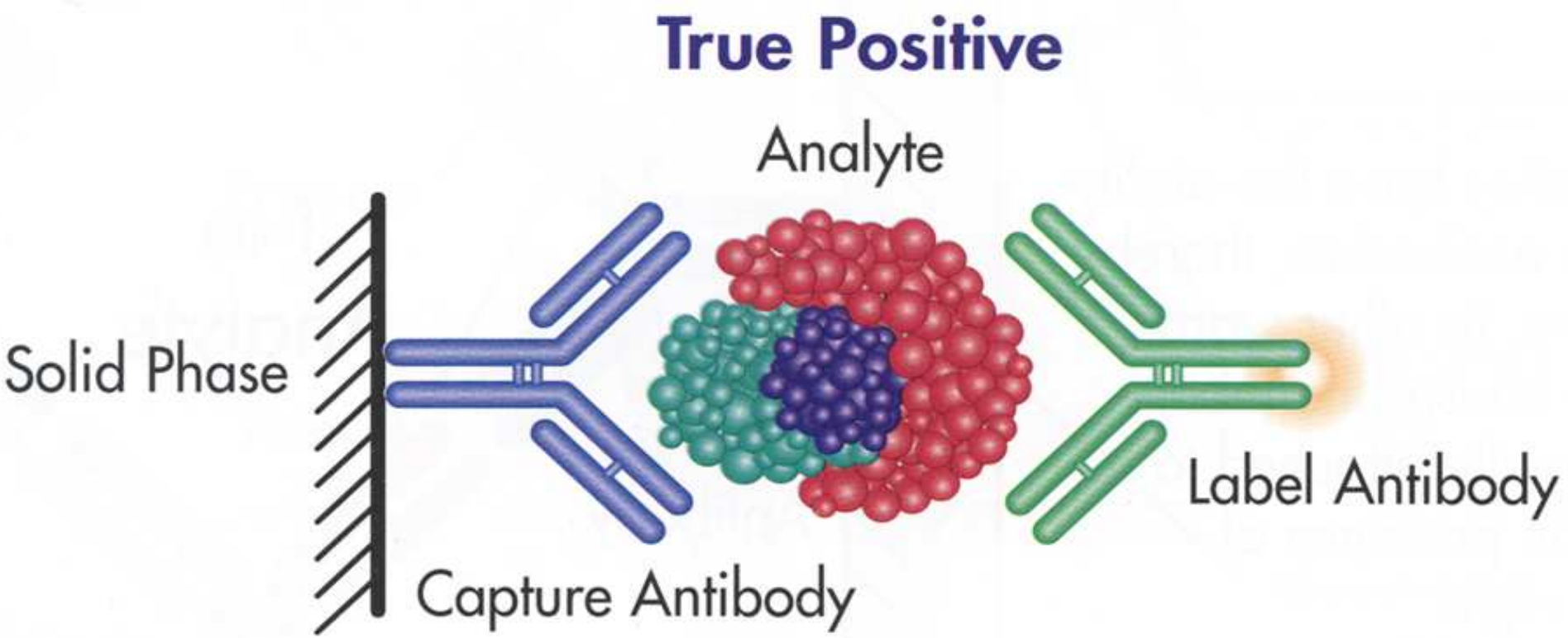
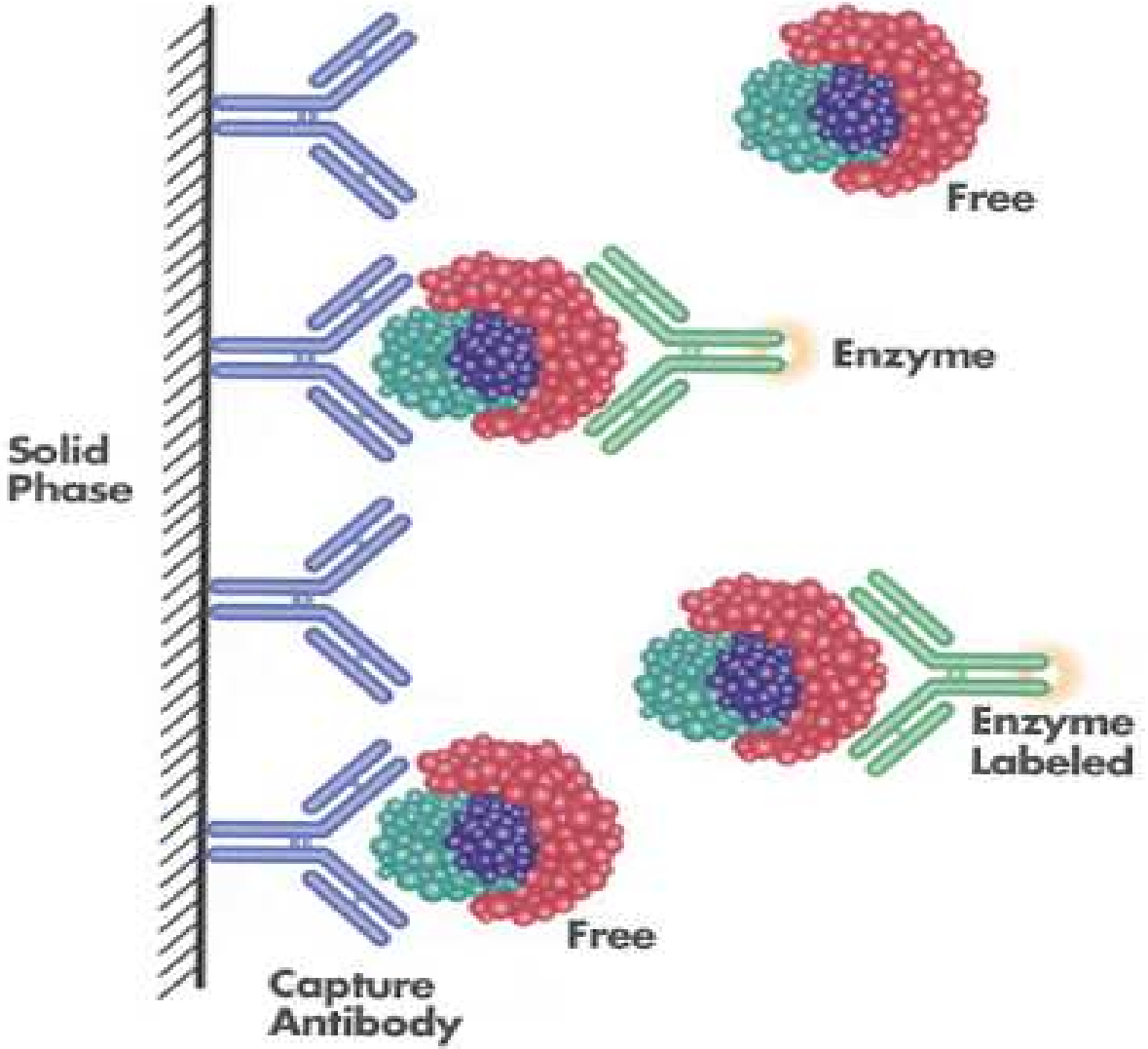
Sandwich Assay (non competitive)

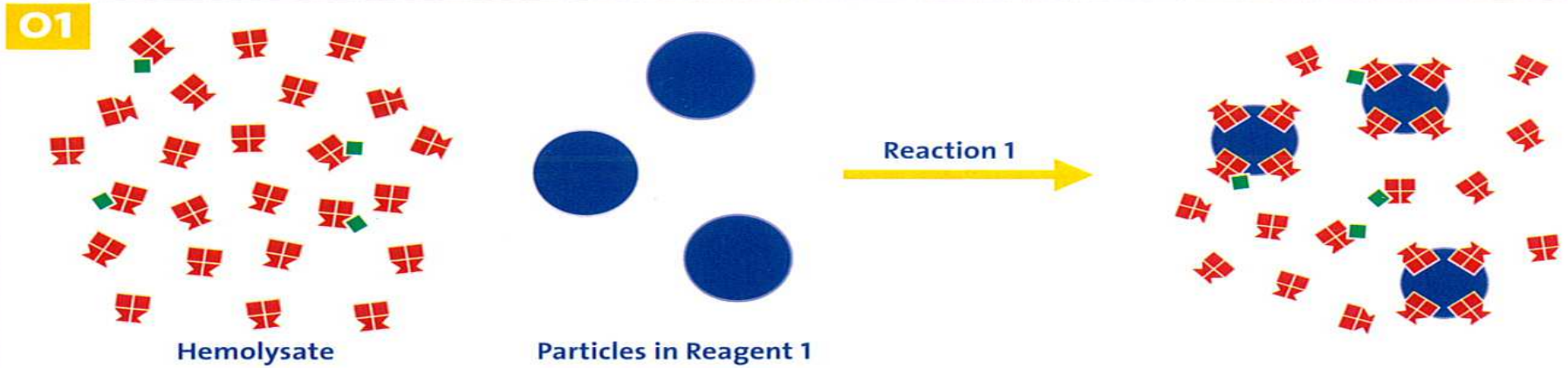


Competitive Assay

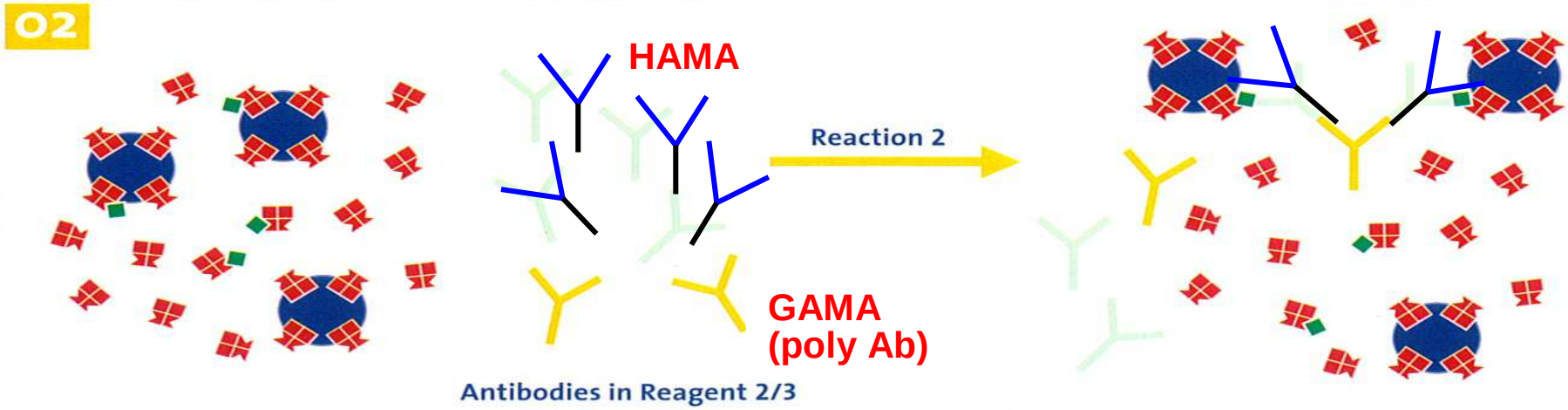


Turbidimetric Assay (HbA1c)

Competitive binding principle



Total Hb and HbA1c in hemolyzed EDTA blood bind with the same affinity to particles in R1. The amount of binding is proportional to the relative concentration of both substances in the blood.



Considerations

- **What is the assay used for?**
  - Detection
  - Quantification
  - Monitoring
- **Stability** (lot-to-lot variation)
- **Are epitopes altered?**

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Standardization

Do immunoassays measure the analyte?

They estimate (quantitatively) by a direct comparison with standard material

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Standardization

- What, Where, How, of Standard Material
- Definitive Method (REAL ‘Reference Method’)
  - **’Well established’ method can be used as reference**
  - **‘Well established’ does NOT mean better**
  - **Need a commonality across the board**
    - **ISO**
    - **NCCLS**
    - **WHO**
    - **NIBSC**
    - **For HbA1c – NGSP / IFCC**

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To be MONOCLONAL or POLYCLONAL?

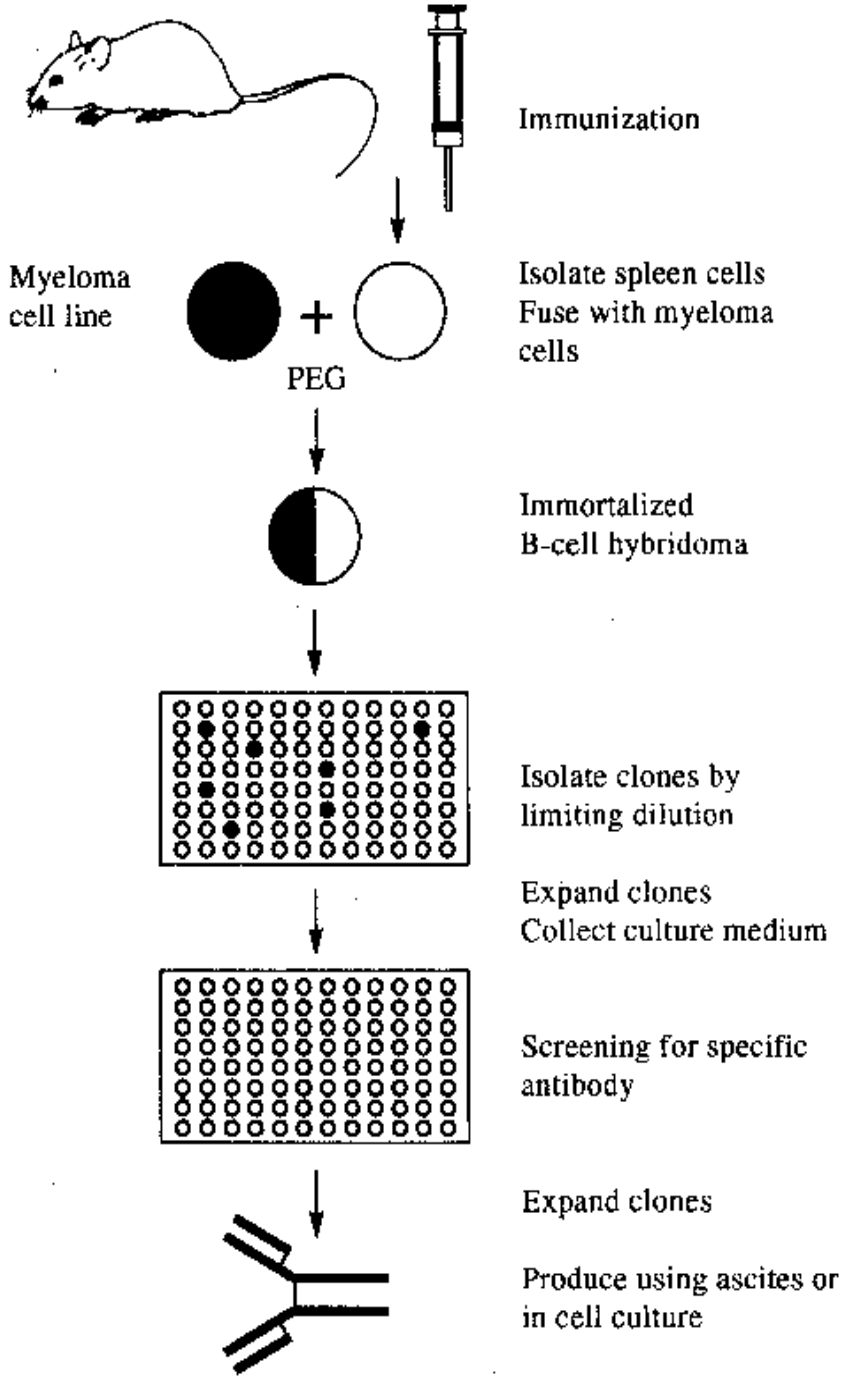
- **Monoclonal Ab** - unending supply of Ab with a **SINGLE SPECIFICITY** = **MONO**

- **HYBRID + OMA**

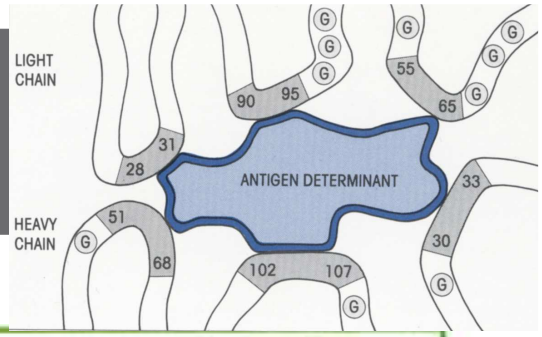
- **IDENTICAL CLONE** that has same IG class, allotype, the same variable region, structure, idiotype, affinity and specificity for a given epitope. (WOW - no wonder it revolutionized immunodiagnostics! (as well as therapeutics)

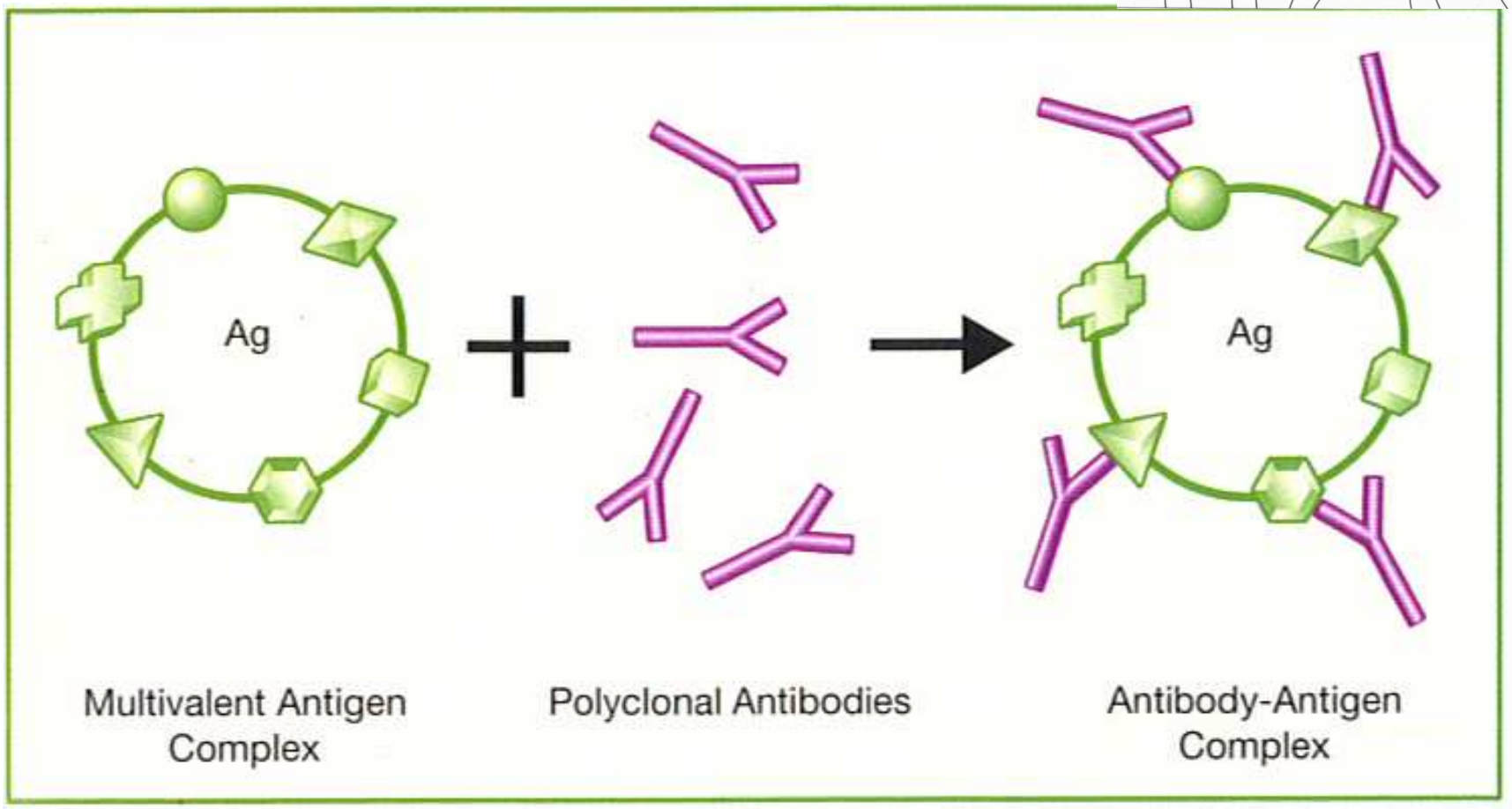
Monoclonal Ab

production scheme



POLYCLONAL Antibodies





POLYCLONAL AB

- **Mixture** of Abs with different affinities can be a '***double edged sword***'

- Some can have low affinity due to nature of polyclonal makeup - can have High Dose Tolerance" (depends on frequency of immunization to the animal) ie. – **cause of Lot-to-lot variation**

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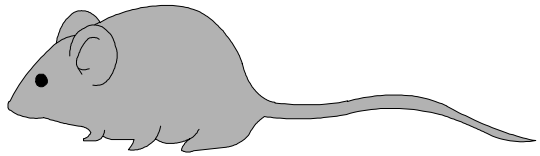
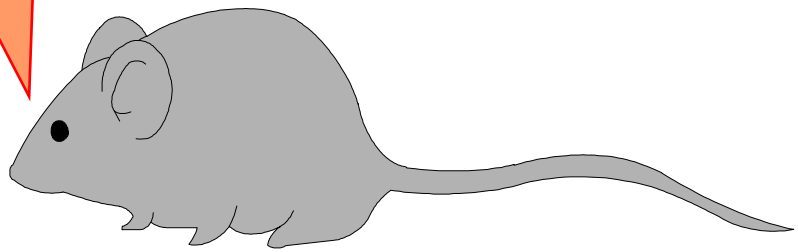
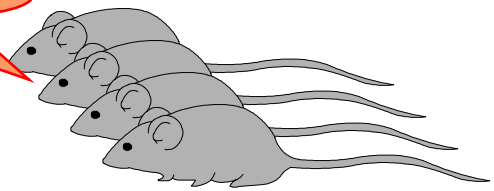
I hope they bleed me at the right time for proper **polyclonal antibody** production





Yo tambien



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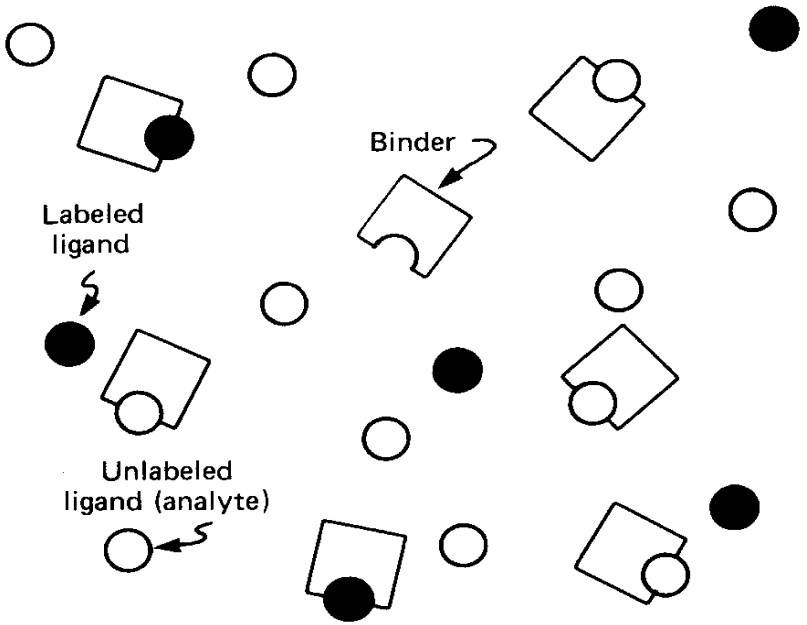
Major Labeling Schemes or Signal Generation

RIA

Radioimmunoassay ^{125}I, ^{14}C



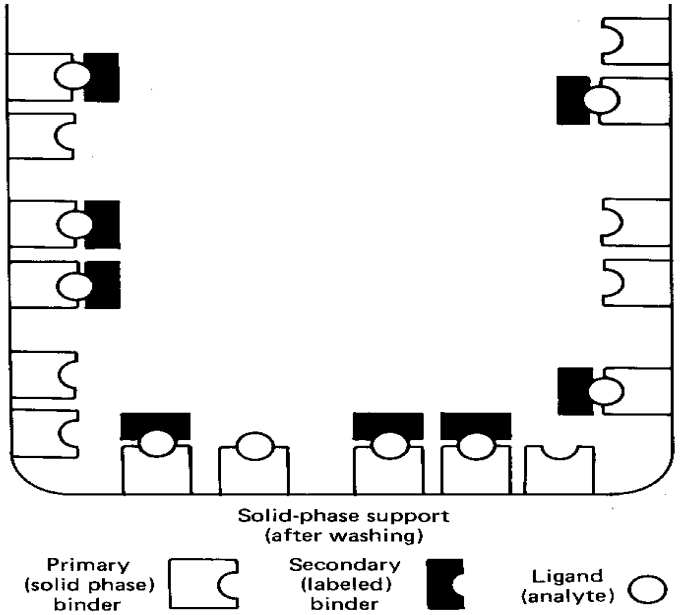
EIA

Enzyme Immunoassay

Alkaline phosphatase

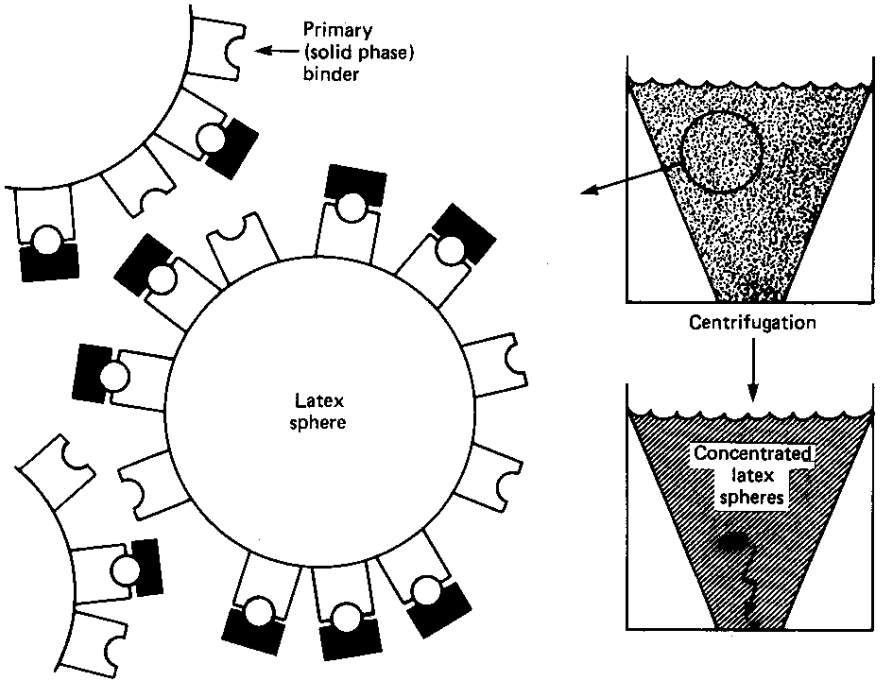
Horseradish peroxidase

They have a free amino left open for conjugation



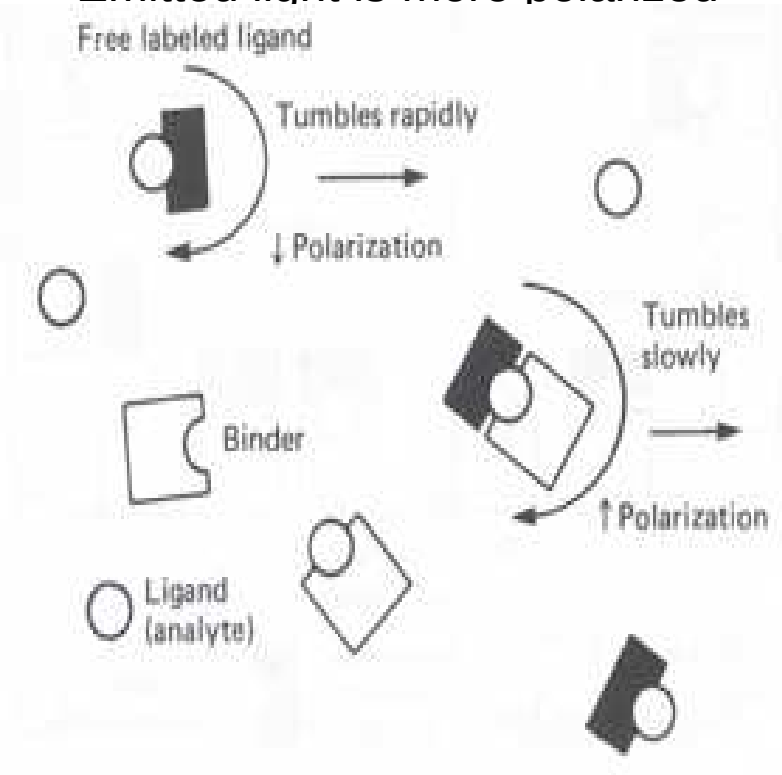
Major Labeling Schemes or Signal Generation

FIA Fluorescent



FPIA Fluorescent Polarization

- Analyte referred to as Ligand
- When tracer is bound to Ab the tumbling is slowed
- Emitted light is more polarized



PRECISION (intrinsic) with IA

- Assay imprecision caused by intrinsic and extrinsic factors
  - **B/F Separation- incomplete separation has been the main cause of imprecise results.**
  - **Detection - How good (stable) is the signal being used? Detector?**
  - **What *flavor* is the Antibody? (MAb or PolyAb)**
  - **Manipulation errors in assay design**

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PRECISION (extrinsic)

- **Cross reactivity**
  - **Steroid Hormones**
- **Interference (The Matrix)**
  - **EDTA**
- **High Dose Hook Effect**
  - **Tumor Markers**
- **HAMAs *'raton'* (SAMAs, '*oveja*' GAMAS *'cabra'*)**
  - **False Pos and/or False Neg – HbA1c**

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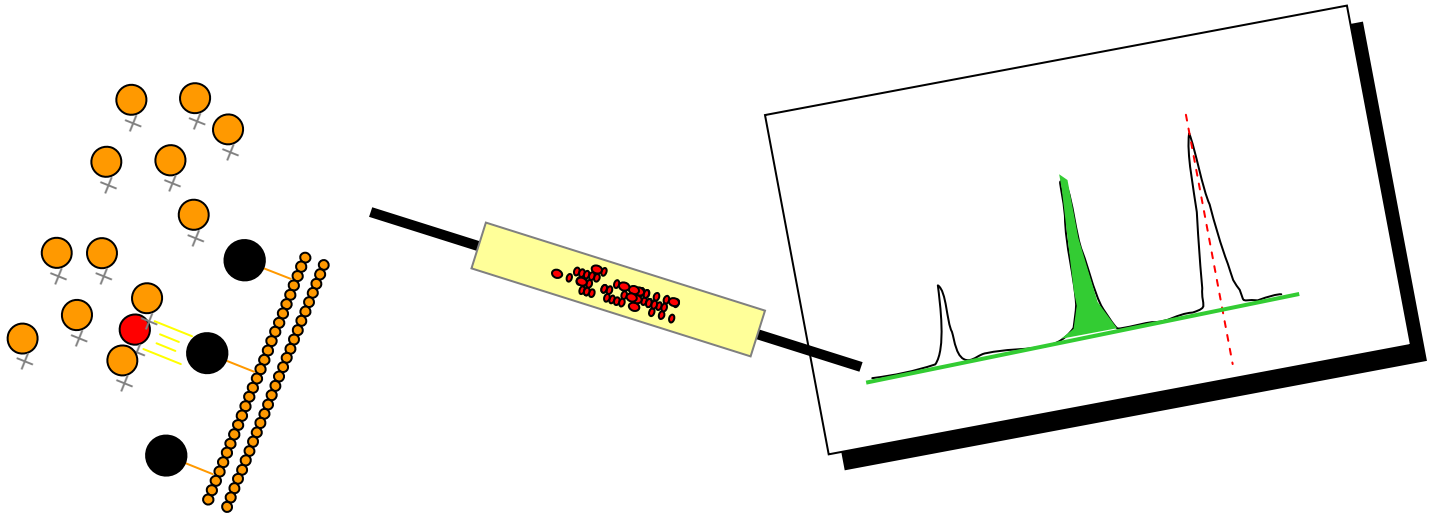
HPLC principle

High Performance Liquid Chromatography



Moisés - el primer cromatógrafo

"and Moses stretched out his hand over the sea…..and the waters were **divided**."

separated

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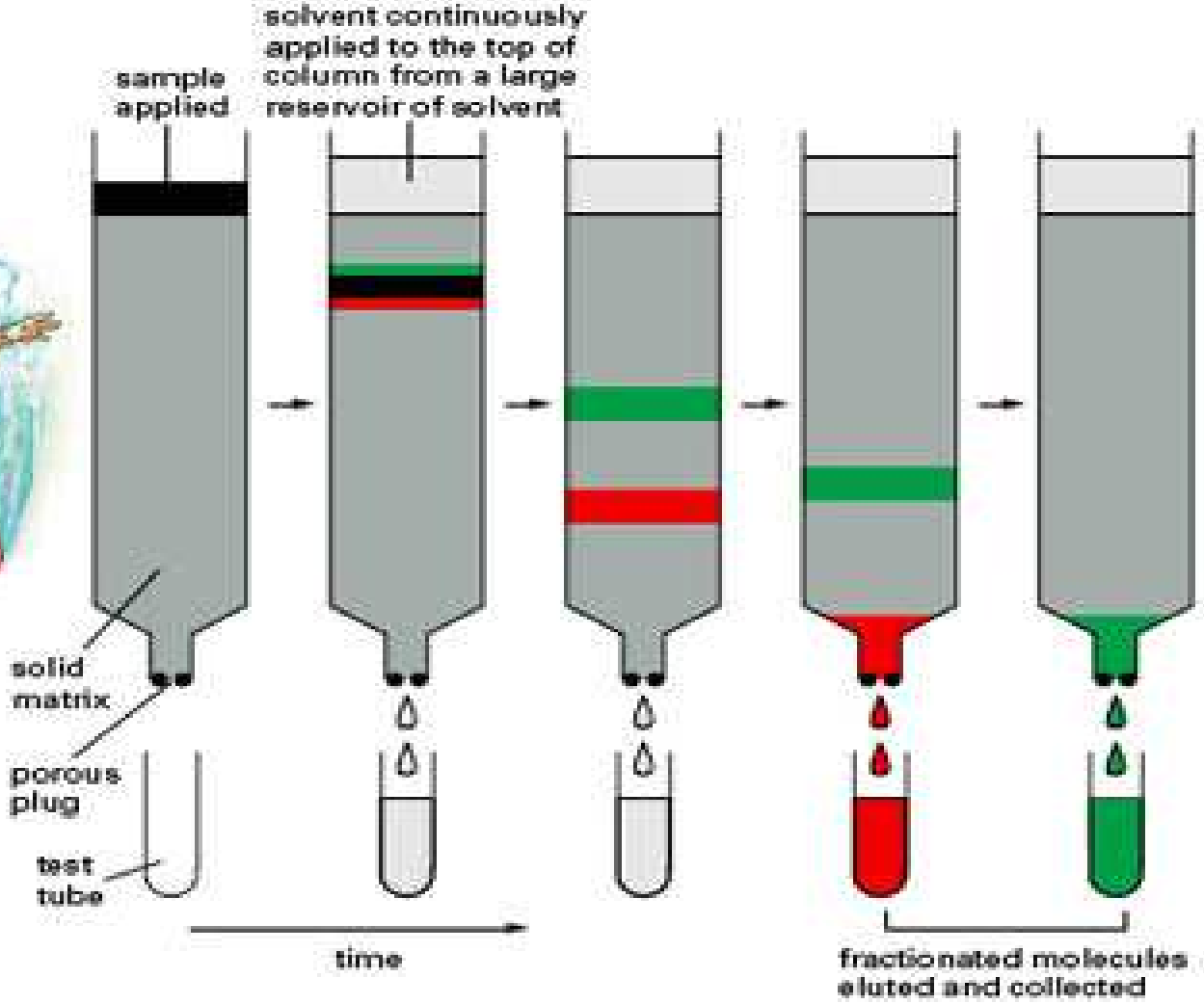
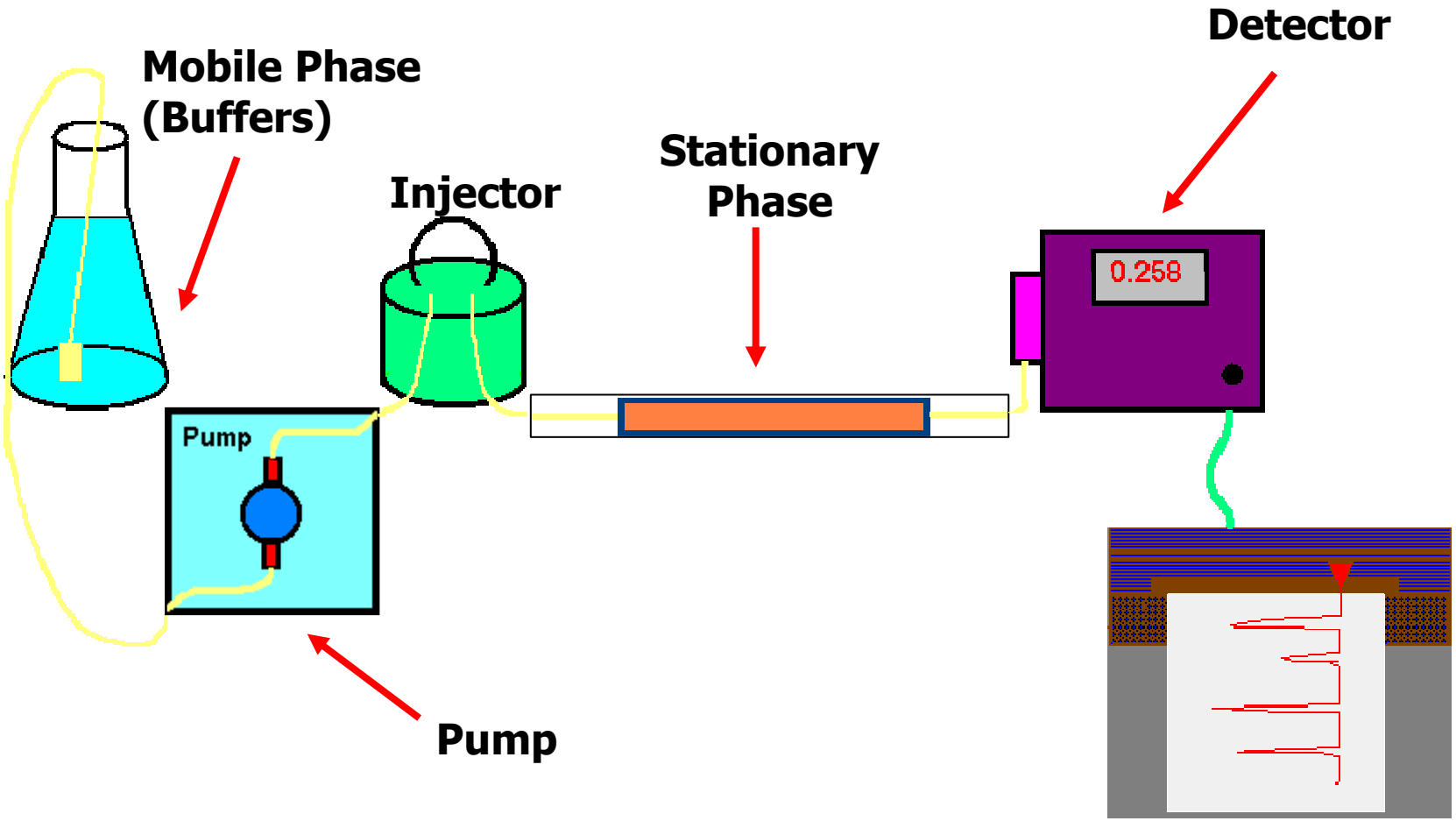
COLUMN CHROMATOGRAPHY

Proteins are often fractionated by column chromatography. A mixture of proteins in solution is applied to the top of a cylindrical column filled with a permeable solid matrix immersed in solvent. A large amount of solvent is then pumped through the column. Because different proteins are retarded to different extents by their interaction with the matrix, they can be collected separately as they flow out from the bottom. According to the choice of matrix, proteins can be separated according to their charge, hydrophobicity, size, or ability to bind to particular chemical groups (see *below*).



Basic Hardware



HPLC principle in plain language

- Separating components of a mixture based on chemical or physical properties:

- In the case of hemoglobin
  - Charge (ion) differences
  - Hence we use “Ion exchange” chromatography **CE – HPLC**
  - Intercambio de cargas

Cation Exchange Chromatography

- Separates Hb based on charge differences
- Positively charged Hb are separated by their absorption on a negatively charged stationary phase in a column
- The cations (positive charge) in the mobile phase (buffers with increasing ionic strength) compete with the absorbed Hb eluting them off
- The fractions are detected optically by a spectrophotometer that measures the concentration of Hgb in each fraction which is quantified by calculating the area under the peaks

HPLC principle in plain language

- The 2 system buffers have different charge
  - Buffer A is low Ionic strength
  - Buffer B is higher Ionic strength
  - Controlled blending generates a “Continuous” gradient of increasing charge – or “Step” gradient

- The analytical resin has a – charge (carboxyl group)
  - Why it is called cation exchange
  - Which creates a competitive binding environment

- Different Hb types have different charges
  - Varying from very weak to very strong
  - Hb Barts very weak, Hb C very strong

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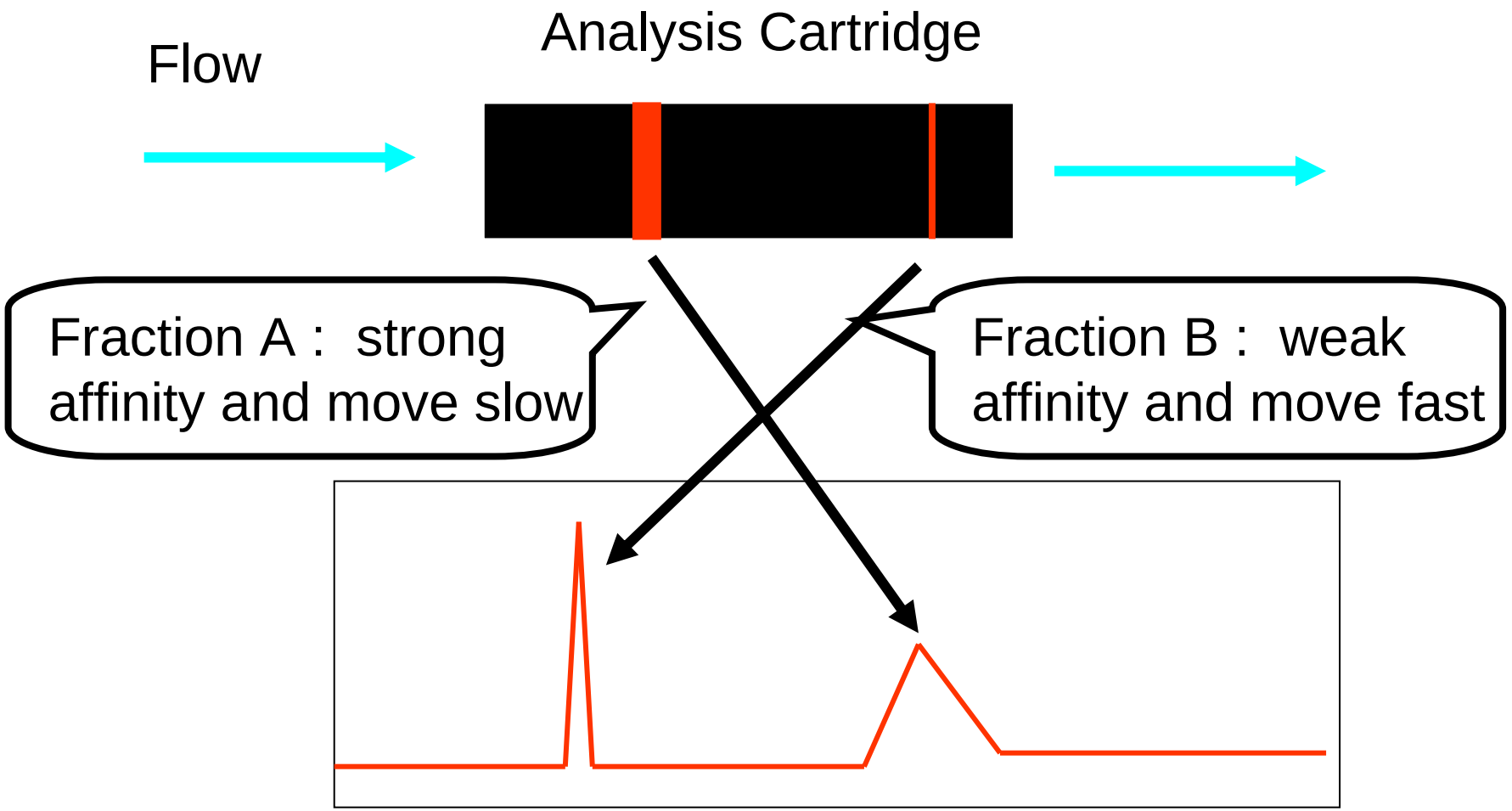
HPLC Separation



HPLC principle in plain language

- HPLC provides a **highly reliable diagnostic tool** provided the environment is ***locked down*** with respect to:

  – Sample integrity
  – Correct calibration
  – Buffer concentration
  – Buffer flow rate
  – Column Temperature
  – Resin stability

  } HPLC needs

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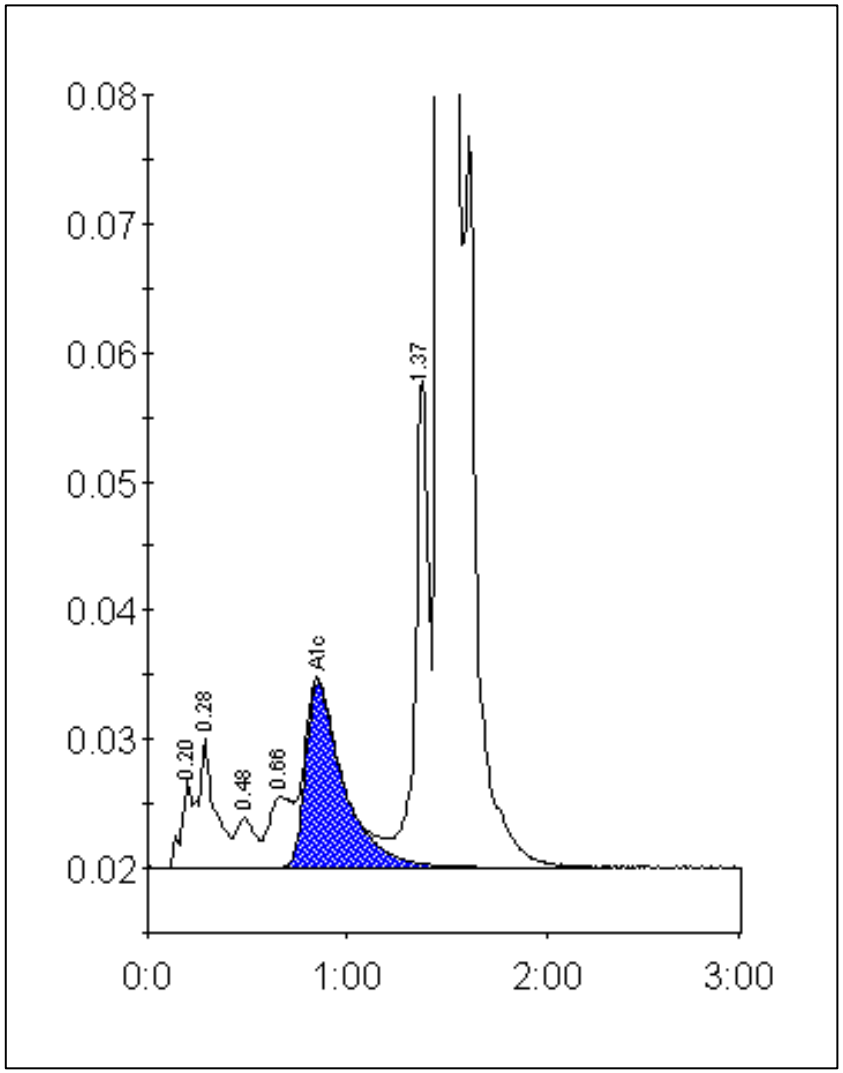
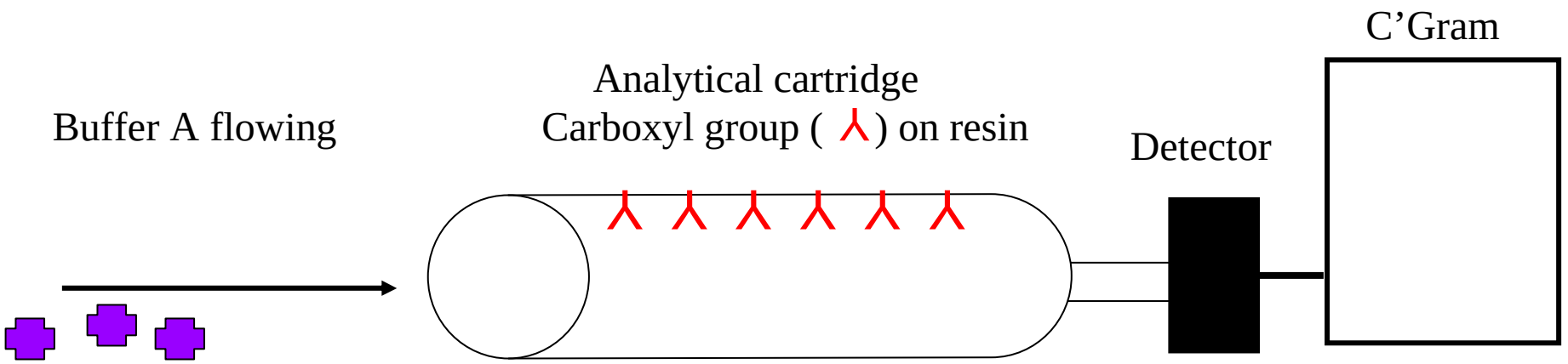
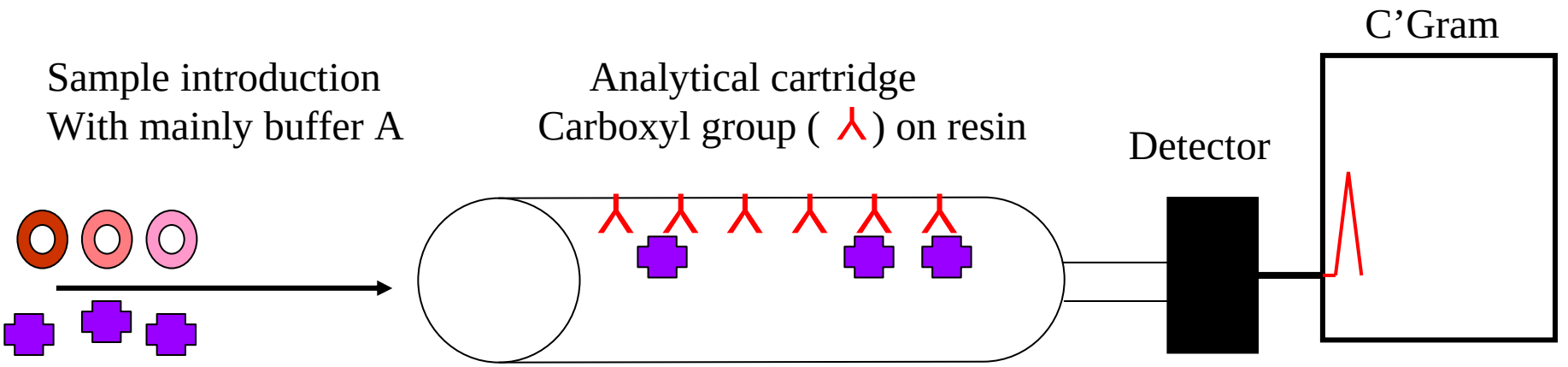
Chromatogram from a Bio-Rad ‘D-10’



The separation process

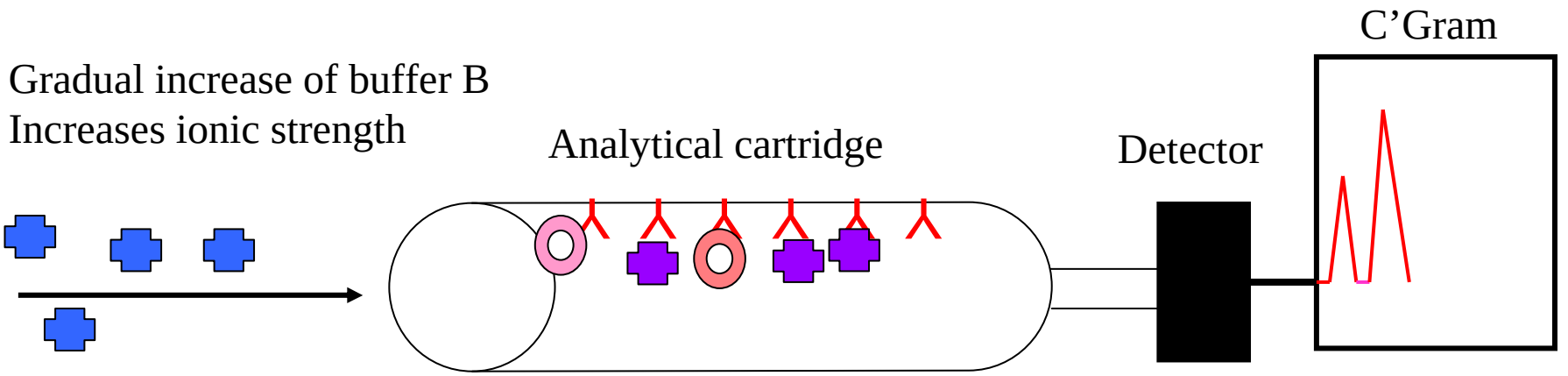


The separation process



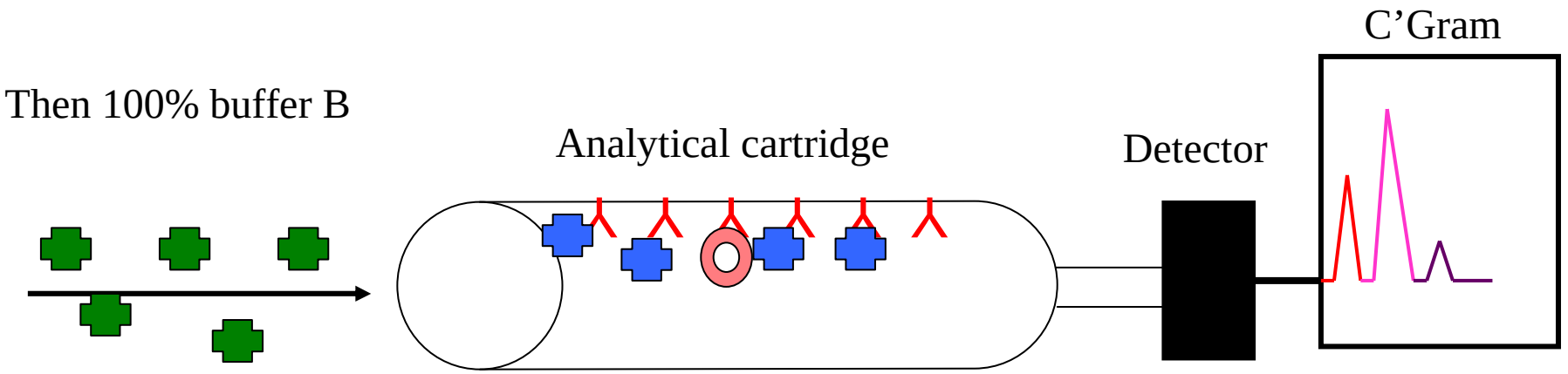
- **Any Hb with ionic strength less than buffer A passes straight through (Barts)**
- **Other Hb's and buffer A bind to Carboxyl group on resin**

The separation process



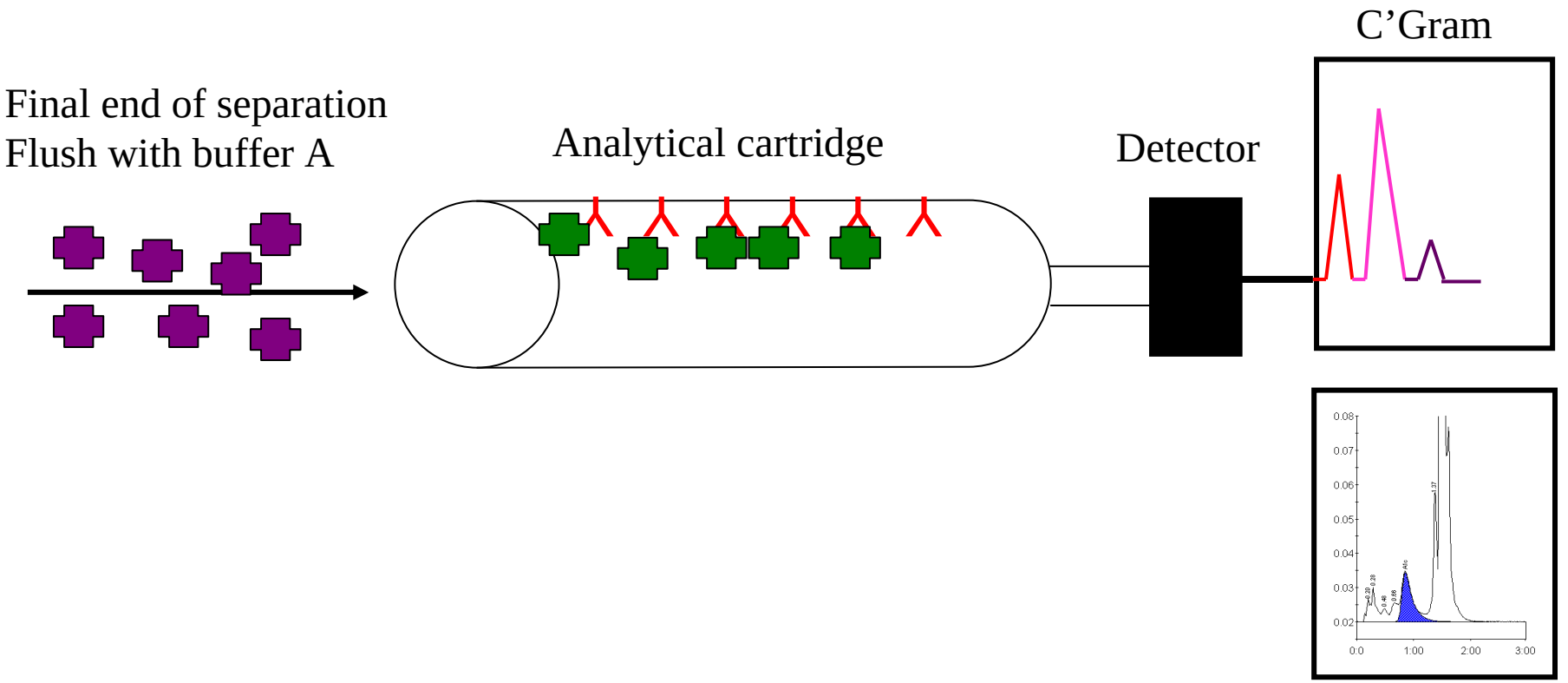
- **Further Hb displacement caused by increased ionic strength of buffer mix**

The separation process

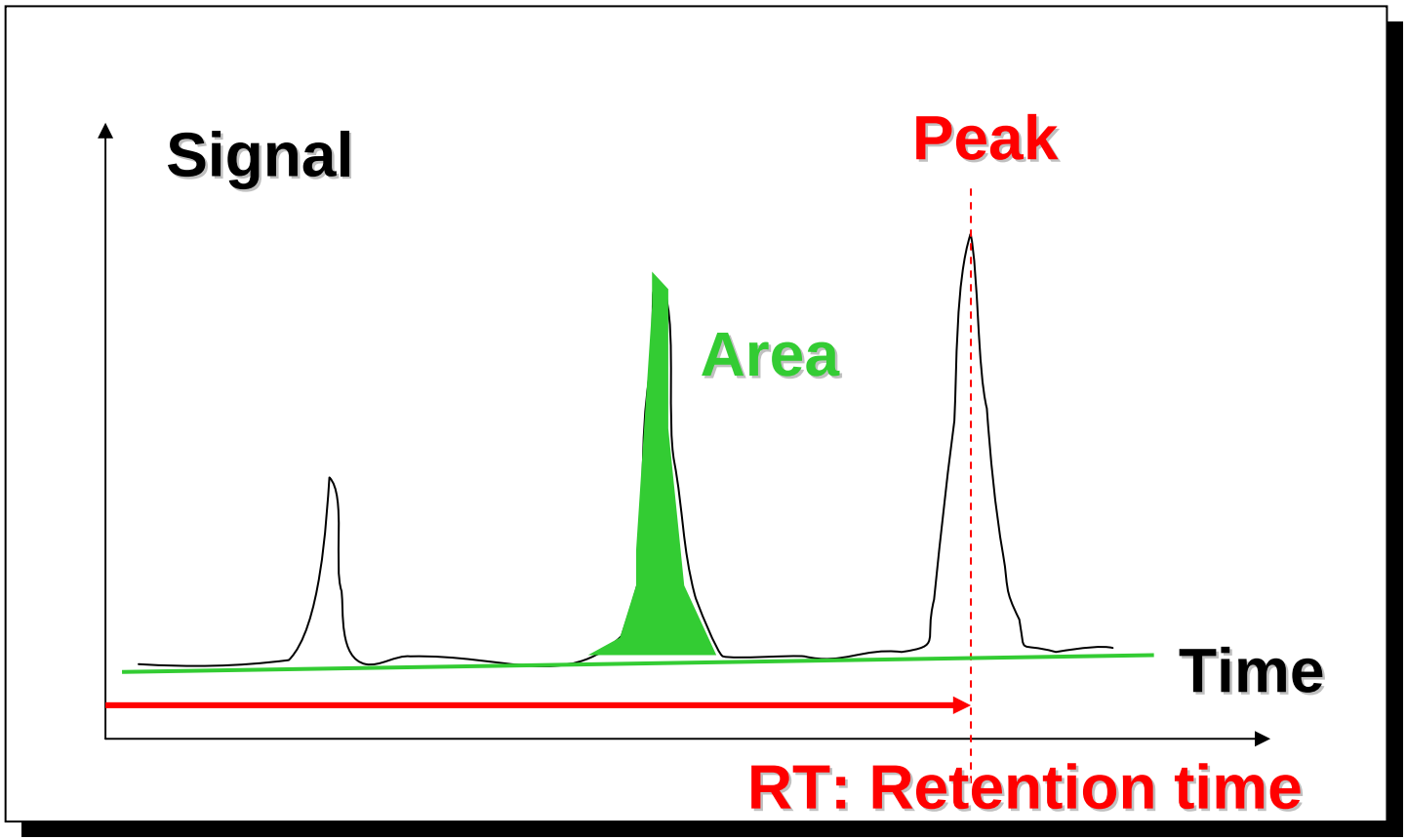


- **Complete Hb displacement along with any residual lower ionic strength buffer**

The separation process



INTEGRATION PARAMETERS



To summarize

- **Good quality resin**
- **Good integration parameters**

- **The separation is driven by tight control of:**
  - **Temperature**
  - **Flow rate**
  - **Increasing buffer strength**

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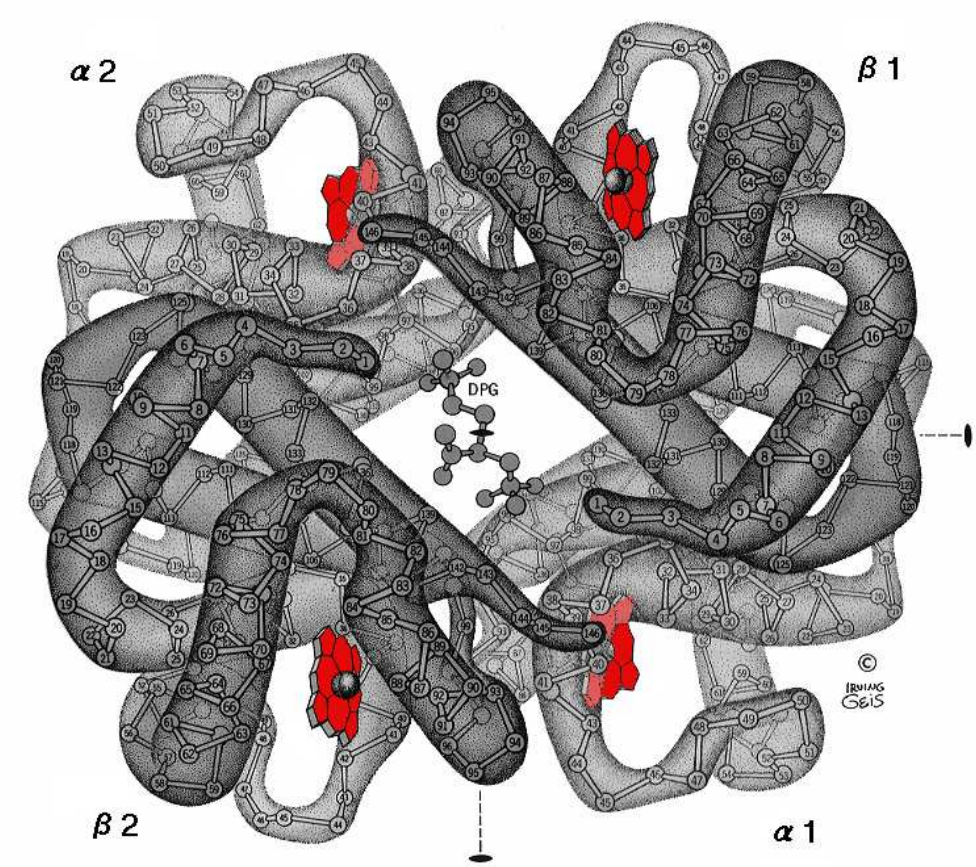
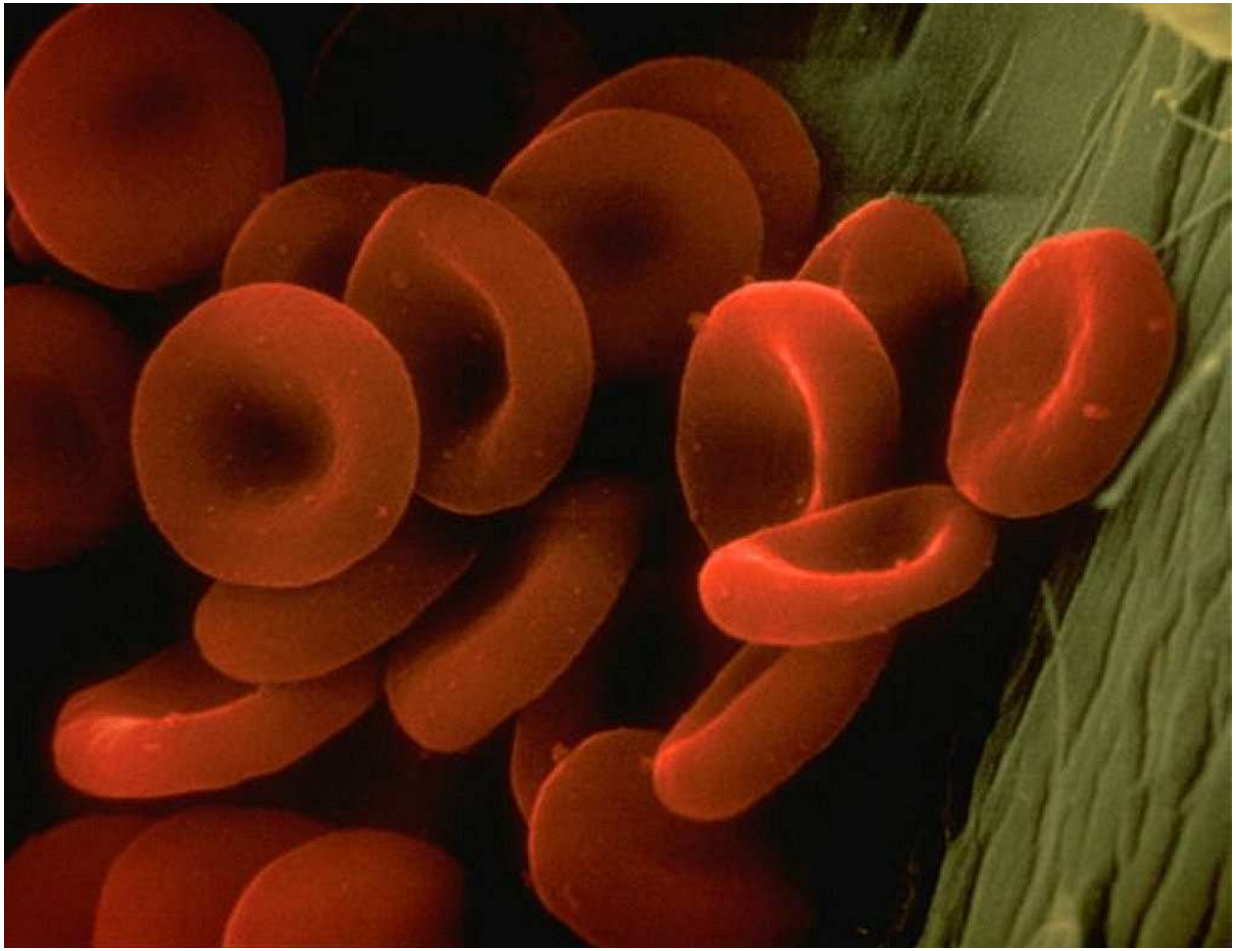
Now……LETS LOOK At HbA1c

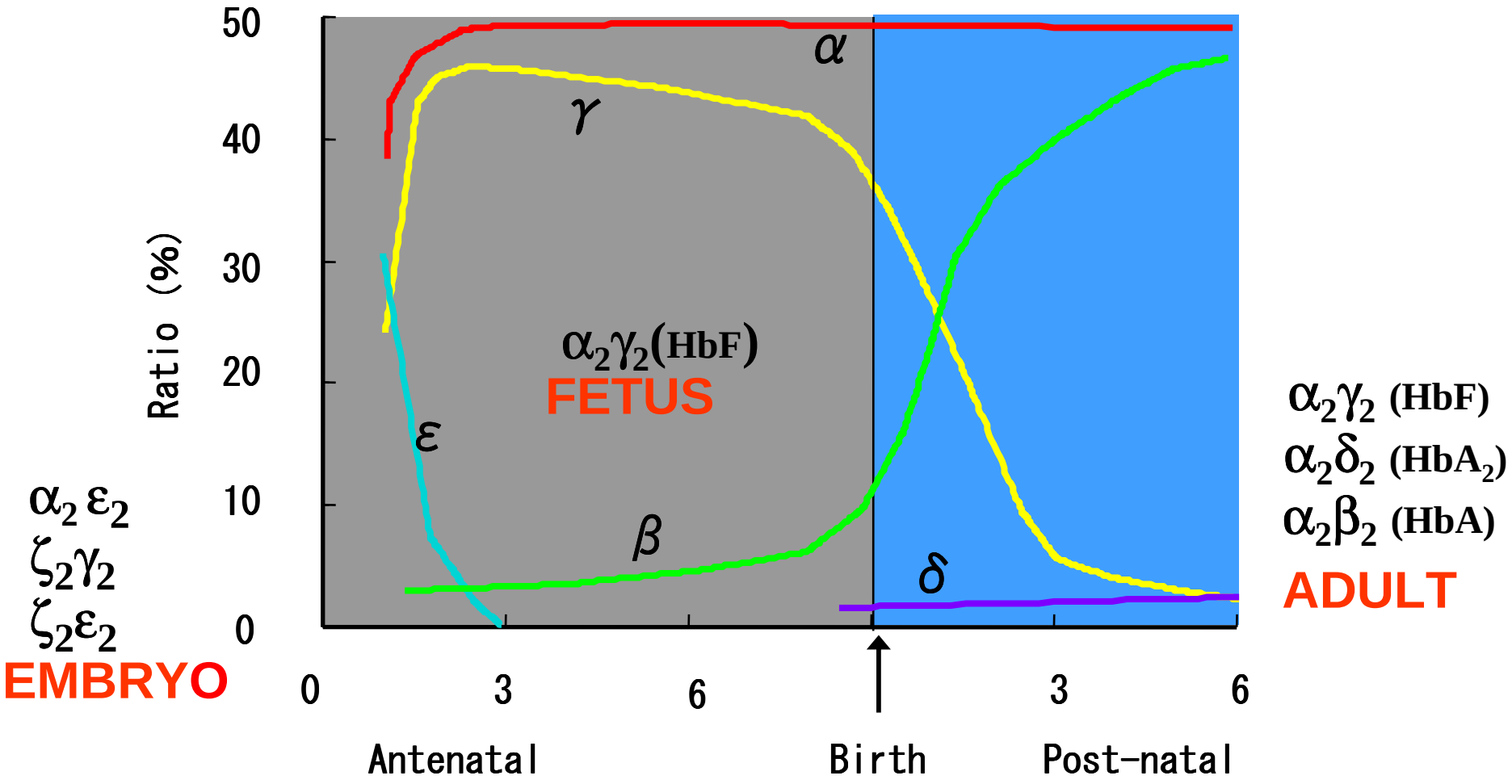


Red Blood Cells



- RBCs play a vital role in oxygen transportation to all the organs of the body and also the removal of carbon dioxide
- **Normal RBCs have a lifespan of 80 - 120 days (A1c)**

Transition of the hemoglobin chains



Normal Hemoglobin Structure

- Composed of 4 subunits:
  2 a and 2 ß chains = Hb A
  2 a and 2 δ chains = Hb A2
  2 a and 2 γ chains = Hb F = **Fetal hemoglobin**
- Normal individual:
  95% HbA
  < 2% HbF
  1.5 - 3.5% HbA2
- A number of chemically modified hemoglobin structures can be present in the blood HbA1c
  Carbamylated hemoglobin
  Acetylated hemoglobin

TODOS TIENEN CARGAS DIFFERENTES

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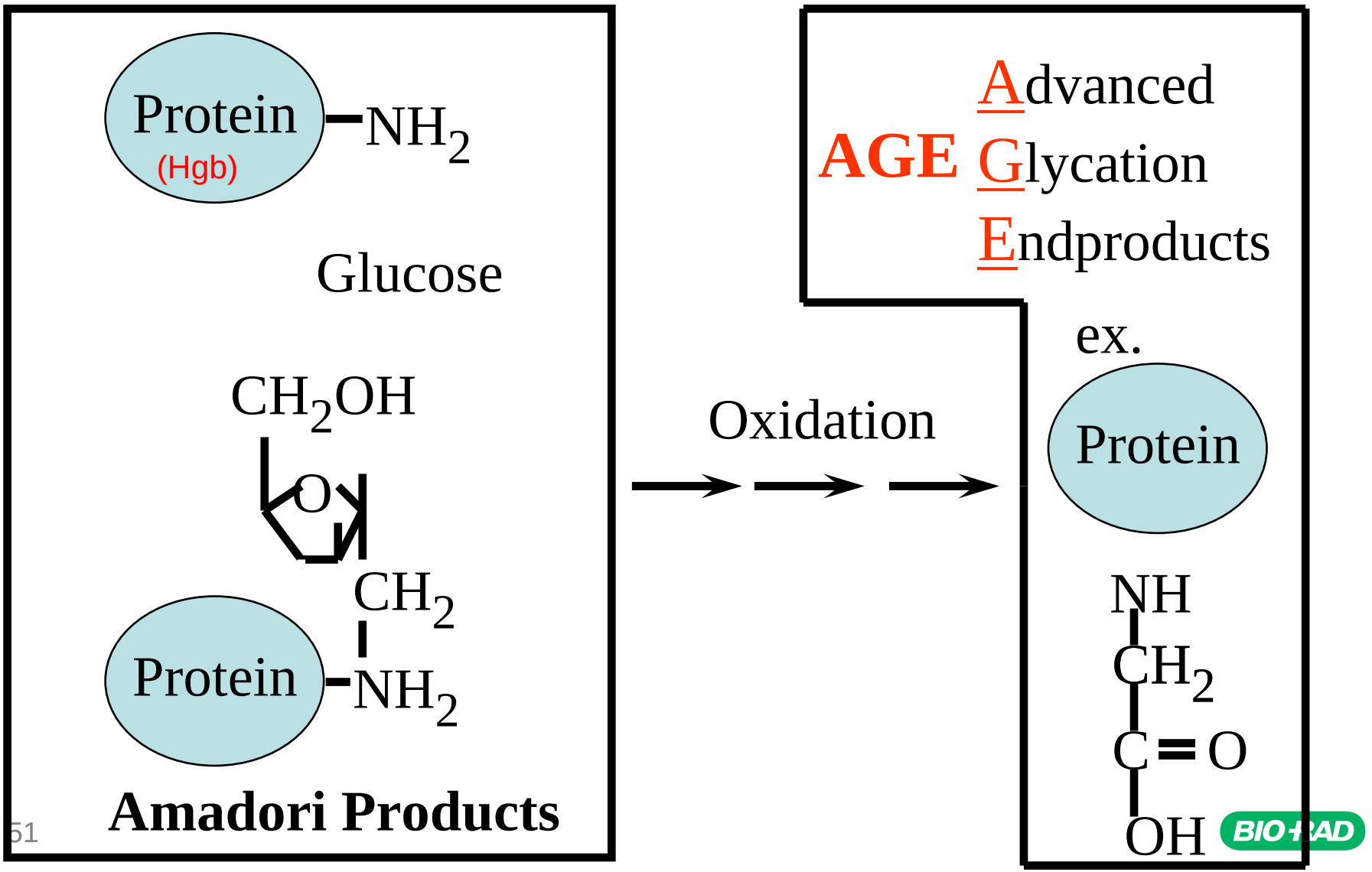
GLYCOHEMOGLOBIN

- Percentage in whole blood depends upon:

1) Duration of glucose exposure to hemoglobin
2) Turnover rate of the RBCs
3) Concentration of glucose
   - **weighted** mean
   - prior 1 - 4 weeks determine 50%
   - prior 5 - 8 weeks determine 25%
   - prior 9 - 18 weeks determine final 25%

PATHWAY FOR THE MALLIARD REACTION



GLYCOHEMOGLOBIN

- Potential glycation sites:
  - N-terminal amino acids of the four polypeptide chains
  - free epsilon-amino groups of lysine within chains
- Most reactive site = N-valine terminal of beta chains (60% of bound glucose)
- **Called HbA1c**

ANALYTICAL METHODS

- **Ion exchange chromatography-** charge differences due to binding with glucose which changes isoelectric point of hemoglobin

- **Immunoassay** - mono or polyclonal antibodies directed against glycated N-terminal group of beta-chain of hemoglobin

- **Affinity chromatography-** reaction between bound glucose and immobilized boronic acid

HPLC - Peak ID & quantification parameters

- Peak area's
  - The area bounded by an individual peak
  - Expressed as a % ratio of "Total area"
  - "Total area" is critical for correct integration
    - The total area indicates the degree of color intensity- or basically the hematocrit (Abs)
    - Since the HPLC detector has a specific dynamic range for absorbance if the total area is too low or too high the operator will be altered
      - **Anemia, Polycythemia**
      - **Short sample**

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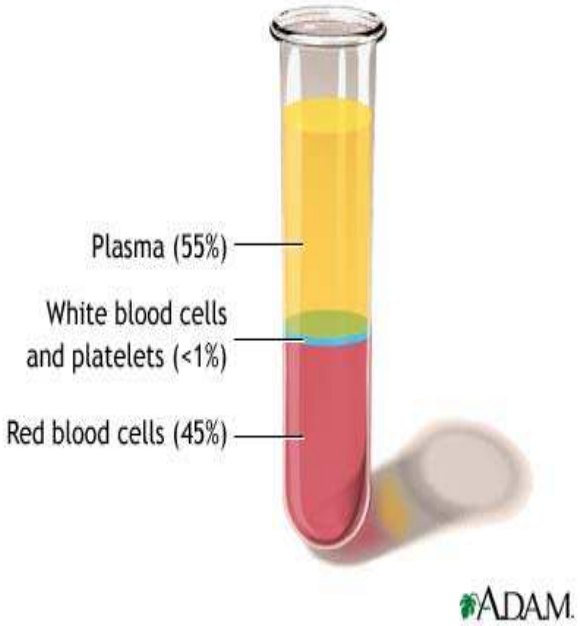
Example from Bio-Rad's HPLC instruments

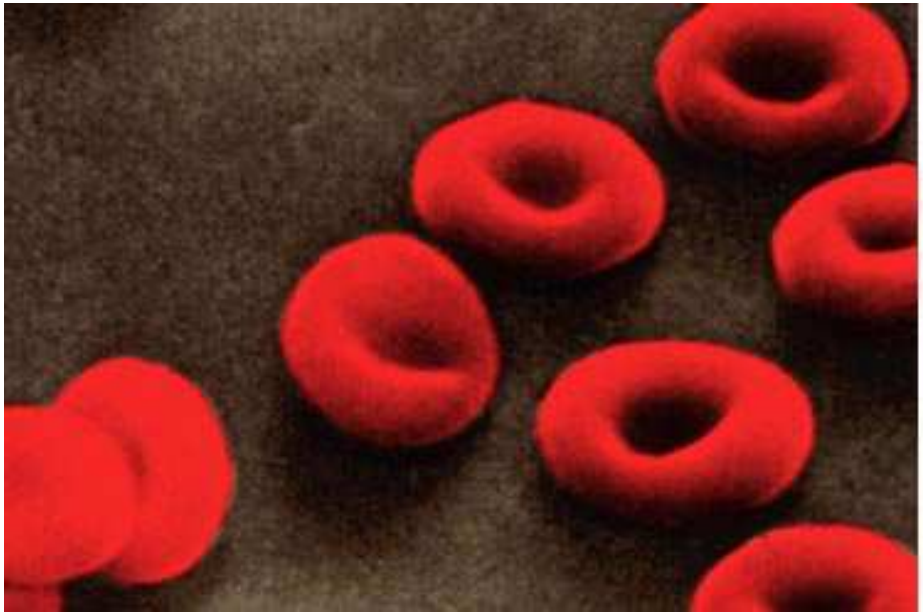
VII beta-Thal	1.0 – 3.0 Million
VII Dual	1.5 – 3.5 Million
D-10	1.0 – 4.0 Million
D-10	1.0 – 5.0 Million
Vnbs	1.0 – 3.5 Million



Mas o menos como hematocrit

BIO-RAD

Hb S



(Source: Internet)

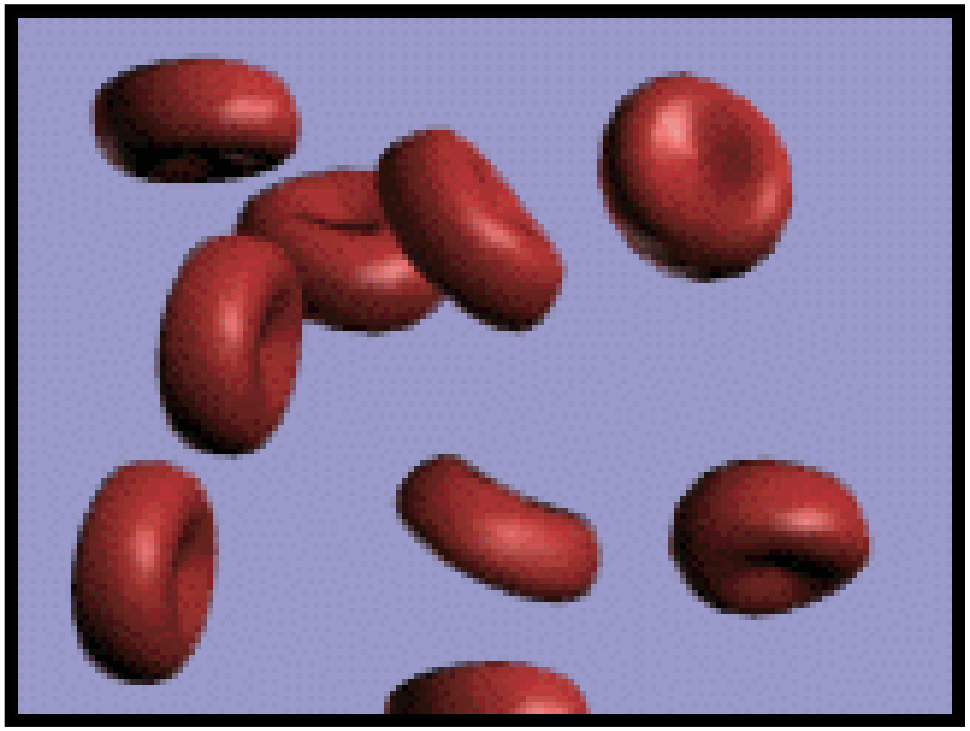
Found throughout Africa- highest in Nigeria, Ghana, Gabon and Zaire

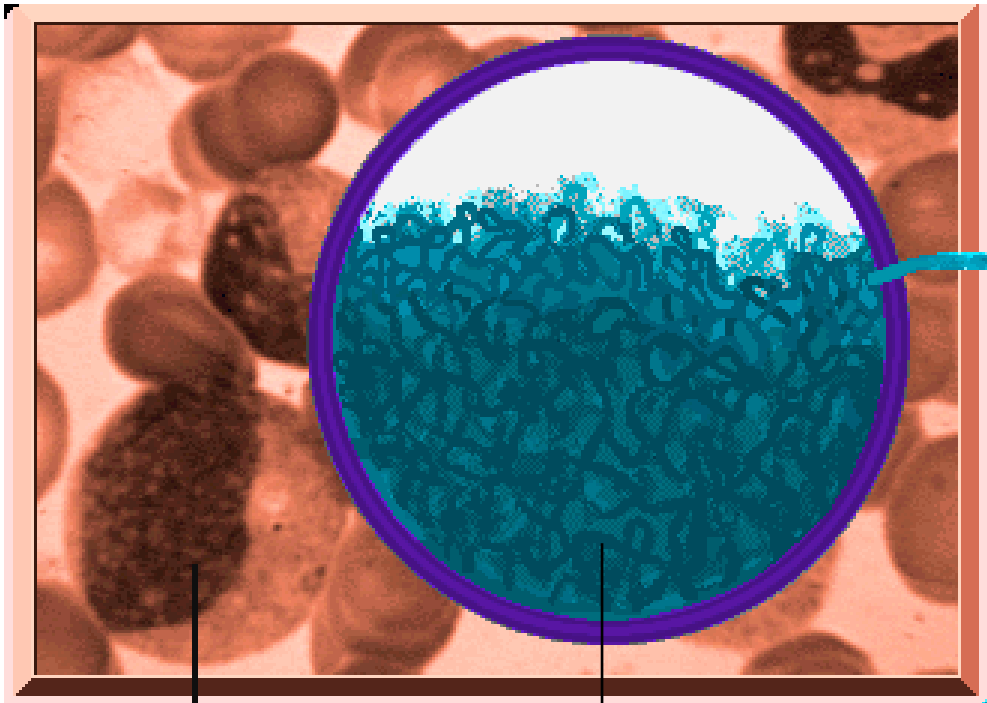
Found in Saudi Arabia and Kuwait

Found in East Central India

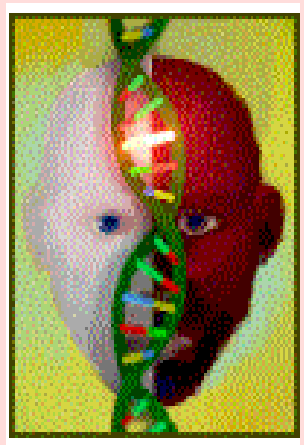
Thru migration/history found in the USA and LA

Hb S





Single nucleotides polymorphisms is expected 1:1000 to 1:100 bases



nucleus

chromosome

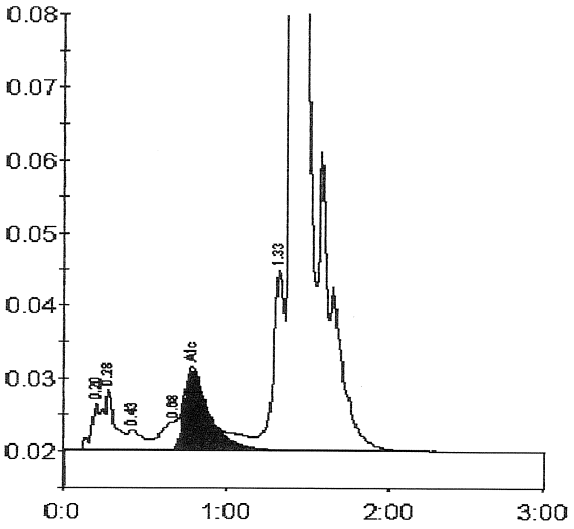
A	G	T	*A*	A	G	G	T	A
T	C	A	*T*	T	C	C	A	T

DNA

A	G	T	C	A	G	G	T	A
T	C	A	G	T	C	C	A	T

Patient report

Bio-Rad	DATE: 08/17/2005
D-10	TIME: 01:17 PM
S/N: #DA3J255004	Software version: 3.07-1
Sample ID:	NP
Injection date	08/17/2005 01:17 PM
Injection N°: 1	Method: HbA1c
Rack N°: 02	Rack position: 1



Peak table - ID: NP

Peak	R.time	Height	Area	Area %
A1a	0.20	6342	20628	0.7
A1b	0.28	8345	41758	1.4
F	0.43	2722	18291	< 0.8
LA1c/CHb-1	0.68	3874	27304	0.9
A1c	0.80	10879	114524	4.1
P3	1.33	24821	122408	4.0
A0	1.41	637907	2716085	88.7
Total Area:	3060997			

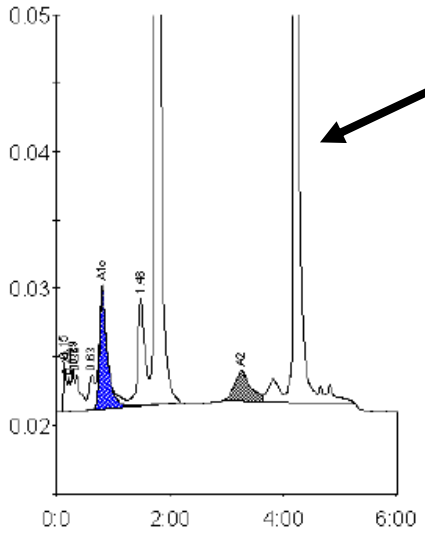
Concentration:	
% A1c	4.1

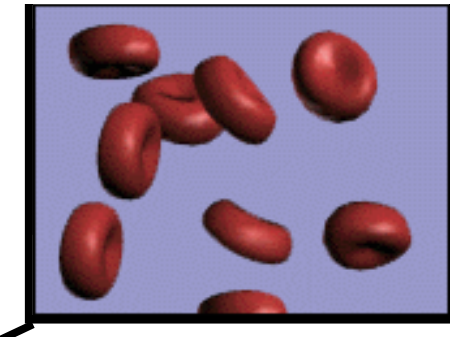
HbA1c% Degree of Glucose Control

> 8	Action Suggested
< 7	Goal
< 6	Non-diabetic level

Patient report

Bio-Rad	DATE: 11/20/2006
D-10	TIME: 11:10 AM
S/N: #DA2G078002	Software version: 3.50-A1
Sample ID:	AS_SAMPLE
Injection date	10/25/2006 05:31 PM
Injection #: 117	Method: HbA2/F
Rack #: E2	Rack position: 7





Entire patient picture with HPLC –

One can 'SEE the DIFFERENCE'

Peak table - ID: AS_SAMPLE

Peak	R.time	Height	Area	Area %
Unknown	0.15	3750	10754	0.7
A1a	0.22	2557	9867	0.7
A1b	0.29	3152	8824	0.6
Unknown	0.36	2646	17199	1.2
LA1c/CHb-1	0.63	2567	18201	1.3
A1c	0.81	8776	84553	11.1
P3	1.48	7783	68800	4.8
A0	1.75	140605	729917	50.8
A2	3.26	2192	43128	3.1
S-Window	4.20	75253	444531	31.0
Total Area:	1435775			

Concentration:	
% A1c	11.1
% A2	3.1

BIO-RAD

SEE the DIFFERENCE

VII Bio-Rad

- Post transfusion
  - **HbSC**
  - **Este persona sin transfusion no tiene Hb A**
  - **solo Hb S y Hb C**
  - **Entonces no HbA1c**

Bio-Rad CDM System 10995 PATIENT REPORT
Bio-Rad Variant II Instrument #1 V2_BThal

Patient Data
Sample ID: Unknown
Patient ID:
Name:
Physician:
Sex:
DOB:

Comments:

Analysis Data
Analysis Performed: 18/04/2001 16:39:38
Injection Number: 196
Run Number: 23
Rack ID: 0006
Tube Number: 6
Report Generated: 18/04/2001 16:46:07
Operator ID:

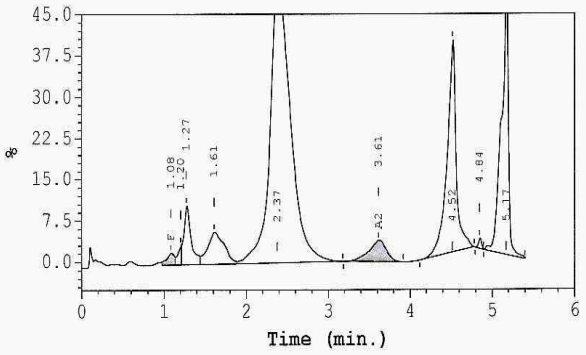
Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	0.8	---	1.08	31822
Unknown	---	0.6	1.20	23746
P2	---	3.9	1.27	151428
P3	---	4.7	1.61	183209
Ao	---	52.3	2.37	2046844
A2	3.8*	---	3.61	132100
S-window	---	17.4	4.52	680827
Unknown	---	0.3	4.84	10371
C-window	---	16.6	5.17	651240

Total Area: 3911587

F Concentration = 0.8 %
A2 Concentration = 3.8* %

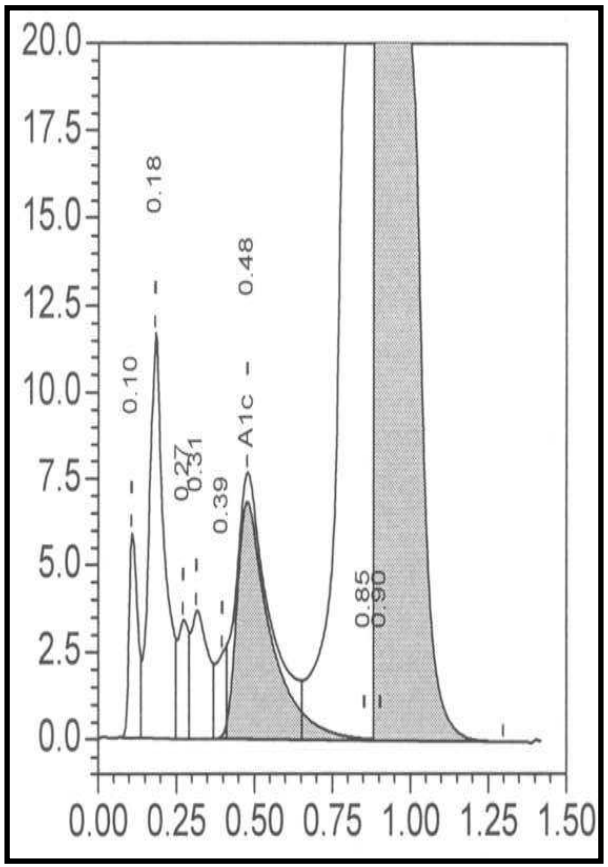
*Values outside of expected ranges

Analysis comments:



Patient with abnormal Hb

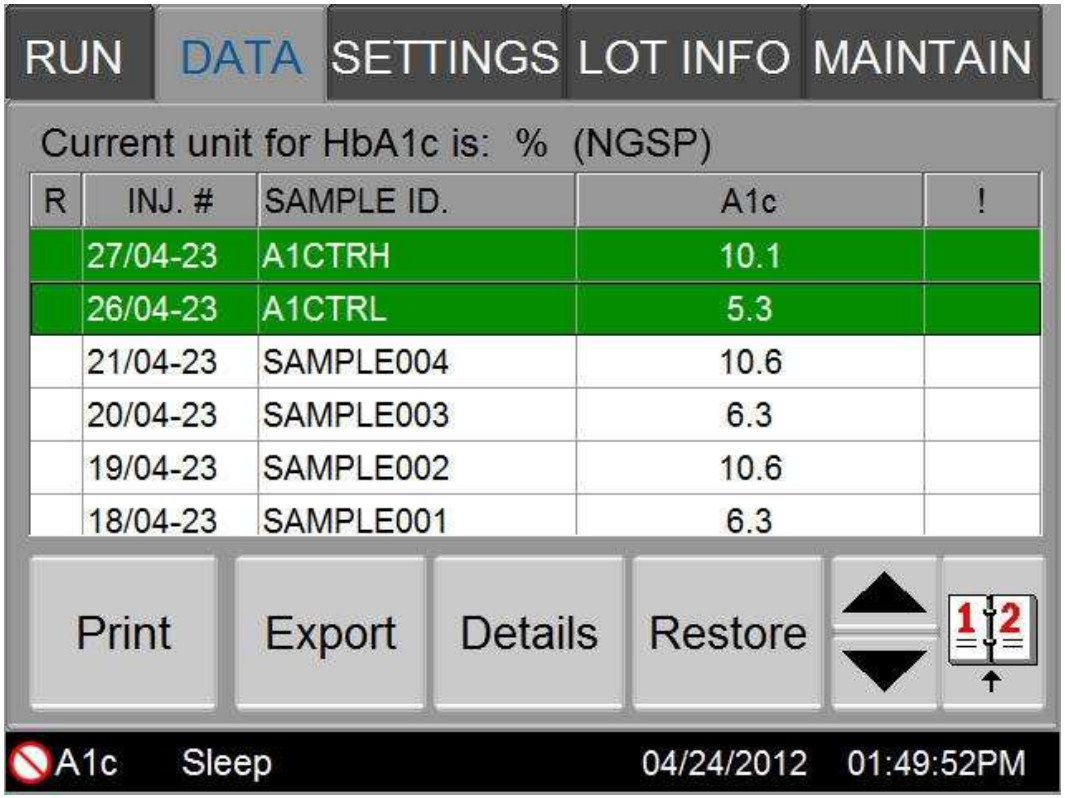
HPLC - Ion Exchange

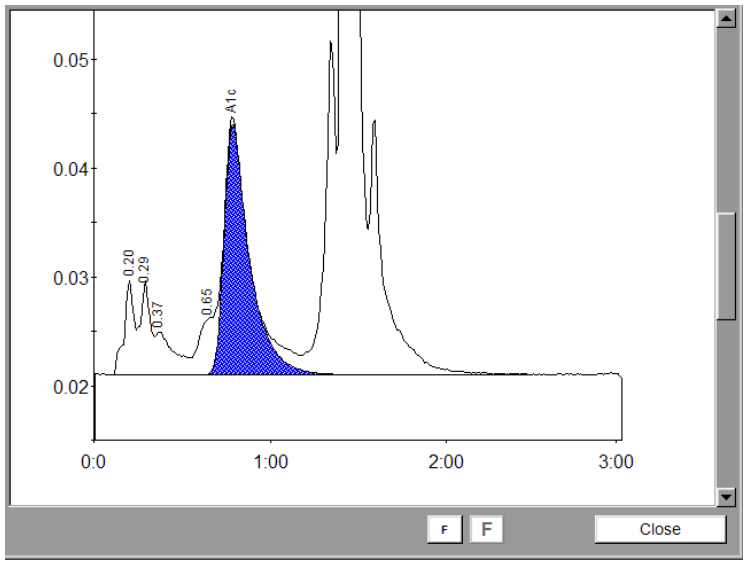


Immunoassay

7.3 % A1c

Una pintura vale mil palabras





Benefits of HPLC for HbA1c testing

Clinical Chemistry 58:2
332–336 (2012)

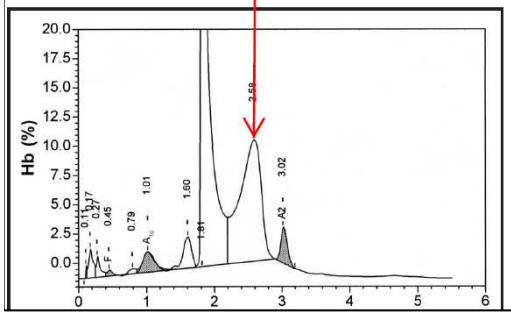
Clinical Case Study

A 14-Year-Old Boy with Chronic Cyanosis, Mild Anemia, and Limited Physical Resistance to Stress

Berndt Zur,[1*] Bernd Mayer-Hubner,[2] Michael Ludwig,[1] and Birgit Stoffel-Wagner[1]

Clinical Case Study

3) The abnormal hemoglobin is clearly visible

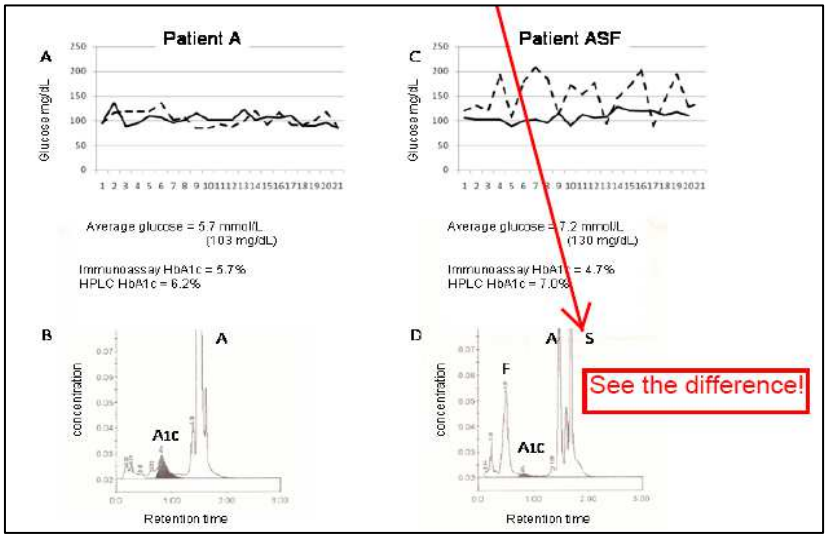


No abnormalities were found. Hemoglobin capillary electrophoresis (Capillarys; Sebia) also revealed no abnormalities. A sample of arterial blood analyzed by cooximetry for oxygen saturation showed a normal oxygen pressure of 94 mmHg (reference interval, 70–100 mmHg) and a decreased arterial oxygen saturation value of 84% (reference interval, >96%). Methemoglobin and carboxyhemoglobin values were normal. The partial pressure of O_2 at which hemoglobin is half-saturated (P_{50}) in whole blood was measured with a blood gas analyzer and found to be increased [39 mmHg; normal, 26 mmHg (*1*)]

CLINICAL PRACTICE

HbA1c Does Not Always Estimate Average Glucose

KRISTINA J BEHAN, JANE MERSCHEN



Clinical Chemistry 57:2
153–157 (2011)

Clinical Case Study

Unexpected Hemoglobin A_{1c} Results

Alina-Gabriela Sofronescu,[1] Laurie M. Williams,[1] Dorinda M. Andrews,[1] and Yusheng Zhu[1*]

BIO-RAD

- **Clin. Lab. 2012;58:821-828**
- **SHORT COMMUNICATION**
- **Advantage of HbA1c Assay by HPLC D-10 Versus Cobas Integra 400 in a Population Carrier for HbS and HbC**

KAHENA BOUZID 1, AFEF BAHLOUS 1, WAFA FERJANI 1, EYA KALAI 1

BIO-RAD

Benefits of HPLC for HbA1c testing

- Only HPLC utilizing ion exchange chromatography measures HbA1c.

- Affinity columns measure any hemoglobin that has glucose attached regardless of it attachment point or its structure because the column binds the glucose portion of the molecule. Any variant hemoglobin that is present will be detected as glycated products.

- Immunoassays also measure more than HbA1c, e.g., the Ab is reactive with HbS1c, HbC1c and HbE1c. Glycated HbF is not detected, for most immunoassays.

Clin Chem 2009; 55(No. 6 Supplement): A92.

BIO-RAD

HbA1c and MBG

- Because the patient could be harboring ahemoglobin variant that interferes with immunologic detection of HbA1c, one cannot know a *priori* whether a patient's HbA1c levels are accurate.

- This situation might be suspected if the level of HbA1c is different than would be expected based on the results of a patient's self monitoring blood glucose (SMBG) levels.

- **If possible, all patients should have at least one HPLC assay for HbA1c to rule out the presence of interfering hemoglobin variants.**

BIO-RAD

6 in the World - MEXICO 16.4 MILLION

Top 10: Countries/territories of number of people with diabetes (20-79 years), 2011 and 2030

	COUNTRY /TERRITORY	2011 MILLIONS
1	China	90.0
2	India	61.3
3	United States of America	23.7
4	Russian Federation	12.6
5	Brazil	12.4
6	Japan	10.7
7	Mexico	10.3
8	Bangladesh	8.4
9	Egypt	7.3
10	Indonesia	7.3

	COUNTRY /TERRITORY	2030 MILLIONS
1	China	129.7
2	India	101.2
3	United States of America	29.6
4	Brazil	19.6
5	Bangladesh	16.8
6	Mexico	16.4
7	Russian Federation	14.1
8	Egypt	12.4
9	Indonesia	11.8
10	Pakistan	11.4