Laboratory Guidelines for Protein C, Protein S, Antithrombin and APC-R Testing

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DISCLOSURES
Richard A. Marlar, PhD

- **Financial Relationships**

  Consultant: **IL & Novo Nordisk**

  Research: **Stago & DMS Pentapharm**

- **Off Label Usage**

  None

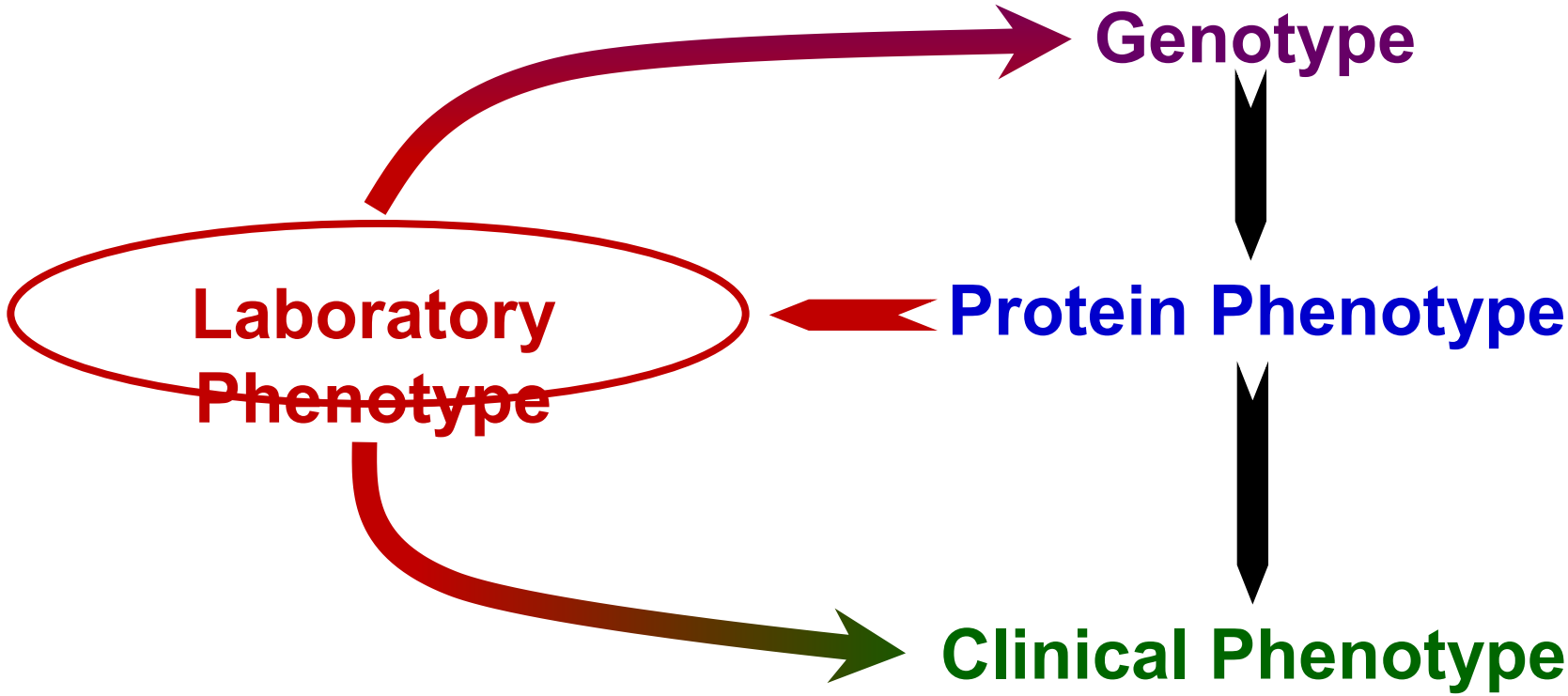
Testing Guidelines for AT, PC, PS and APC-R Assays

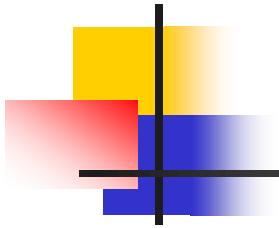
Outline

- Concept of Laboratory Phenotype
  - How it relates to Genotype & Clinical Phenotype
- General Recommendations
  - Pre-analytical & Post-analytical issues
- Protein C Testing
- Protein S Testing
- Antithrombin Testing
- APC-Resistance Testing

- Algorithms for Testing

Role of Laboratory Phenotype in Coagulation





General Recommendations for PC, PS, AT and APC-R Testing

General Recommendations for AT, PC and PS Assays

- **Pre-Analytical Variables must be controlled:**
  - Correct timing with respect to patient history
  - Testing during pathology or treatment
  - Specimen collection, processing & storage
- **Post-Analytical Processes must be appropriate:**
  - Interpretation of result is a COMPARATIVE DECISION-MAKING PROCESS
  - Correct Reference Interval
- **Repeat abnormal results**

Protein C Assays

Major Issues Associated with Testing

Protein C Deficiency
Quantitative and Qualitative Defects

- **Quantitative Defects (Type I):**
  - Major gene defect
  - Decrease of both activity and antigen
- **Qualitative Defects (Type II):**
  - Due to point mutation
  - Decrease of activity with normal antigen
- **Type II sub-types:**
  - Type IIa- defect in activation or active site
  - Type IIb- defect in cofactor or PL binding

Protein C Clotting & Chromogenic Assays

- **PC Clotting Assay:**
  - Measures activation, activity & cofactor/PL interactions
  - Clotting time proportional to PC concentration
  - Assay reagents vary significantly- may affect results
- **PC Chromogenic Assay:**
  - Measures activation & enzymatic activity only
  - Generated color proportional to PC concentration
  - Less interfering effects

Marlar & Gausman, IJLH 36:289, 2014

Type II Protein C Deficiency
Genetic Defects in Function

- 8-10% of Protein C deficiencies are Type II deficiencies.
- Clotting Assay: Most Type II defects are identified.
- Chromogenic Assay: 85-90% of Type II defects are identified.
  - Will miss defects in cofactor and PL binding.

Diagnosis of PC Deficiency
Testing and Genetic Defects

	Clotting Assay	Chromogenic Assay	PC Antigen
Normal	96%	98%	95%
Type I	42%	46%	40%
Type IIa	58%	53%	98%
Type IIb	48%	89%	94%

Protein S Assays

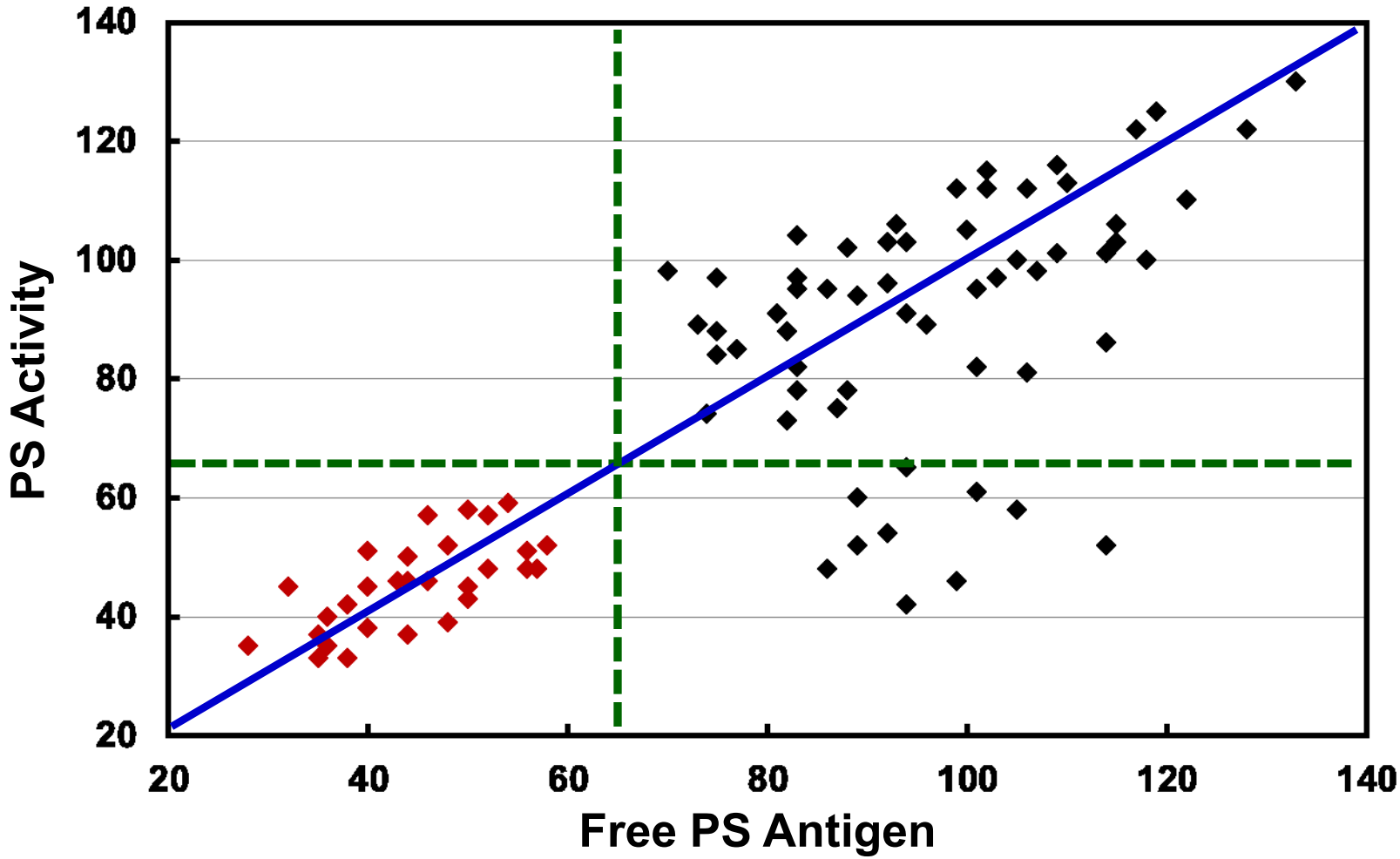
Major Issues Associated with Testing

Clinical and Laboratory Aspects of Protein S

- Complex interactions and functions of Protein S
- Deficiency classification is also complex
  - Type I, Type II and Type III
- 3 Types of Assays
  - Protein S Activity Assay: Clot Based
  - Free Protein S Antigen Assay: Monoclonal Antibody
  - Total Protein S Antigen Assay: Polyclonal Antibody

Marlar & Gausman, Am J Hematol 86:418, 20111
Marlar & Gausman, IJLH 36:289, 2014

Spurious Protein S Activity Levels in Non-PS Deficient Patients

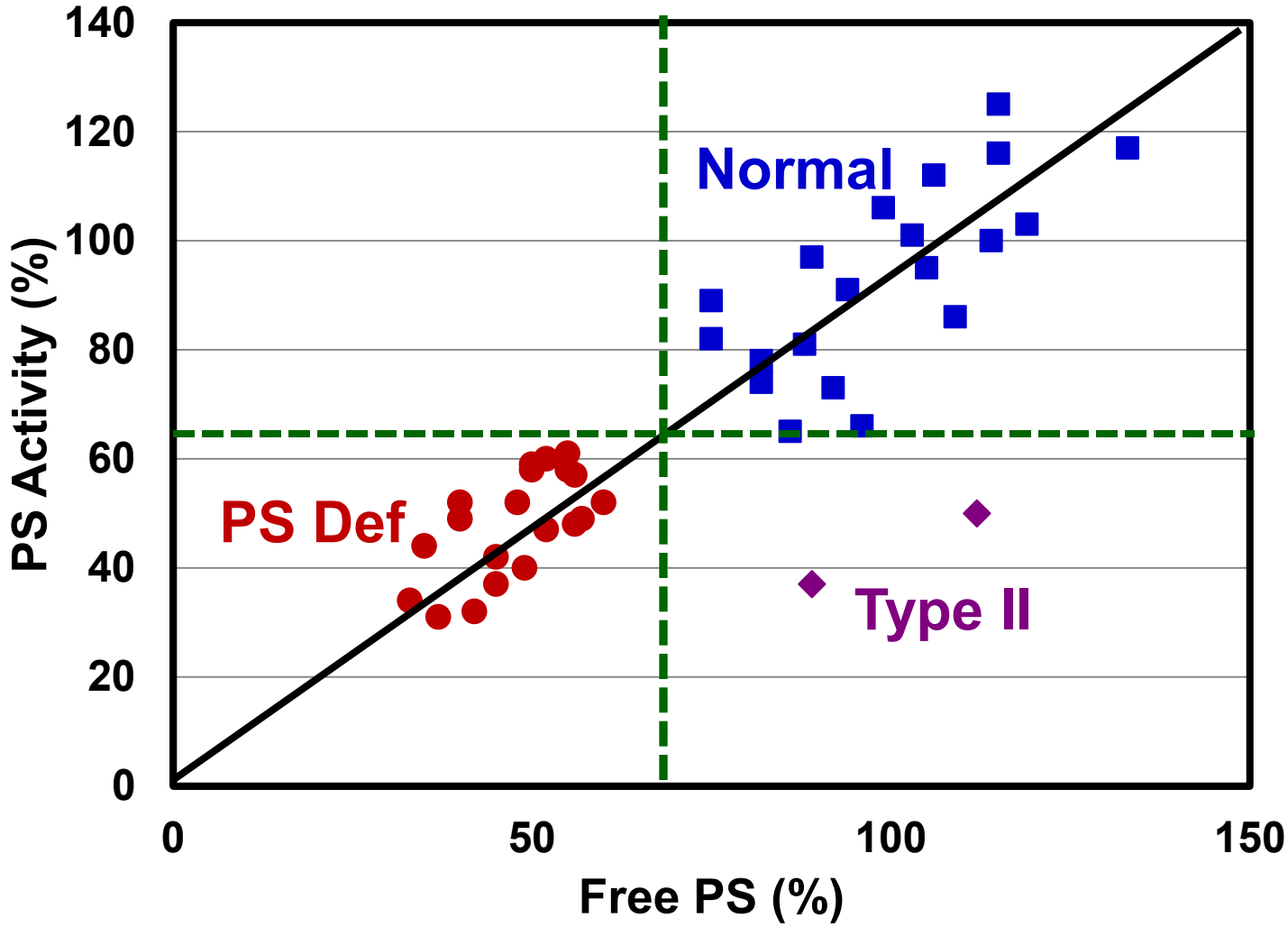


Protein S Activity Assays
Assay Inconsistencies

- False low PS activity compared to Free PS Ag
  - Assay #1: 11% of samples
  - Assay #2 14% of samples
  - Assay #3 16% of samples
  - Assay #4 19% of samples
- Unknown Cause of Decreased PS Activity
- On repeat, PS Act will normalize to Free PS value

Marlar, et al. J Clin Ligand, 2005

Comparison of Free PS Levels with PS Activity



Issues for Free PS Antigen Assay

- Free PS Antigen usually correlates with PS activity
- Potentially will miss Type II PS molecules (dysfunctional molecule)
- Estimated 1-3% of Protein S deficiencies

Issues for Total PS Antigen Assay

- Poor correlation of Total PS Assay with PS Act or Free PS Ag assays.
- Will miss numerous PS deficiencies.
- Increased cost with little or no additional clinical or mechanistic information.

Antithrombin Assays

Major Issues Associated with Testing

Diagnosis of Antithrombin Deficiency
Type I and Type II Defects

Quantitative Defects (Type I):

- Major gene defect- decreased activity & antigen (Ratio 0.7-1.3)
- Both thrombin & FXa assays detects Type I deficiencies

Qualitative Defects (Type II): (May be Difficult to Diagnose)

- Point mutation- decreased Act & normal Ag (Ratio 0.3-0.7)
- Values can be different between thrombin and Factor Xa
- Can lead to wrong diagnosis of "normal"
- Multiple sub-types

Laboratory Assays for Antithrombin

- Both Activity and Antigenic AT assays available
- Activity assays are chromogenic methodology
- Activity based using one of two enzymes
  - Factor Xa
  - Thrombin (human or bovine)
- Majority use heparin
- Assay Variables: incubation time, enzyme, buffers

Cooper, et al IJLH 33:227, 2011
Marlar & Gausman, IJLH 36:289, 2014

Comparison of Enzymes for Antithrombin Deficiencies

Enzyme and Antigen Levels

	Human Thrombin	Bovine Thrombin	Human FXa	AT Antigen
Normal	115%	99%	101%	102%
Type I	69%	52%	54%	54%
Type II-Reactive Site	--	67%	93%	87%

Cooper, et al IJLH 33:227, 2011
Marlar & Gausman, IJLH 36:289, 2014

APC- Resistance Assays

Major Issues Associated with Testing

APC-Resistance
Mechanism

- Inability of patient's plasma to be inhibited by Activated Protein C (APC)
- Major genetic mechanism is: Factor V_{Leiden}
- Other mechanisms of APC-R:
  - Elevated factor VIII levels
  - Elevated factor II and fibrinogen levels
  - Decreased PS levels
  - Minor genetic mutations in factor V

Factor V_{Leiden}
Genetic Mechanism

- **Molecular Cause:**

  Mutation of an APC cleavage site in Factor V

- **Mutation Site:** (DNA and protein sequence)

  Amino Acid: Arg_{506} → Gln_{506}

  Nucleotide: CGA → CAA

- **Molecular Function:**

  APC cleaves Arg_{506} - Gly_{507} bond.

  Mutation: Gln_{506} - Gly_{507} bond not cleaved.

  FVa remains active generating more thrombin.

APC-Resistance Clotting Assay

2nd Generation Assay

- Patient plasma diluted in FV def plasma to make specific.
- Either APTT or RVVT based assay.
- Add APC and saline to diluted plasma.
- Perform clotting assay.
- Normalized ratio of APC/saline clotting times determined.
- Results:
  - Wild type factor V ratio: >2.0
  - Factor V_{Leiden} ratio: <2.0

APC-R and Factor V_{Leiden} Second Generation Test

	APC-R Test *Positive*	APC-R Test *Negative*
FV_{Leiden} DNA *Positive*	123	0
FV_{Leiden} DNA *Negative*	0	985

Summary of Recommendations

PC, PS, AT and APC-R Testing
Cost Effective Protocol

- Protocol should be based on:
  - Prevalence of deficiency
  - Understanding of the mechanisms
- Must know the status of patient
- Draw & process the samples properly
- Know the assay limitations
  - What is the measured molecule or domain?
  - What interfering substances affect results?
- Use appropriate assays:
  - Activity, Antigen or Genetic

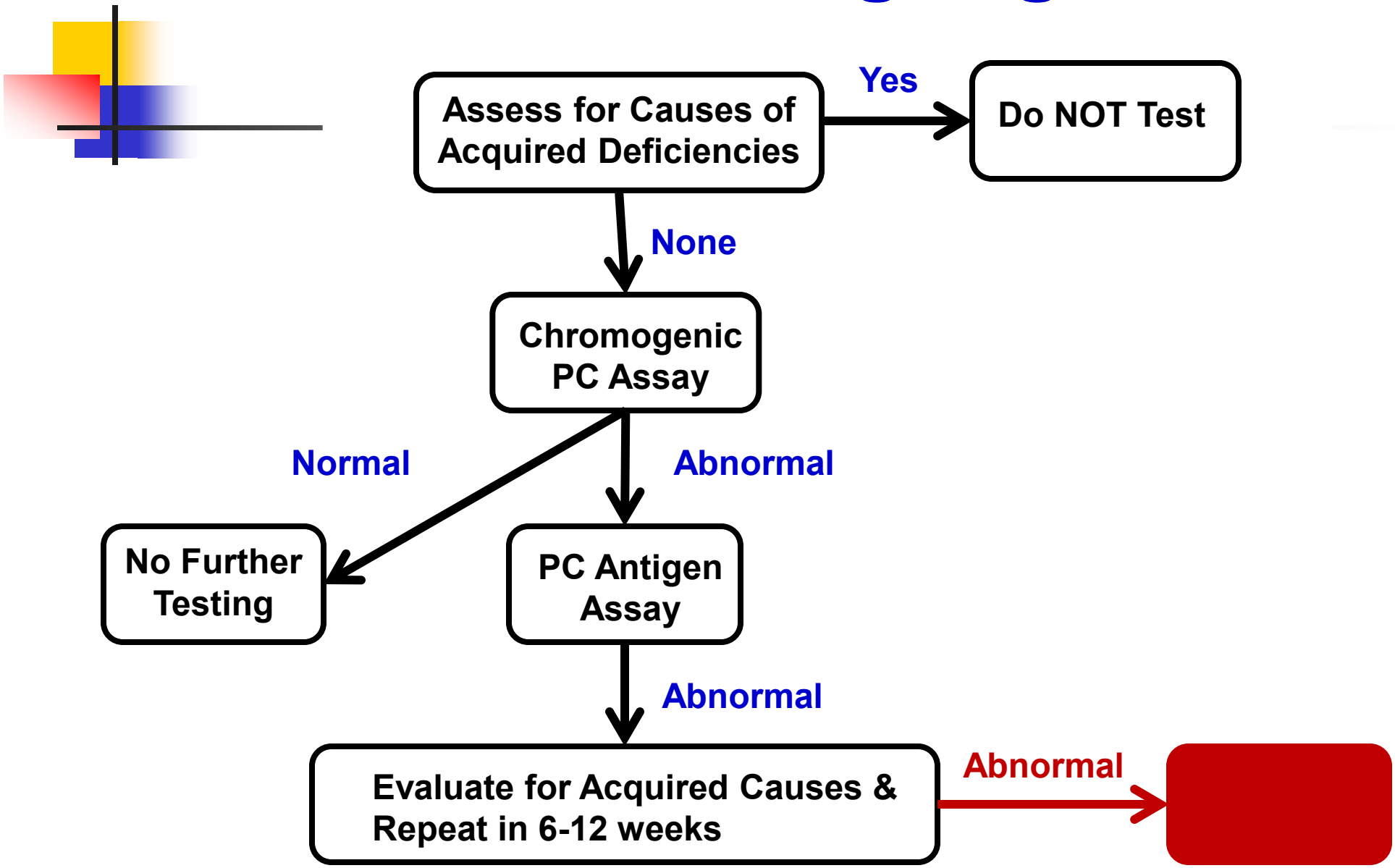
PC, PS, AT and APC-R Testing: Recommendations

- All test kits measure the same analyte.
- However, not all measure analyte the same.

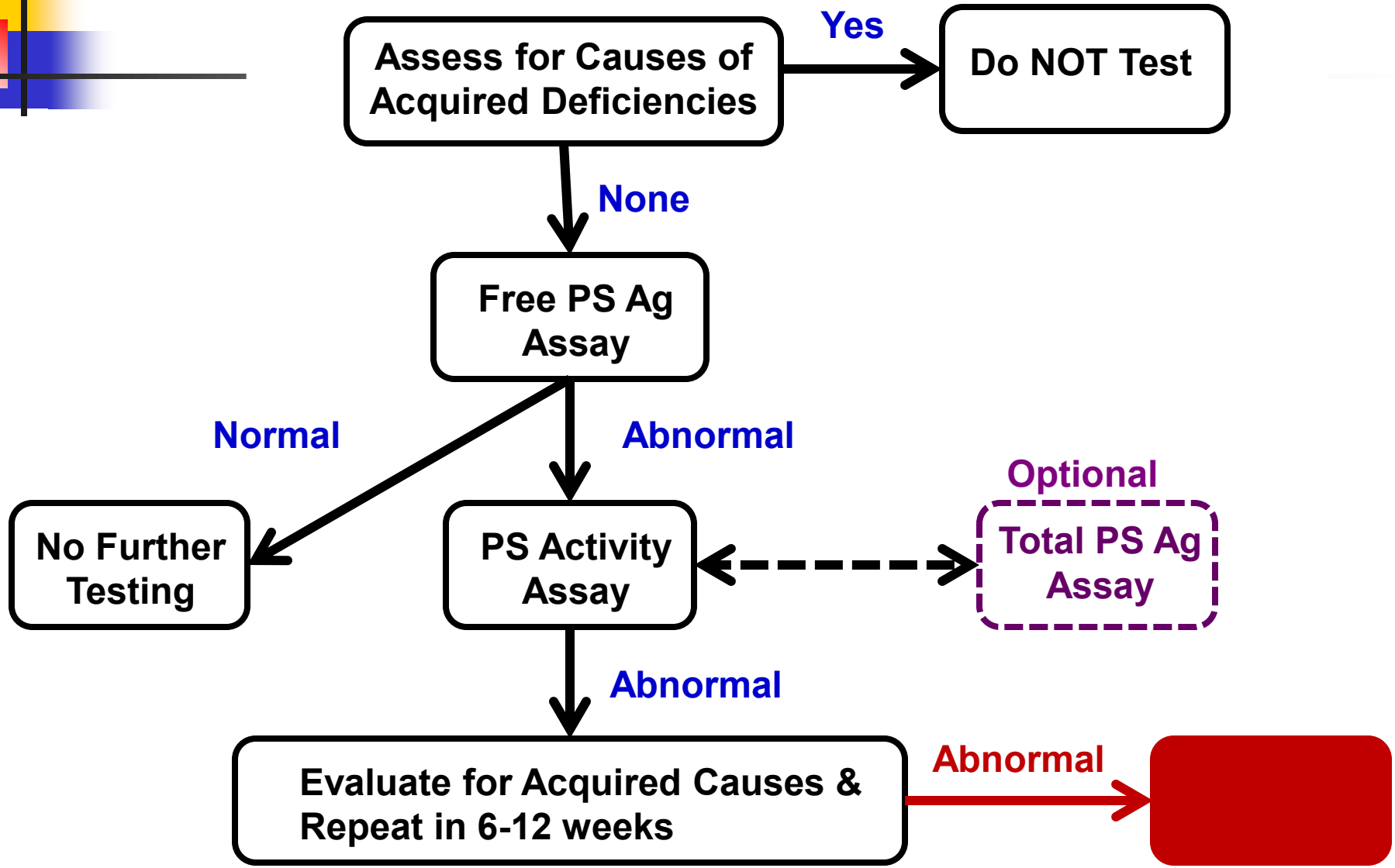
So…..

- Question an assay result:
  - If borderline normal.
  - Has variability on duplicates or repeating.
- Repeat questionable results.

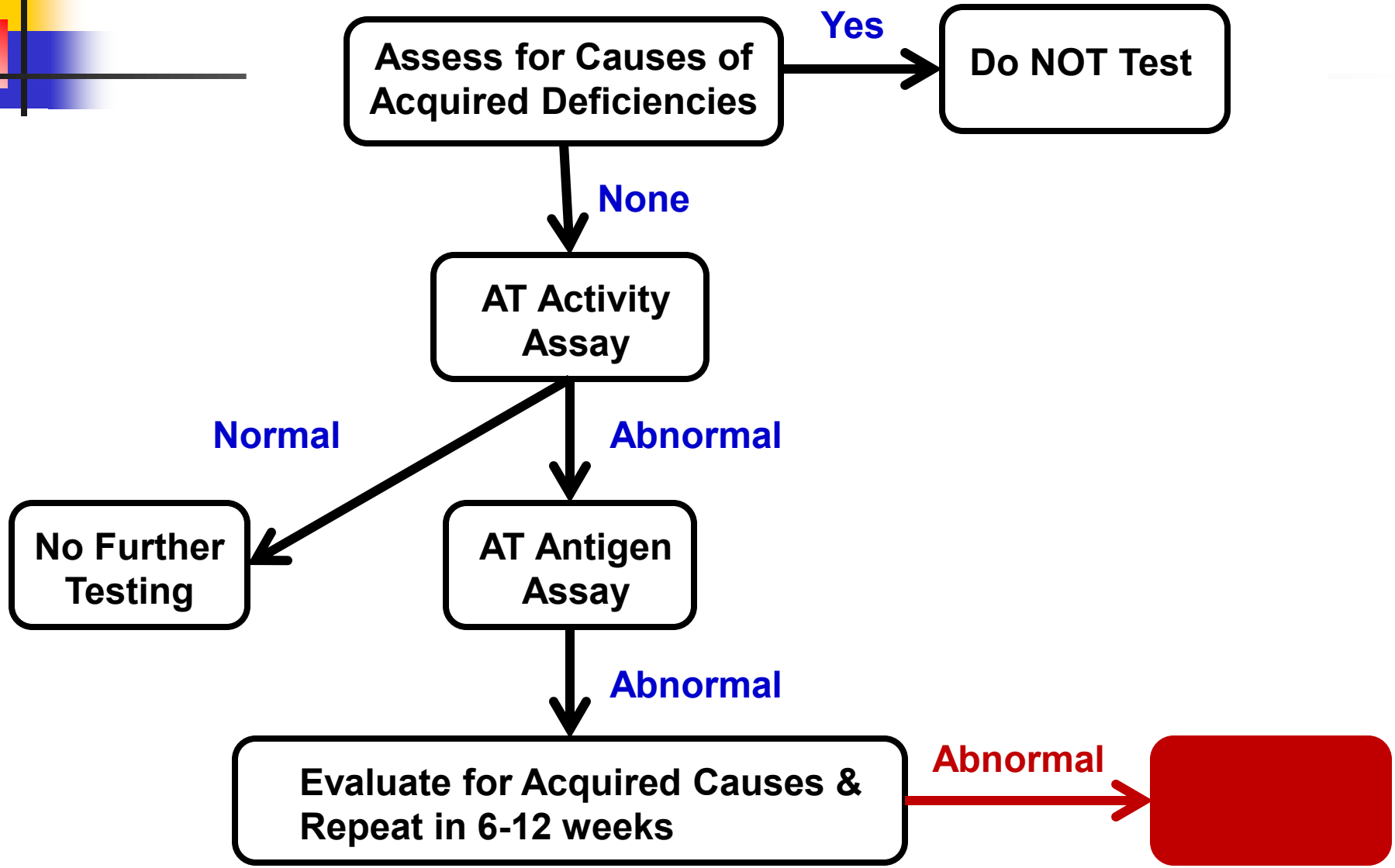
Protein C Testing Algorithm



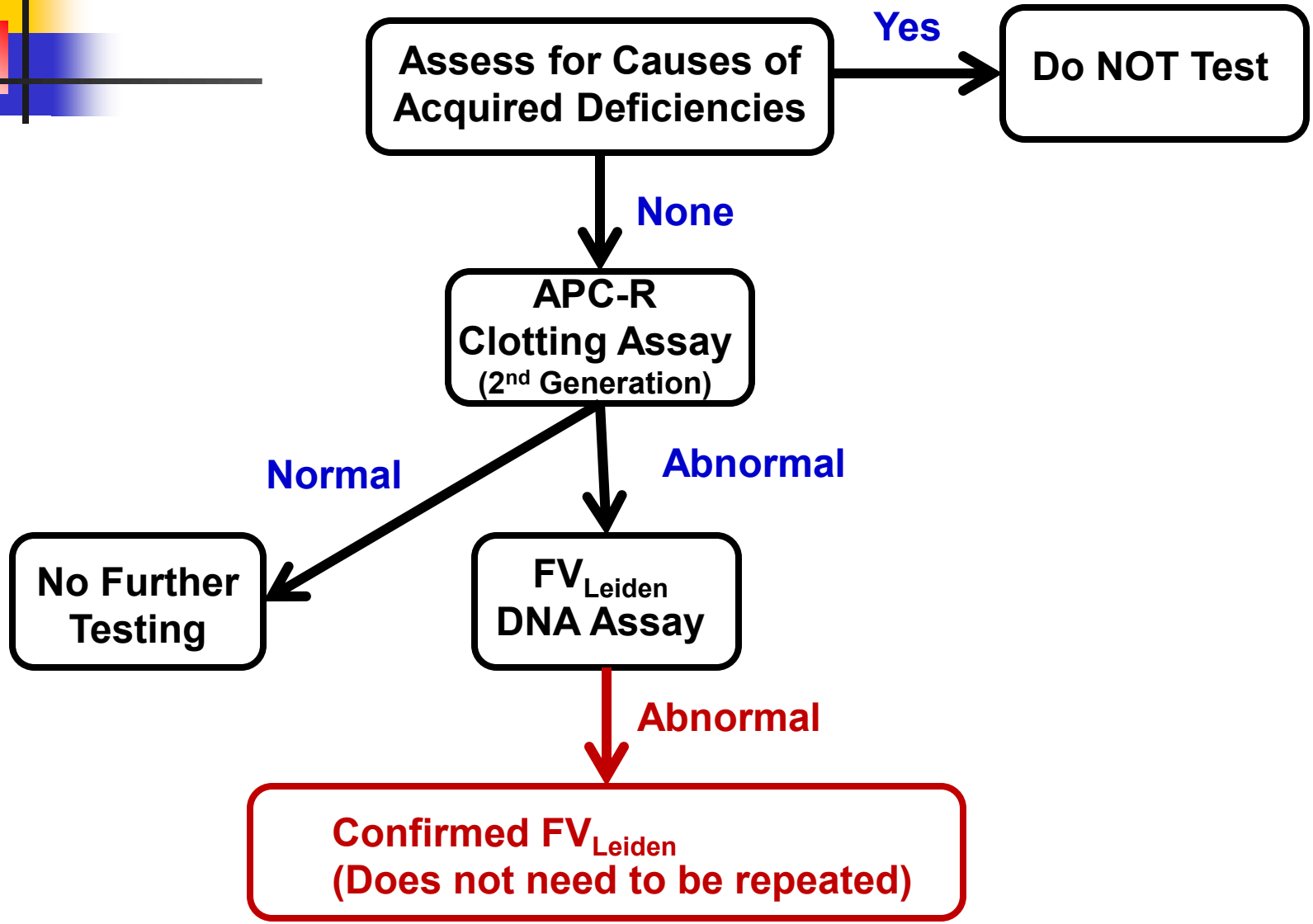
Protein S Testing Algorithm



Antithrombin Testing Algorithm



APC-R Testing Algorithm



Will Deficiencies Be Missed With Proposed Protocols?

- **Protein C Assays:**
  - **Chromogenic: 1.2% of deficiencies**
  - **Clotting: 0.5% of deficiencies**
  - **Antigen: 14.0% of deficiencies**

  Clotting Assay is more costly and more interfering substances

- **Protein S Assays:**
  - **Free Protein S Ag: 1.5% of deficiencies**
  - **Protein S Activity: 0.7% of deficiencies**
  - **Total Protein S Ag: 18.0% of deficiencies**

  PS Activity Assay has 10-15% false positive and more costly

Will Deficiencies Be Missed With Proposed Protocols?

- **Antithrombin Assay:**
  - **Bovine Thrombin:** 0.5% of deficiencies
  - **Factor Xa:** 0.7% of deficiencies
  - **Human Thrombin:** 2.5% of deficiencies

  Human Thrombin has interference by Heparin Cofactor II

- **APC-Resistance Assay:**
  - **2nd Generation method:** <0.2% of mutations
  - **1st Generation method:** 6% of mutations

  1st Generation has too many interfering substances

Testing Panel for Thrombophilia
Initial Tests and Reflex Tests

- **Protein C-** → **PC Chromogenic Assay**
  - If abnormal: → PC Antigen Assay
- **Protein S-** → **Free PS Antigen Assay**
  - If abnormal: → PS Activity Assay
- **Antithrombin-** → **AT Activity Assay**
  - If abnormal: → AT Antigen Assay
- **APC-R-** → **2nd Generation Assay**
  - If abnormal: → FV_{Leiden} DNA Analysis

Testing for Thrombophilia
Bottom Line

- Must know your patient
- Must have specimen & sample integrity
- Use the appropriate initial assay
- Must know the assay limitations
- NO assay is 100% diagnostic
- Must know Reference Interval
- Must confirm abnormal results

Questions??