MAYO CLINIC
Mayo Medical Laboratories

Molecular Testing Applications in Coagulation

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- **Relevant Financial Relationship(s): NONE**
- **Off Label Usage: NONE**

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Objectives

- Basic introduction of molecular genetics related to coagulation
- Algorithmic approach and molecular testing in thrombosis
- Algorithmic approach and molecular testing in bleeding

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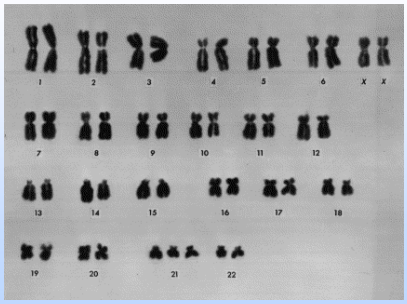
Human Genome – Chromosome Analysis



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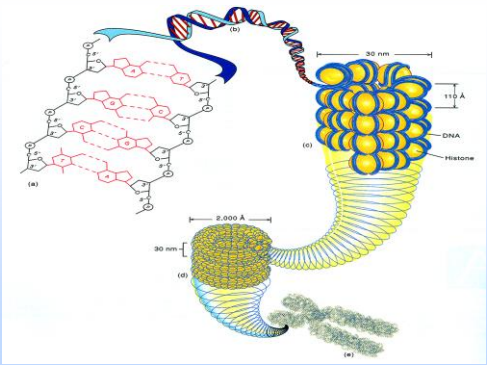

Zubay. Biochemistry. 3rd Ed., Wm. C. Brown, 1993

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Genetic Mutations

- Alterations in DNA sequence
  - Neutral – Does NOT cause disease
    - Polymorphisms
    - Intron / non-coding regions
  - Deleterious – Disease causing
    - Affect structure or function
    - Exon / coding regions

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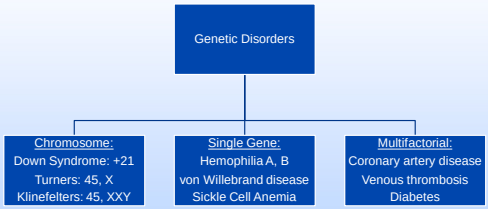
Types of Genetic Disorders



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Mutation Types

- Missense

WT: Gly Gly Ser Cys (amino acid)
GGG GGC AGT TGT (DNA)
Mut: Gly Ser Ser Cys
GGG AGC AGT TGT

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Mutation Types

- Missense
- Nonsense

WT: Gly Gly Ser Cys (amino acid)
GGG GGC AGT TGT (DNA)
Mut: Gly Ser Ser Stop
GGG AGC AGT TGA

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Mutation Types

- Missense
- Nonsense
- Deletions

WT: Gly Ser Ser Cys (amino acid)
GGG AGC AGT TGT (DNA)
Mut: Gly Ser Val Gly
GGG AGC GTT GTG

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Mutation Types

- Missense
- Nonsense
- Deletions
- Insertions

WT: Gly Ser Ser Cys (amino acid)
GGG AGC AGT TGT (DNA)
Mut: Gly Ser Ser Trp
GGG AGC AGT TGG T

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Mutation Types

- Missense
- Nonsense
- Deletions
- Insertions
- Inversions

WT: Pro Glu Glu Cys Gly (amino acid)
GGT CTC CTC ACG CCA (DNA)
Mut: Pro Gly Glu Cys Gly
GGT CCT CTC ACG CCA

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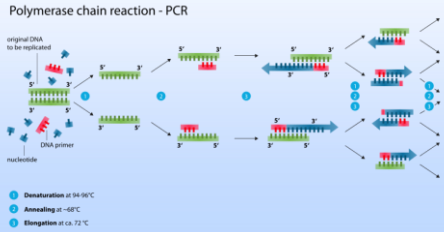
Molecular Testing Methods

- Utilize Polymerase Chain Reaction (PCR) to detect alterations at DNA level



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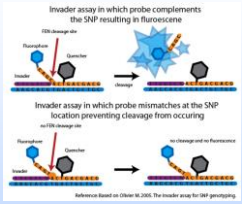
Molecular Testing Methods

- Single Nucleotide Polymorphisms
- Flap endonuclease (FEN) - Invader Plus chemistry
- Measure fluorescence ratios



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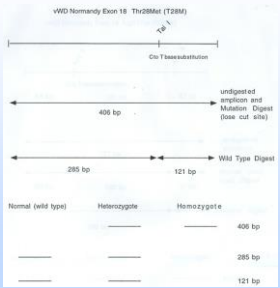
Molecular Testing Methods

- Single Nucleotide Polymorphisms
- Restriction Fragment Length Polymorphism (RFLP)
- Gel Electrophoresis detection



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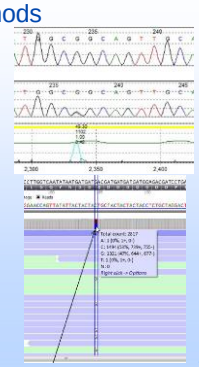
Molecular Testing Methods

- Full Gene Sequencing
- SNPs, small deletions/duplications/ insertions
- Sanger sequencing or next-generation sequencing (NGS)



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Molecular Testing Methods

- Deletion and Duplication Analysis
- Identifies large, exonic or whole-gene deletions
- Multiplex ligation-dependent probe amplification (MLPA)

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Genetic Inheritance Patterns

- Autosomal Dominant - *Factor V Leiden (FVL)*
  - Each affected person has an affected parent
  - Occurs in every generation
- Autosomal Recessive - *von Willebrand Disease Type 2N*
  - Both parents of an affected person are carriers
  - Not typically seen in every generation
- X-Linked Dominant - *Rett syndrome*
  - Females more frequently affected
  - Can have affected males and females in same generation
- X-Linked Recessive - *Hemophilia A/B*
  - Males more frequently affected
  - Affected males often present in each generation

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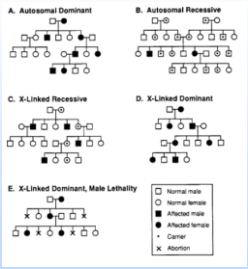
Pedigree



Recommend genetic counseling prior to any genetic testing

Fred Levine, in Fetal and Neonatal Physiology (Third Edition), 2004.

Thrombophilia

- Venous thrombosis (VT)
- Estimated 60,000 deaths each year
- US incidence of VT is estimated to be 1.2 cases per 1,000 persons per year
- Approximately 200,000 new cases of VT are diagnosed in the US each year
- 30-day mortality with pulmonary embolism (PE) is 12%, Deep vein thrombosis (DVT) is 6%

Risk Factors in Thrombosis

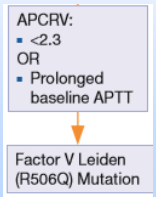
Acquired	Hereditary
• Pregnancy	• Factor V Leiden
• Major surgery	➢ Up to 20% w/ DVT
• Oral contraceptives	• Prothrombin G20210A
• Immobilization	➢ Up to 6% w/ DVT
• Smoking	• Antithrombin, Protein C, Protein S deficiency
• Obesity	➢ Very low frequency

When to consider thrombophilia testing?

- If you have a family history of blood clots or known mutational status
- If you have had one or more blood-clotting incidents without an apparent cause
  - Spontaneous venous thromboembolism (VTE) if you are under the age of 50
- Selective screening or at the presentation of VTE generally not recommended

Factor V Leiden (FVL)

- Start with Activated Protein C Resistance (APCR) testing
- Reflex to FVL mutation testing when APCR ratio is abnormal
- Approximately 90% of APC resistance patients have FVL
- Most common inherited risk factor for VT



Prothrombin G20210A

- Perform mutation testing on patients with clinically suspected thrombophilia
- Associated with a 3-fold increased risk of VTE
- No other methods of detecting risk
- G20210A is a common polymorphism in the *F2* gene
  - Affects 1.5%-3% of Caucasian Americans
  - Uncommon in African Americans

Antithrombin/Protein C/Protein S

- Perform testing when:
  - Protein activity levels are reduced (75-80% from normal)
  - Acquired causes have been excluded
- Hereditary deficiencies are rare
- AT deficiency = up to 16-fold increase in risk of VTE and may manifest heparin resistance
- Acquired deficiencies of protein C and protein S may occur in association with vitamin K deficiency

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Thrombosis Molecular Testing

Deficiency / Gene	Mutations Detected	Method
Factor V Leiden	R506Q - SNP	FEN Invader Plus
Prothrombin/*F2* gene	G20210A - SNP	FEN Invader Plus
Antithrombin/ *SERPINC1*	SNP, insertions, deletions	Sequencing
Protein C	SNP, insertions, deletions	Sequencing
Protein S	SNP, insertions, deletions	Sequencing

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Bleeding Disorders

- Hemophilia is a bleeding disorder that slows the blood clotting process
- Serious complications can result from bleeding into the joints, muscles, brain, or other internal organs
- Major types of hemophilia include hemophilia A (factor VIII) deficiency and hemophilia B (factor IX) deficiency with X-linked inheritance
  - Hemophilia A – *F8* gene
  - Hemophilia B – *F9* gene

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When to consider hemophilia testing?

- Protein studies show decreased activity levels
- When you have a symptomatic male patient with low factor VIII or factor IX levels
  - Soft tissue bleeding and articular hemorrhage
  - Deep-muscle bleeding or intracranial bleeding
  - Prolonged oozing after surgery
  - Abnormal activated partial thromboplastin time (aPTT)
- When you have a female patient with a confirmed or reported family history of hemophilia or abnormally low factor VIII or factor IX levels

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Hemophilia A

- X-linked recessive bleeding disorder leading to a deficiency of clotting factor VIII
- Affects approximately 1 in 5,000 males
- There are three levels of severity: Factor VIII levels of:
  - 6-40% is mild
  - 1-5% is moderate
  - <1% is severe

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Hemophilia A Mutation Analysis Assays

- 98% of patients have a mutation in *F8* gene
  - Approximately 50% of severe Hemophilia A cases have a mutation breaking intron 22
  - Approximately 5% of severe Hemophilia A cases have a mutation breaking intron 1
  - Smaller point mutations cause 43% of severe mutations
  - Large gene deletions represent 6% of severe mutations

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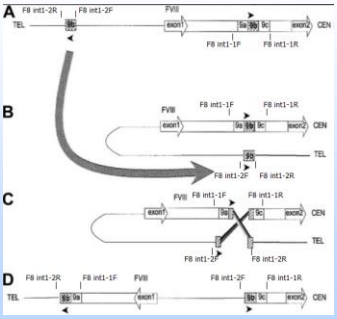
F8 Intron 22/1 Inversion Mutation Analysis



Bagnall et al. 2002

Hemophilia A (*F8*) Testing Algorithm

- Males w/ Severe HA
- Females w/ family history of Severe HA or unkn severity

- Males w/ Mild/Moderate HA
- Females w/ family History of Mild/Moderate HA

F8 Int22/1 Inv Analysis

Positive → Stop, Report Results

Negative, Report Results → *F8* Gene, Sanger/NGS

Positive → Stop, Report Results

Negative, Report Results → MLPA (Del/Dup) for *F8* Gene → Report Results

Hemophilia B

- Hemophilia B (factor IX deficiency) is an X-linked recessive bleeding disorder with an incidence of ~1 per 30,000 live male births
- Classified into severe, moderate and mild
  - Mild 6-40%
  - Moderate 2-5%
  - Severe has FIX activity levels <1%
- Severity depends on the molecular alteration detected

Hemophilia B Mutation Analysis

- Inhibitors to factor IX activity occur in 5-8% of patients and correlates with genotyping
- Sequencing utilized to detect the majority of *F9* mutations
- Del/Dup analysis will detect the remaining approximately 3% of mutations in hemophilia B

von Willebrand Disease (VWD)

- Caused by quantitative or qualitative defects in von Willebrand factor (VWF)
- VWF is a carrier protein for factor VIII
  - Exclude the possibility of VWD with reduced factor VIII activity
  - Often misdiagnosed as having hemophilia A
- VWD is classified into three types:
  - Type 1 is a mild bleeding disorder
  - Type 2 is of variable severity
  - Type 3 is a severe disorder

von Willebrand Type 2 Normandy

- Type 2 is the most common type
  - Further differentiated into 4 subclasses: 2A, 2B, 2M and 2N
- Inherited as an autosomal recessive disorder
- Three mutations in the FVIII binding domain of VWF account for 96% of all mutations associated with VWD type 2N
  - Exon 18, 19 and 20
  - Perform RFLP

Bleeding Molecular Testing

Deficiency / Gene	Mutations Detected	Method
Hemophilia A / *F8*	Inv22/1, SNP, insertions, deletions, duplications	IS-PCR, Sequencing, MLPA
Hemophilia B / *F9*	SNP, insertions, deletions, duplications	Sequencing, MLPA
von Willebrand Factor type 2 Normandy	SNP, insertions, deletions, duplications	RFLP, Sequencing (rare)

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Questions & Discussion

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