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PEARLS OF LABORATORY MEDICINE

Targeted Mutation Analysis

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Outline

I. Purpose of targeted mutation analysis

II. Disorder types for molecular mutation testing

III. Molecular mutation testing specimen types

IV. Genetic variant types and molecular mutation testing methods

Purpose of targeted mutation analysis

Diagnostic testing

- Confirm a suspected diagnosis

Predisposition testing

- Determine likelihood of developing a disease

Carrier testing

- Detect carrier mutations in unaffected individuals

Predictive testing

- Predict response to therapy

Prognostic testing

- Characterize the course of a disease in an untreated individual

Prenatal or Preimplantation testing

- Detect mutations in a fetus or embryo

Disorder types for molecular mutation testing

Inherited disorders

- Cystic fibrosis
- Fragile-X
- Congenital hearing loss
- …etc

Acquired disorders

- Hematopoietic malignancies
- Lymphoid malignancies
- Solid tumor malignancies

Molecular mutation testing specimen types

Inherited disorders

- Peripheral blood
- Saliva (buccal cells)
- Aminocytes
- Skin

Acquired disorders

- Tissue
  - Formalin-fixed paraffin embedded (FFPE)
  - Fresh/Frozen
- Cell-free DNA (cfDNA) and circulating tumor DNA (ctDNA)
- Bone marrow
- Fluid
- Peripheral blood

- **Issues**: compatible anticoagulants (EDTA/ACD); tumor percentage; heterogeneity; integrity

Genetic variant types and molecular mutation testing methods

Single nucleotide variants

- PCR, NGS/Sanger sequencing

Small insertions/deletions

- PCR, NGS/Sanger sequencing

Large insertions/deletions

- MLPA, Southern blot hybridization, microarrays, NGS

Structural variants

- MLPA, Reverse-Transcription PCR, microarrays, NGS

Molecular mutation testing methods: Outline

I. Polymerase Chain Reaction (PCR)

- Allele-Specific PCR
- Reverse Transcription PCR
- Real-Time PCR
- Melting curve analysis
- Methylation-sensitive PCR
- Multiplex Ligation-Dependent Probe Amplification (MLPA)

II. Southern blot hybridization

III. Microarray

- Comparative Genome Hybridization

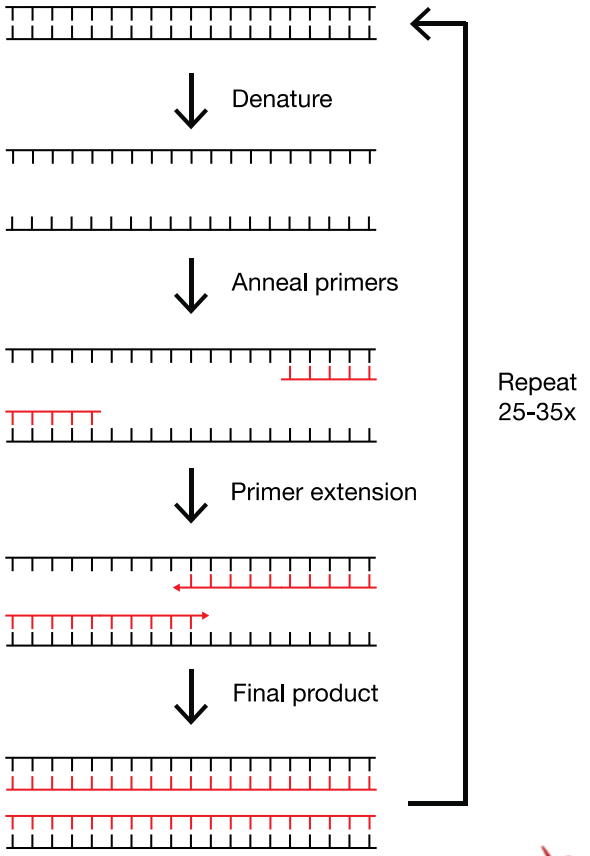
IV. Sequencing

- Sanger sequencing
- Next Generation Sequencing (NGS)

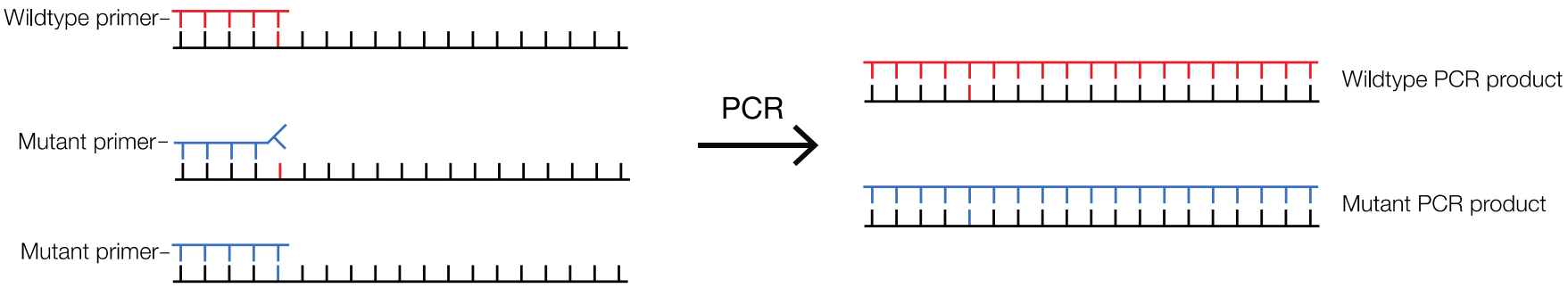
Molecular mutation testing methods: Polymerase Chain Reaction (PCR)

Components

- Template
  - DNA/cDNA
- Oligonucleotide primers
  - 18-22bp in length
  - Typically anneal to flank target of interest on opposite template strands
- Deoxynucleotide triphosphates
  - dATP, dTTP, dCTP, dGTP
- Taq DNA polymerase
  - Thermostable enzyme

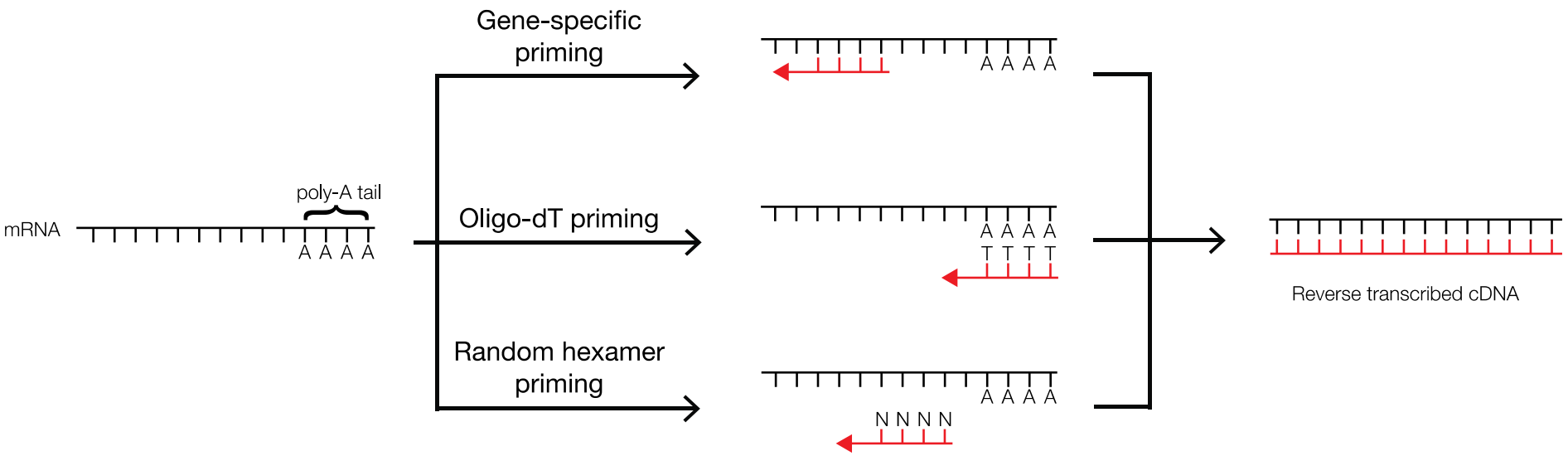


Molecular mutation testing methods: Allele-Specific PCR (AS-PCR)



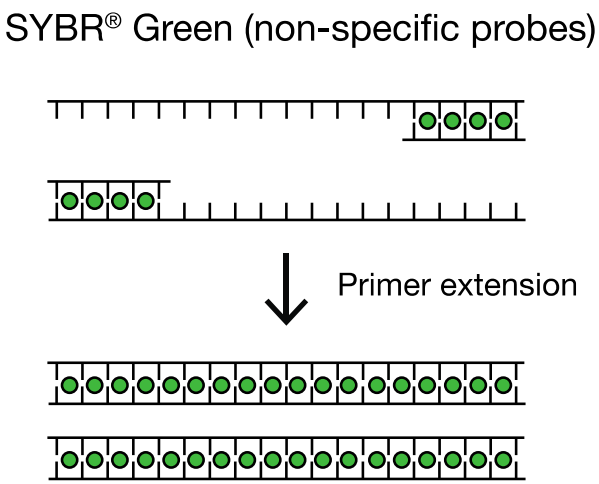
- Used for single nucleotide variant detection; also known as amplification refractory mutation system (ARMS)
- Target DNA is amplified with two primer pairs that share a single common primer and an allele-specific primer (normal or mutant)
- 3’ mismatch of the primer with the DNA template will prevent PCR amplification

Molecular mutation testing methods: Reverse Transcription PCR (RT-PCR)



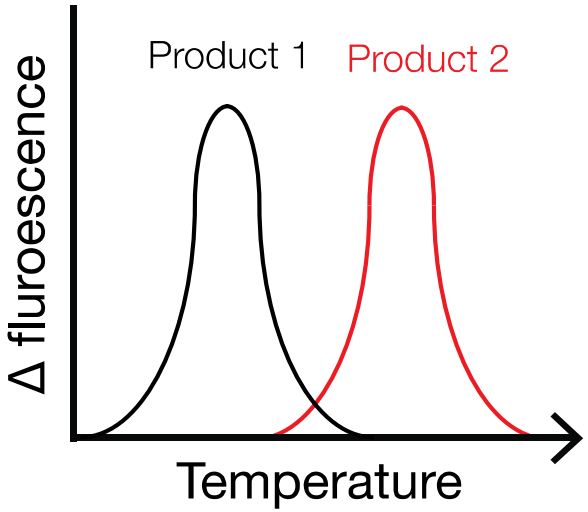
- Method to detect RNA transcripts
- Converts RNA to complimentary DNA (cDNA) using ‘reverse transcriptase’
- cDNA is used as a template for downstream PCR amplification

Molecular mutation testing methods: Real-Time PCR (quantitative PCR/qPCR)



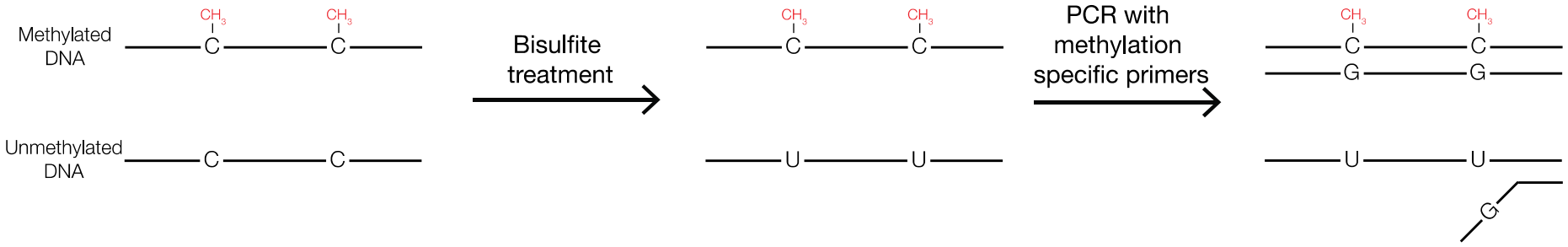
- Measures abundance of DNA/cDNA/RNA molecules in “real time” during PCR
- Special thermocyclers monitor amount of product during amplification
- Common approaches include: 1) non-specific dyes that intercalate with DNA; 2) sequence specific fluorescently labelled probes

Molecular mutation testing methods: Melting curve analysis



- Method to detect PCR products from different alleles using fluorescent probes binding to sequence of interest
- Regions lacking probe complementarity denature at lower melting temperatures (T_m)

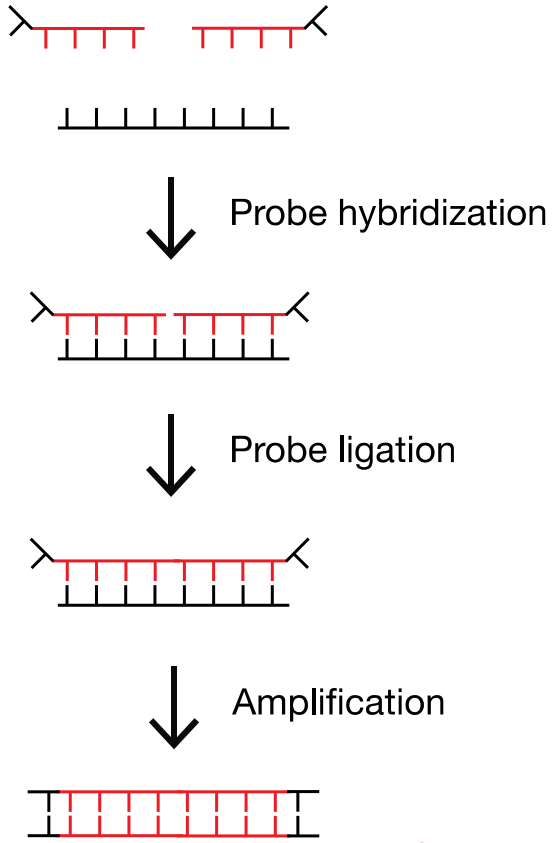
Molecular mutation testing methods: Methylation-sensitive PCR



- Method for detecting methylated DNA
- DNA treated with sodium bisulfite converts cytosine to uracil, but leaves 5-methylcytosine unchanged
- Methylation specific PCR primers can be used to detect methylation status

Molecular mutation testing methods: Multiplex Ligation-Dependent Probe Amplification (MLPA)

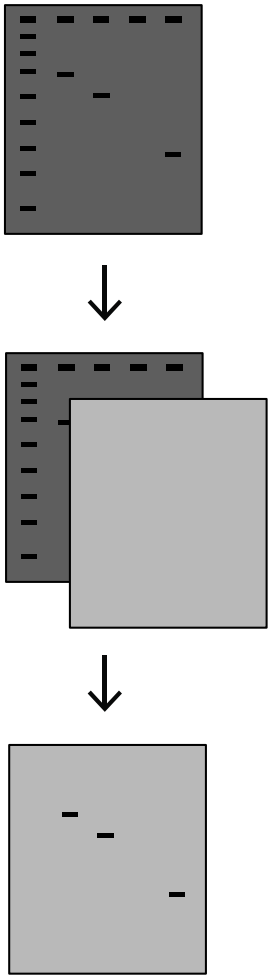
- A PCR variant used to detect large INDELs and structural variants
- dsDNA is denatured and hybridized with immediately-adjacent probes for the region of interest
- Adjacent probes are ligated, and PCR amplified using fluorescently labelled primers
- Only regions containing hybridized adjacent probes are detected



Molecular mutation testing methods: Southern blot hybridization

- Method to detect large structural variants too big for normal PCR

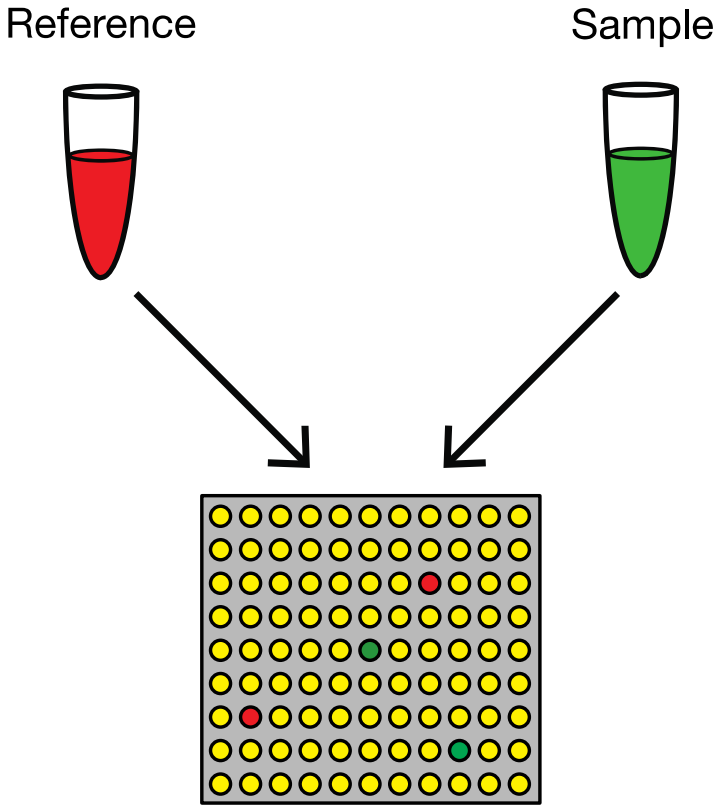
1. Digest high molecular weight DNA with restriction enzymes
2. Run digested DNA on an agarose gel
3. Denature to ssDNA
4. Transfer DNA to membrane with sodium hydroxide, and affix with heat or UV radiation
5. Hybridize membrane with labelled probes, wash, and expose to film



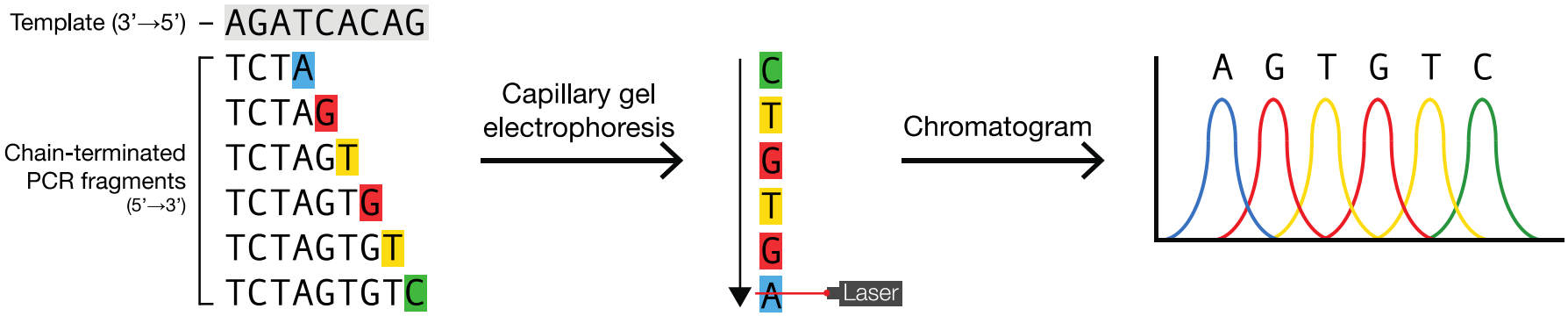
Molecular mutation testing methods: Microarrays

- Fixed DNA probes are bound to array; complementary DNA molecules from sample bind to probes and fluoresce
- Array-Based Comparative Genomic Hybridization (aCGH):

1. Specimen and reference DNA hybridized to microarray with known genomic probes
2. Ratio of specimen:reference intensities used to determine copy number



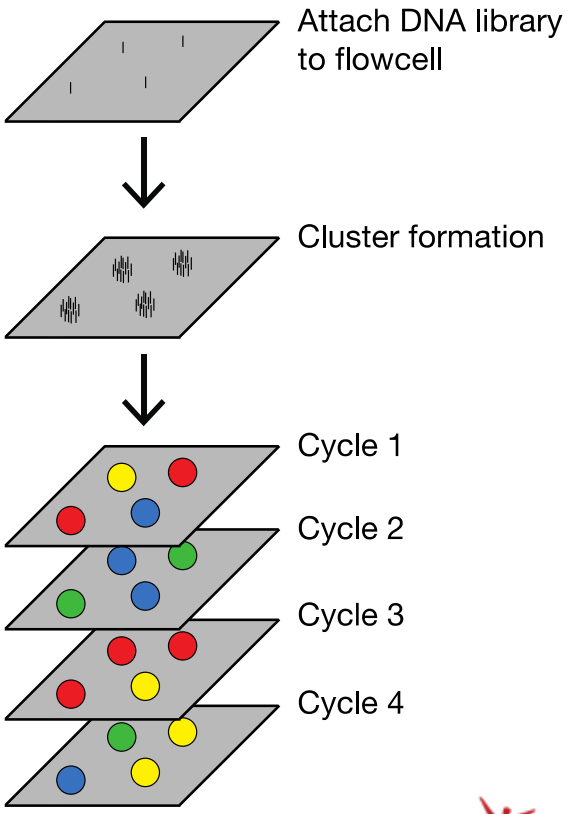
Molecular mutation testing methods: Sanger sequencing



- DNA is PCR amplified with unlabelled dNTPs and fluorescently-labelled ddNTP terminators, producing PCR fragments of varying length
- PCR products are fractionated via electrophoresis in capillary tube and detected as they sequentially pass by laser
- Chromatogram peaks represent each PCR product of a specific length

Molecular mutation testing methods: Next Generation Sequencing (NGS)

- Several NGS methods exist to sequence millions of DNA molecules simultaneously in parallel; Illumina is the most prevalent
- Illumina: DNA sequencing libraries are bound to a sequencing flow cell, amplified into identical clusters, and sequenced by incorporating fluorescent dNTPs in real time
- Short sequencing reads are mapped to the human genome followed by variant calling



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