PCR Basics and Applications

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➢Genetic Materials

DNA double helix

2 nm

Core of eight histone molecules

"Tails" protrude from histones and allow them to interact with other molecules in the nucleus.

Nucleosomes form "beads" on a DNA "string."

Histone H1

"Linker" DNA

Nucleosome

Nucleosomes pack into a coil that twists into another larger coil, and so forth, producing condensed, supercoiled chromatin fibers.

DNA wraps around histones, forming a vast number of nucleosomes.

30 nm

Chromatin

300 nm

The fibers fold to form loops.

700 nm

The loops coil even further, forming a chromosome.

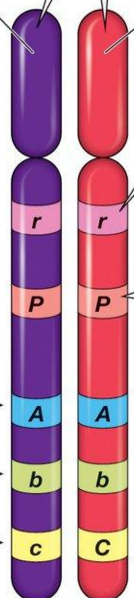
1400 nm

Metaphase chromosomes

In a pair of homologous chromosomes, one is inherited from the male parent, and the other from the female parent.

Paternal homologue

Maternal homologue



A genetic locus is the location of a particular gene on a chromosome.

At each genetic locus, an individual has two alleles, one on each homologous chromosome.

AA = Homozygous dominant

bb = Homozygous recessive

Cc = Heterozygous

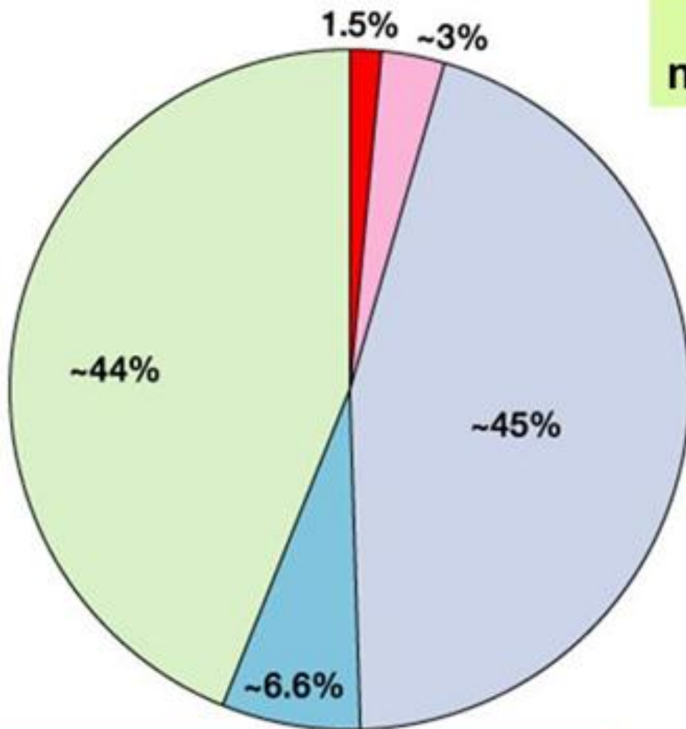
Three gene pairs at three different loci

➢ History of Genes and Related Techniques

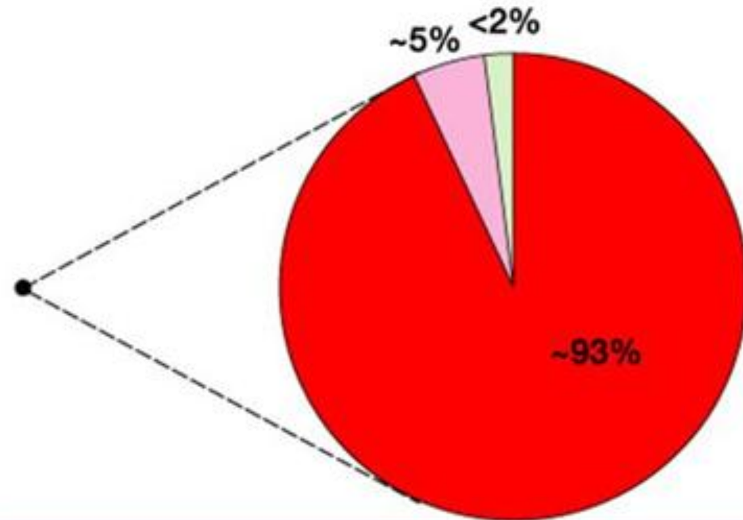
- **1865:** Hereditary Transmission, Mendel
- **1869:** DNA isolated, Miescher
- **1879:** Mitosis, Flemming
- **1909:** Gene, Johannsen
- **1953:** Double DNA Helix, Watson and Crick
- **1955:** Human Chromosomes, Tjio
- **1966:** Genetic Code
- **1977:** Sanger Sequencing
- **1980:** First Genome Sequenced
- **1983:** PCR
- **1992:** Digital PCR
- **1995:** Microarray
- **2005:** Next Generation Sequencing

➢ Human Genome

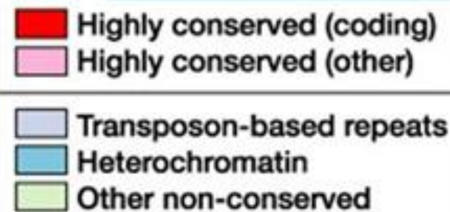
Human genome =
nuclear genome + mitochondrial genome



HUMAN
NUCLEAR GENOME
24 chromosomes (haploid)
3200 Mbp
30,000 genes



Mitochondrial genome
16569 bp
37 genes



Mitochondial Genome consists of 2 rRNA, 22 tRNA and 13 polypeptides genes

➢ DNA vs RNA

Cytosine

Guanine

Adenine

Thymine

Nitrogenous Bases

ATCG's

Base Pair

Sugar Phosphate Backbone

DNA

Deoxyribonucleic Acid

AUCG's

RNA

Ribonucleic Acid

Cytosine

Guanine

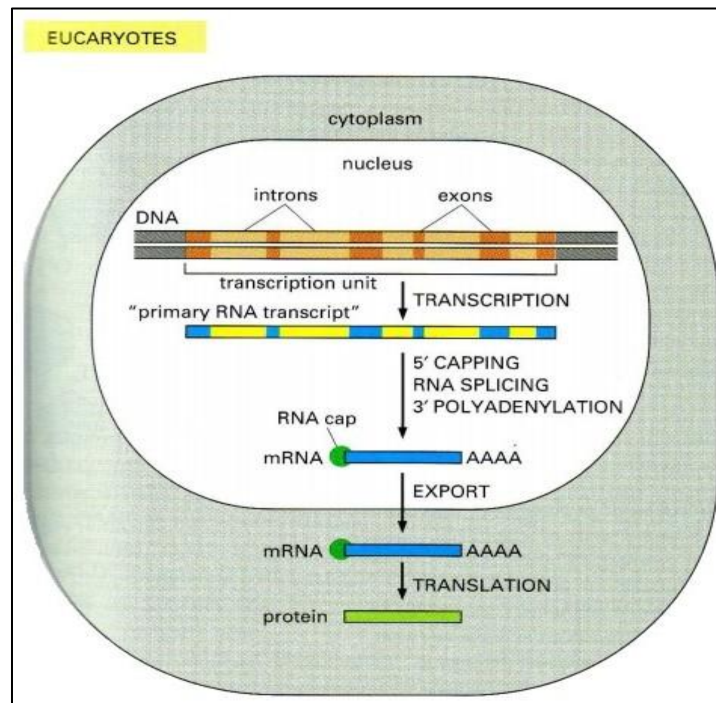
Adenine

Uracil

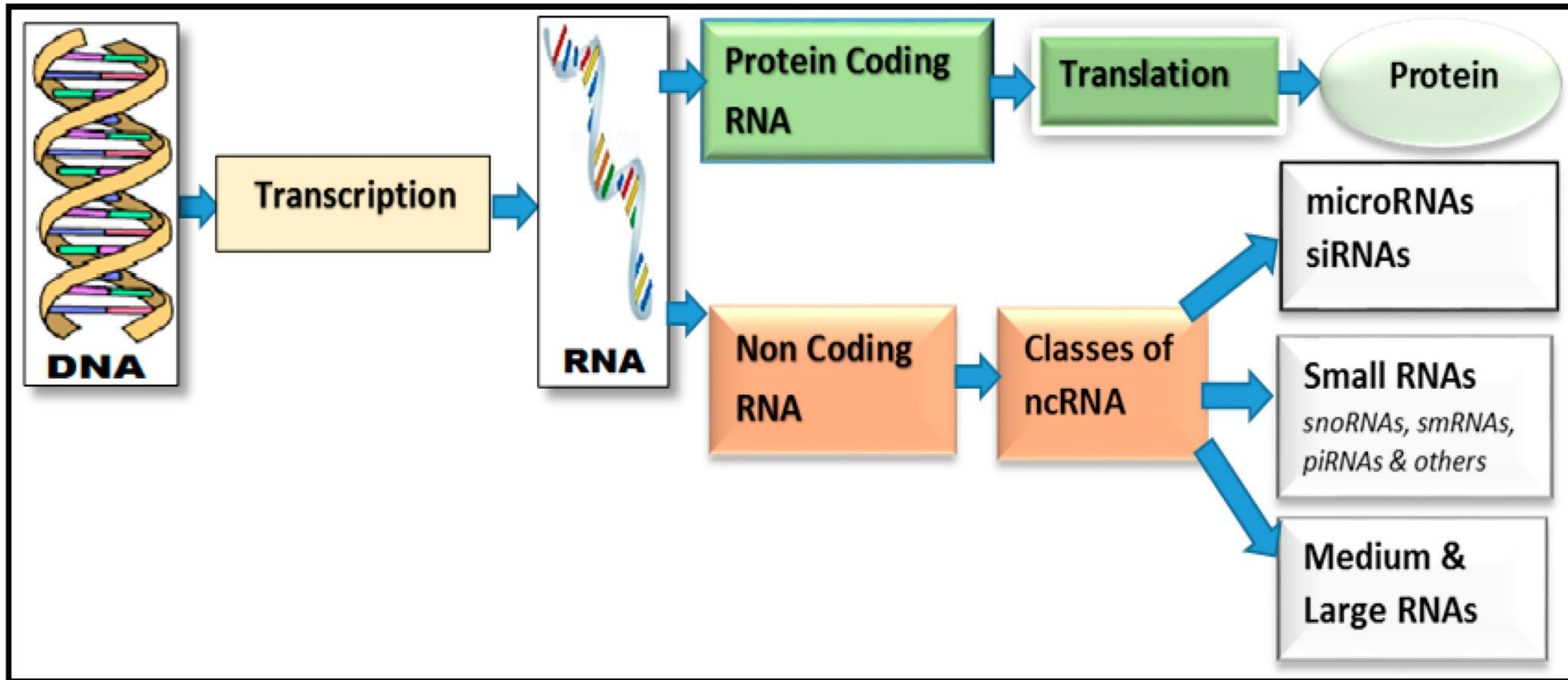
Replaces Thymine in RNA

Nitrogenous Bases

➢DNA, RNA and Protein

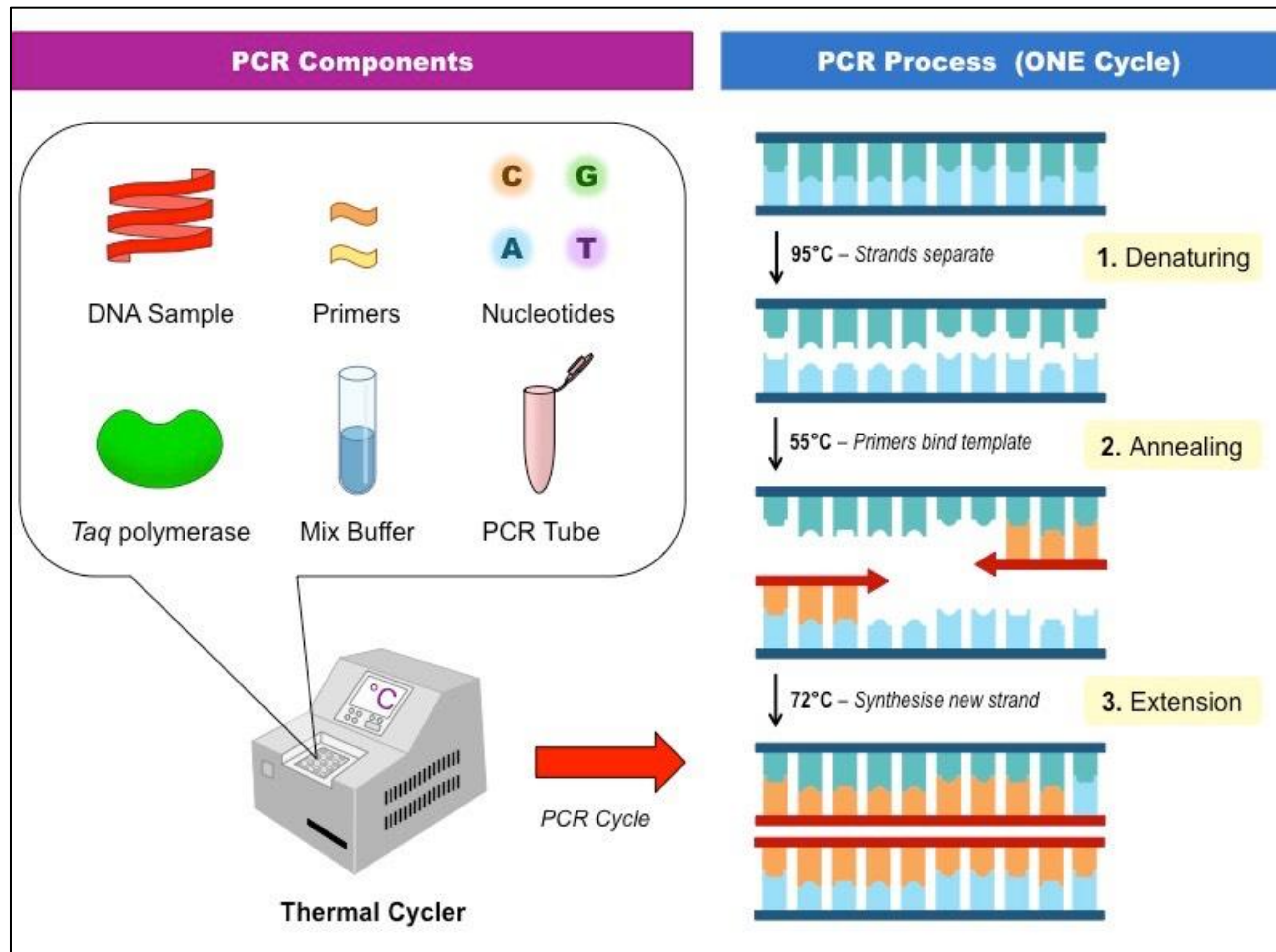


➢ RNAs



➢Polymerase Chain Reaction (PCR)

It's a means of selectively amplifying a particular segment of DNA.
i.e. <u>It is a molecular photocopier of DNA</u>.
Invented by Kary Mullis in 1983 (get Noble Prize in 1993).



➢Polymerase Chain Reaction (PCR)

Requirements:

- ➢ Template DNA,
- ➢ Primers
- ➢ Reaction buffer (Tris, ammonium ions (and/or potassium ions), magnesium ions, bovine serum albumin),
- ➢ Nucleotides (dNTPs),
- ➢ DNA polymerase (usually Taq) and
- ➢ PCR Controls
- ➢ a thermocycler.

PCR Cycles:

30–35 cycles each comprising:

– denaturation (95°C), 30 sec.

– annealing (55–60°C), 30 sec.

– extension (72°C), time depends on product size.

➢PCR template (starting material)

PCR template is DNA. If RNA is the starting material, it must be converted to cDNA by a reverse transcriptase and this is called **RT-PCR**. RT-PCR could be One-Step OR two-Step (cDNA step separated)

Steps:

A- Storage: Samples (5-10 mg tissue, 10 µl to 1 ml if fluid) must be used freshly or rapidly stored either in RNA Later OR flash frozen in liquid nitrogen then stored at -70.

B- Cell disruption: by homogenization, sonication or vigorous vortexing with or without enzymes (Ex. Lysozyme) and detergents.

C- Removal of proteins by digestion with proteinase K.

D- Nucleic acids (DNA/RNA) extraction:

1- Organic (Ex. Phenol) extraction (Ex. Trizol reagent).

2- Column-based procedures (binding to anion exchange or silica glass-fiber filters in the presence of chaotropic salts (Ex. guanidine isothiocyanate or phosphate).

3- Salting out.

➢PCR template (starting material)

E- DNA recovery: by precipitation using ethanol or isopropanol OR elution.

F- Sample Purification:
Ex. DNAse treatment for RNA extract to avoid contaminant DNA.

G- Check Sample Purity, integrity and quantify:
1- A260/A280 ratio (1.8-2 for pure DNA/RNA)
2- Formaldehyde agarose gel electrophoresis (for RNA).
3- Quantification by measuring A260.

H- Purified DNA/RNA storage:
Purified RNA may be stored at –20°C or –70°C in water for 1 year.
Purified DNA may be stored at –20°C or –70°C in TE buffer for 1 year.

I- If RT-PCR:
cDNA syntheis using dNTPs, RNase inhibitor, Reverse Transcriptase, and primer (random or anchored poly dT).

➢PCR Primers Guidelines

- Primers should be ~20 (18-24) bases long.
- The G/C content should be 40-60%.
- The Tm of all primer pairs should be similar
- Optimal annealing temperature ~ 5°C below the calculated Tm
- The primers must not base pair with each other or with themselves or form hairpins.
- Primers must avoid repetitive DNA
- Tm calculation: 2°C x (A+T) + 4°C x (G+C)
- Ensure primer sequence is unique for the template sequence (BLAST)
- Use a concentration of 0.1–1.0 µM of each primer. For many applications, a primer concentration of 0.2 µM will be sufficient
- Primers differ according to the type of PCR Ex. , qPCR and multiplex PCR

➢PCR Enzymes

- **Taq DNA polymerase**, isolated from the eubacterium *Thermus aquaticus*, is the most commonly used enzyme for standard end-point PCR. However, as this enzyme is active at room temperature, it is necessary to perform reaction setup on ice to avoid nonspecific amplification.

- **Hot-start polymerase** Taq DNA polymerase activity can be inhibited at lower temperatures with chemical modifiers leading to complete inactivation of the polymerase until the covalent bonds are broken during the initial heat activation step.

- **High-fidelity polymerase (Proof-reading)** high-fidelity PCR enzymes generally provide a 3' to 5' exonuclease activity for removing incorrectly incorporated bases. High-fidelity PCR enzymes are ideally suited to applications requiring a low error rate, such as cloning, sequencing, and site-directed mutagenesis. (Error 1 in 10^5 / 10^6 vs 1 10^3 / 10^4 for Taq polymerase)

➢PCR Controls

- **NTC:** No template control
- **Reference or internal or housekeeping control** (Ex. B-actin, GAPDH, 18S ribosomal RNA, ……..)
- **Positive control**

Note:

Conventional PCR reaction is an end-point. After the 35 cycles, the PCR products are transferred to and isolated using **gel electrophoresis** (with ethidium bromide or SYBR green or EVA green) and visualized (with UV or Flourescense) and compared to a DNA Ladder for Size.

➢Real-time PCR

- Real-time PCR (also known as quantitative or qPCR) allows accurate quantification of starting amounts of DNA, cDNA, and RNA targets.

- Fluorescence is measured during each cycle, which greatly increases the dynamic range of the reaction, since the amount of fluorescence is proportional to the amount of PCR product.

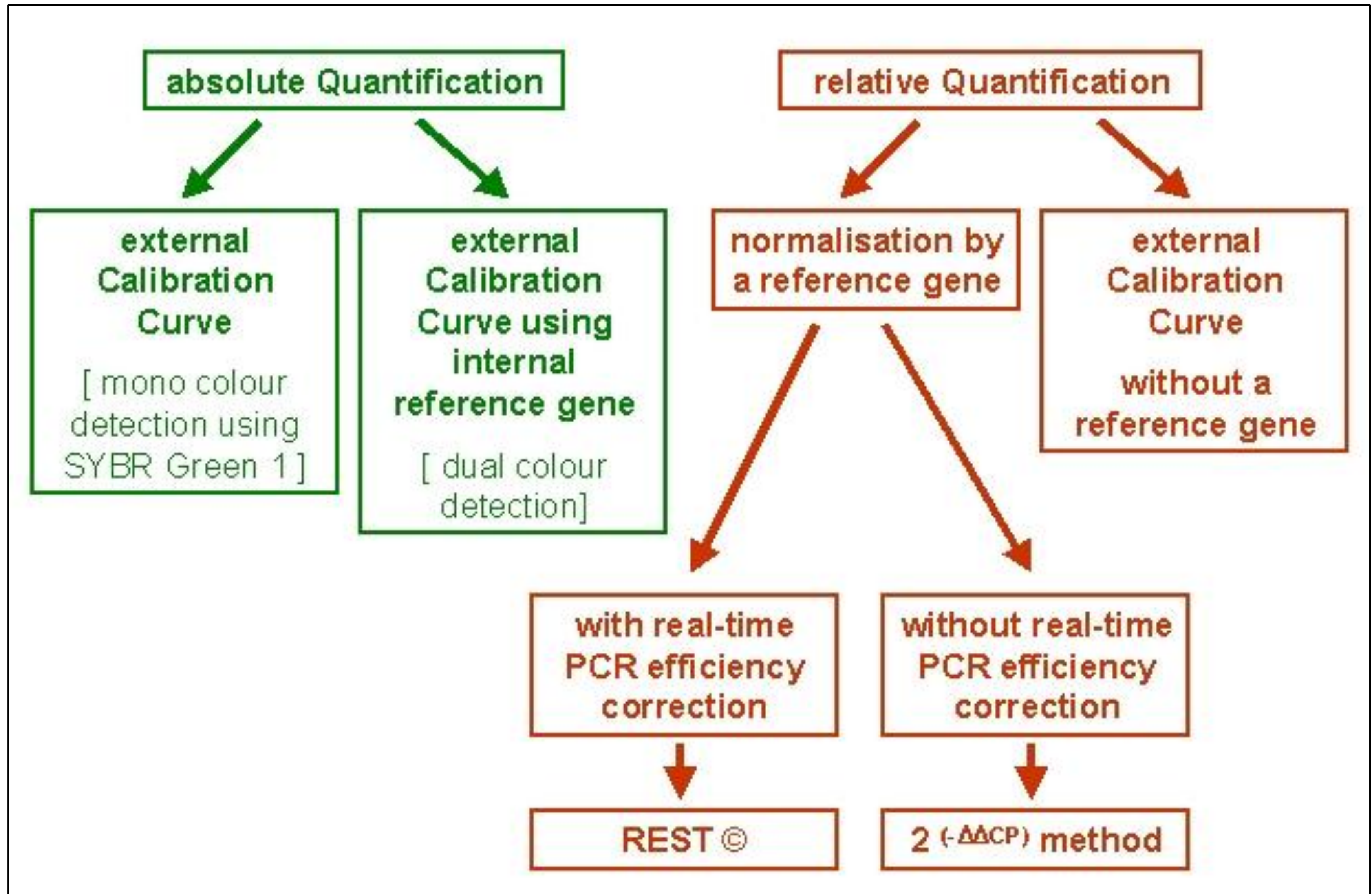
PCR products can be detected using:

- Fluorescent dyes (Ex. SYBR Green or Eva Green) that bind to double-stranded DNA , less specific as they bind to any double stranded DNA

  OR

- Fluorescently labeled sequence-specific probes (Ex., TaqMan probes, FRET Probes,.)…. More specific but more expensive

➢Quantification by PCR



➢Gene Information/sequence

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National Center for Biotechnology Information

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Gene | Gene | iNOS Search

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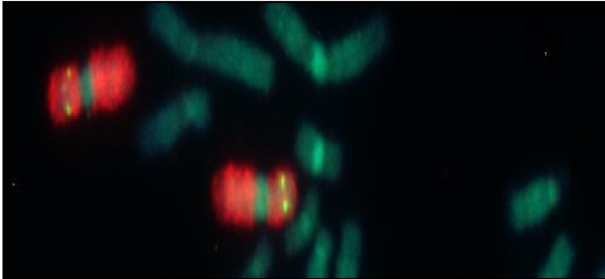

Gene

Gene integrates information from a wide range of species. A record may include nomenclature, Reference Sequences (RefSeqs), maps, pathways, variations, phenotypes, and links to genome-, phenotype-, and locus-specific resources worldwide.

Using Gene

- Gene Quick Start
- FAQ
- Download/FTP
- RefSeq Mailing List
- Gene News
- Factsheet

Gene Tools

- Submit GeneRIFs
- Submit Correction
- Statistics
- BLAST
- Genome Workbench
- Splign

Other Resources

- OMIM
- RefSeq
- RefSeqGene
- Protein Clusters

➢ **Note: Bioinformatics helps identify genes within a long DNA sequence. This technique locates a gene simply by analyzing sequence data (by similarity or prediction) using a computer (in silico).**

➢Gene Information/sequence

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Gene sources
Genomic
Mitochondria
Organelles
Plasmids

Categories
Alternatively spliced
Annotated genes
Non-coding
Protein-coding
Pseudogene

Sequence content
CCDS
Ensembl
RefSeq
RefSeqGene

Status clear
✓ Current

Clear all

Show additional filters

Tabular ▾ 20 per page ▾ Sort by Relevance ▾   Send to: ▾

See Inos myo-inositol-1-phosphate synthase in the Gene database
inos in Drosophila melanogaster Homo sapiens (2) All 33 Gene records

Search results

Items: 1 to 20 of 2587

<< First < Prev Page 1 of 130 Next > Last >>

See also 28 discontinued or replaced items.

Name/Gene ID	Description	Location	Aliases	MIM
Inos ID: 35671	myo-inositol-1-phosphate synthase [*Drosophila melanogaster* (fruit fly)]	Chromosome 2R, NT_033778.4 (7454922..7458874, complement)	Dmel_CG11143, CG11143, Dmel\CG11143, INOS, bs36h12.y1	
Nos2 ID: 18126	nitric oxide synthase 2, inducible [*Mus musculus* (house mouse)]	Chromosome 11, NC_000077.7 (78811613..78851052)	MAC-NOS, NOS-II, Nos-2a, i-NOS, iNOS, Nos2	
NOS2 ID: 4843	nitric oxide synthase 2 [*Homo sapiens* (human)]	Chromosome 17, NC_000017.11 (27756766..27800529, complement)	HEP-NOS, INOS, NOSA, NOS2	163730
Nos2 ID: 24599	nitric oxide synthase 2 [*Rattus norvegicus* (Norway rat)]	Chromosome 10, NC_051345.1 (63815308..63851208)	Nos2a, iNos	
Nos ID: 34495	Nitric oxide synthase [*Drosophila melanogaster*	Chromosome 2L, NT_033779.5 (10804274..10837511	Dmel_CG6713, CG6713, DNOS, DNOS1, Dmel\CG6713, NOS, NOS1	

Hide sidebar >>

Filters: Manage Filters

Results by taxon

Top Organisms [Tree]
Mus musculus *(252)*
Homo sapiens *(185)*
Rattus norvegicus *(129)*
Drosophila melanogaster *(72)*
Caenorhabditis elegans *(23)*
All other taxa *(1926)*
More...

Find related data

Database: Select

Find items

Search details

```
iNOS[All Fields] AND alive[prop]
```

➢ mRNA information

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Nucleotide

The Nucleotide database is a collection of sequences from several sources, including GenBank, RefSeq, TPA and PDB. Genome, gene and transcript sequence data provide the foundation for biomedical research and discovery.

Using Nucleotide

- Quick Start Guide
- FAQ
- Help
- GenBank FTP
- RefSeq FTP

Nucleotide Tools

- Submit to GenBank
- LinkOut
- E-Utilities
- BLAST
- Batch Entrez

Other Resources

- GenBank Home
- RefSeq Home
- Gene Home
- SRA Home
- INSDC

➢ mRNA information

Items: 1 to 20 of 273

<< First < Prev Page 1 of 14 Next > Last >>

1. **Rattus norvegicus nitric oxide synthase** (NOS2) mRNA, complete cds
   3,444 bp linear mRNA
   Accession: U26686.1 GI: 886072
   Protein PubMed Taxonomy
   GenBank FASTA Graphics

2. **Rattus norvegicus nitric oxide synthase 2** (Nos2), mRNA
   3,793 bp linear mRNA
   Accession: NM_012611.3 GI: 148298710
   Protein PubMed Taxonomy
   GenBank FASTA Graphics

3. Scenedesmus sp. PABB004 contig_2, whole genome shotgun sequence
   2,759,038 bp linear DNA
   Accession: JABVCE010000002.1 GI: 1918025517
   Assembly BioProject BioSample Protein Taxonomy
   GenBank FASTA Graphics

➢Primer Design Using PubMed BLAST

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GenBank | Send to:

Rattus norvegicus nitric oxide synthase 2 (Nos2), mRNA

NCBI Reference Sequence: NM_012611.3

FASTA Graphics

Go to:

```
LOCUS       NM_012611              3793 bp    mRNA    linear
DEFINITION  Rattus norvegicus nitric oxide synthase 2 (Nos2),
ACCESSION   NM_012611 XM_220732
VERSION     NM_012611.3
KEYWORDS    RefSeq; RefSeq Select.
SOURCE      Rattus norvegicus (Norway rat)
  ORGANISM  Rattus norvegicus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Euarchontoglires; Glires; Rodentia; Myomorpha;
            Muroidea; Muridae; Murinae; Rattus.
REFERENCE   1  (bases 1 to 3793)
  AUTHORS   He A, Guo Y, Xu Z, Yan J, Xie L, Li Y, Lv D and Luo M.
  TITLE     Hypoglycaemia aggravates impaired endothelial-dependent
            vasodilation in diabetes by suppressing endothelial nitric oxide
            synthase activity and stimulating inducible nitric oxide synthase
            expression
  JOURNAL   Microvasc Res 146, 104468 (2023)
   PUBMED   36513147
  REMARK    GeneRIF: Hypoglycaemia aggravates impaired endothelial-dependent
```

Design and test primers for this sequence using Primer-BLAST.

Change region shown

Customize view

Analyze this sequence

- Run BLAST
- Pick Primers
- Highlight Sequence Features
- Find in this Sequence
- Show in Genome Data Viewer

Articles about the Nos2 gene

- Post-translational modification of manganese superoxide dis [J Heart Lung Transplant. 2005]
- Monomeric inducible nitric oxide synthase localizes to pe [Proc Natl Acad Sci U S A. 2005]
- Increased rat alveolar macrophage expression of functional iNOS induced I [Nitric Oxide. 2005]

See all...

➢Primer Design Using Primer-BLAST

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Primer-BLAST — A tool for finding specific primers

Finding primers specific to your PCR template (using Primer3 and BLAST).

Primers for target on one template | Primers common for a group of sequences

Retrieve recent results | Publication | Tips for finding specific primers

PCR Template

Enter accession, gi, or FASTA sequence (A refseq record is preferred) ? [Clear]

NM_012611.3

Or, upload FASTA file [Browse...] No file selected.

Range ? [Clear]

	From	To
Forward primer		
Reverse primer		

Primer Parameters

Use my own forward primer (5'->3' on plus strand) ? [Clear]

Use my own reverse primer (5'->3' on minus strand) ? [Clear]

	Min	Opt	Max	Max T_m difference
PCR product size	70		1000	
# of primers to return	10			
Primer melting temperatures (T_m)	57.0	60.0	63.0	3

Exon/intron selection

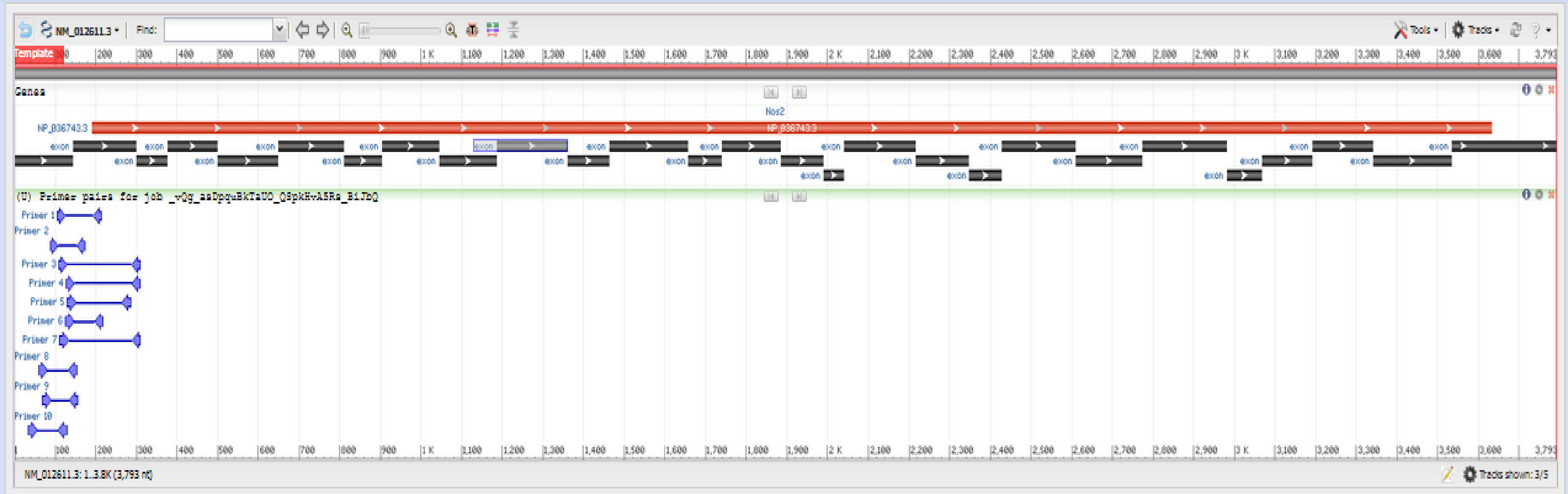
A refseq mRNA sequence as PCR template input is required for options in the section ?

Exon junction span No preference ?

Exon junction match Min 5' match | Min 3' match | Max 3' match

➢Primer Design Using Primer-BLAST

Graphical view of primer pairs



Detailed primer reports

Primer pair 1

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TCCTCAGGCTTGGGTCTTGT	Plus	20	105	124	60.77	55.00	5.00	0.00
Reverse primer	AGAAACTTCCAGGGGCAAGC	Minus	20	213	194	60.54	55.00	4.00	2.00
Product length	109								

Products on intended targets

>NM_012611.3 Rattus norvegicus nitric oxide synthase 2 (Nos2), mRNA

```
product length = 109
Forward primer  1    TCCTCAGGCTTGGGTCTTGT  20
Template        105  ....................  124

Reverse primer  1    AGAAACTTCCAGGGGCAAGC  20
Template        213  ....................  194
```

➢Primer Analysis Using NetPrimer

Free web based tool to analyze primers

Oligo Analysis		
Name	:	Rat iNOS
Description	:	

Sequence #1 (5' to 3')		
Length	:	20
TCCTCAGGCTTGGGTCTTGT		

Sequence #2 (5' to 3')		
Length	:	20
AGAAACTTCCAGGGGCAAGC		

Reaction Conditions			
Oligo Concentration	:	250.0	pM
Monovalent Ion Concentration	:	50.0	mM
Free Mg++ Ion Concentration	:	1.5	mM
Total Na^{+} Equivalent	:	204.92	mM
Temperature for Free Energy Calculation	:	25.0	°C

➢Primer Analysis Using NetPrimer

Analysis Results #1: TCCTCAGGCTTGGGTCTTGT

Rating	:	100.0	
Molecular Wt	:	6106.04	
Tm	:	59.32	°C
GC%	:	55.0	
GC Clamp	:	1	
nmol/A_{260}	:	5.7	
ug/A_{260}	:	34.81	
ΔG	:	-34.47	kcal/mol

3' end stability	:	-6.84	kcal/mol
ΔH	:	-151.7	kcal/mol
ΔS	:	-0.39	kcal/°K/mol
5' end ΔG	:	-7.82	kcal/mol
Self Dimer (ΔG)	:		kcal/mol
Hairpin (ΔG)	:		kcal/mol
Repeats (# of pairs)	:		kcal/mol
Run (# of bases)	:	3	kcal/mol

Analysis Results #2: AGAAACTTCCAGGGGCAAGC

Rating	:	91.0	
Molecular Wt	:	6160.1	
Tm	:	60.82	°C
GC%	:	55.0	
GC Clamp	:	2	
nmol/A_{260}	:	4.99	
ug/A_{260}	:	30.74	
ΔG	:	-36.62	kcal/mol

3' end stability	:	-8.64	kcal/mol
ΔH	:	-163.1	kcal/mol
ΔS	:	-0.42	kcal/°K/mol
5' end ΔG	:	-7.06	kcal/mol
Self Dimer (ΔG)	:	-4.54	kcal/mol
Hairpin (ΔG)	:	-0.44	kcal/mol
Repeats (# of pairs)	:		kcal/mol
Run (# of bases)	:	4	kcal/mol

Cross Dimer (ΔG)	:	-9.64	kcal/mol

Print

➢BLAST

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BLAST®

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Basic Local Alignment Search Tool

BLAST finds regions of similarity between biological sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance. Learn more

NEWS

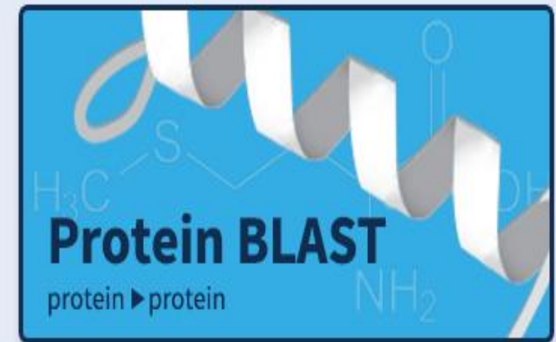
BLAST+ 2.14.0 is here!

BLASTP, BLASTX, and TBLASTN are faster than before.

Fri, 28 Apr 2023

More BLAST news...

Web BLAST



➢BLAST

Standard Nucleotide BLAST

blastn | blastp | blastx | tblastn | tblastx

BLASTN programs search nucleotide databases using a nucleotide query. more...

Enter Query Sequence

Enter accession number(s), gi(s), or FASTA sequence(s) ? Clear

TCCTCAGGCTTGGGTCTTGT

Query subrange ?

From

To

Or, upload file Browse... No file selected. ?

Job Title

Enter a descriptive title for your BLAST search ?

☐ Align two or more sequences ?

Choose Search Set

Database ◉ Standard databases (nr etc.): ○ rRNA/ITS databases ○ Genomic + transcript databases ○ Betacoronavirus

New ○ Experimental databases

Try experimental taxonomic nt databases Download

For more info see What are taxonomic nt databases?

Reference RNA sequences (refseq_rna) ?

Organism
Optional

Rattus norvegicus (taxid:10116) ☐ exclude Add organism

Enter organism common name, binomial, or tax id. Only 20 top taxa will be shown

➢BLAST

Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments Download Select columns Show 100

select all *100 sequences selected* GenBank Graphics Distance tree of results MSA Viewer

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	Rattus norvegicus nitric oxide synthase 2 (Nos2), mRNA	Rattus norvegicus	40.1	40.1	100%	0.002	100.00%	3793	NM_012611.3
☑	PREDICTED: Rattus norvegicus NFKB inhibitor delta (Nfkbid), transcript variant X3, mRNA	Rattus norvegicus	30.2	30.2	75%	1.5	100.00%	2932	XM_039093857.1
☑	PREDICTED: Rattus norvegicus NFKB inhibitor delta (Nfkbid), transcript variant X2, mRNA	Rattus norvegicus	30.2	30.2	75%	1.5	100.00%	2999	XM_039093856.1
☑	Rattus norvegicus potassium channel tetramerization domain containing 17 (Kctd17), transcript variant 1, mRNA	Rattus norvegicus	30.2	30.2	75%	1.5	100.00%	1740	NM_001395732.1
☑	PREDICTED: Rattus norvegicus potassium channel tetramerization domain containing 17 (Kctd17), transcript v...	Rattus norvegicus	30.2	30.2	75%	1.5	100.00%	1536	XM_006241982.4
☑	PREDICTED: Rattus norvegicus potassium channel tetramerization domain containing 17 (Kctd17), transcript v...	Rattus norvegicus	30.2	30.2	75%	1.5	100.00%	1612	XM_039078953.1

➢ Other Useful Websites

For Primer Design and Analysis

- **BatchPrimer3:**
- **IDT PrimerQuest:**
- **OligoPerfect by ThermoFisher:**

For Gene/mRNA/Exon-Intron/BLAST Information:

- **Ensembl:**

➢Multiplex PCR

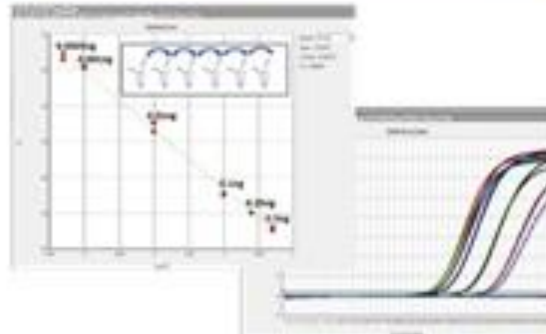
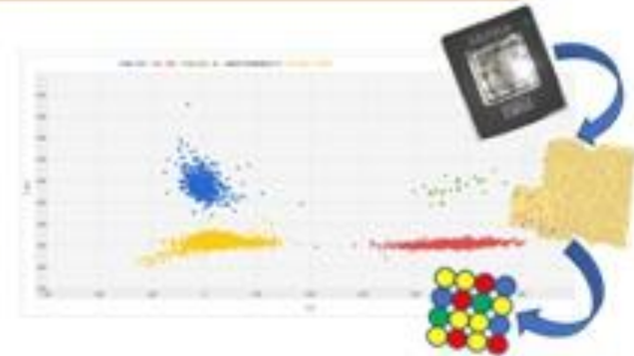
➢ Multiplex PCR is a variant of real-time PCR in which more than one target sequence can be amplified by including more than one pair of primers in the same reaction.

➢ Each primer pair for a target will have a specific fluorescent dye different than the others

➢ Multiplex PCR has the potential to produce considerable savings of time and effort within the laboratory without compromising test utility.

➢ It requires special design for primers and extensive optimization of buffers, temperatures, ….. To avoid interaction between different primers and their amplicons (products)

➢ Types of PCR – According to Detection Method

PCR qualitative	Real Time PCR relative	Digital PCR absolute
		
• End point quantification • Qualitative or semi quantitative • Detection by agarose gel	• Detection during the reaction progress • Relative quantification • Requires normalization and a standard curve • Variations in amplification efficiency may affect the results	• End point quantification • Absolute quantification • Precise • Highly sensitive • No standard curve needed • High tolerance to PCR inhibitors

➢ Digital PCR

➢ Digital PCR (or 3rd generation PCR), is a new method for highly sensitive detection and absolute quantification of nucleic acids.

➢ Compared with traditional PCR, dPCR adds the operation of partitioning the reaction system, separating the reaction system (10 μl) into tens of thousands of tiny independent reaction systems.

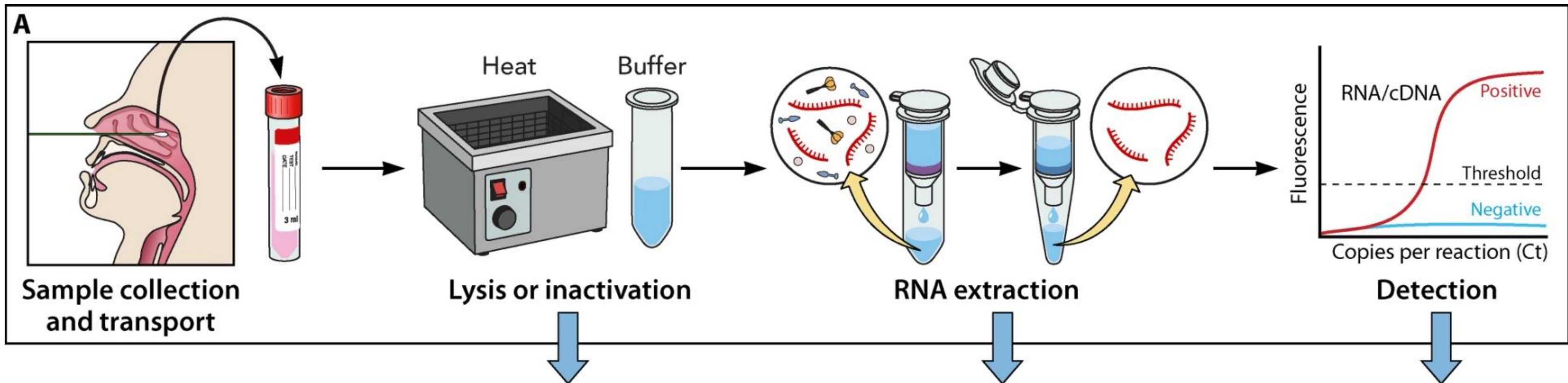
➢ Partitioning uses oil/water partitioning into droplets (ddPCR) either through droplet generator or through a chip.

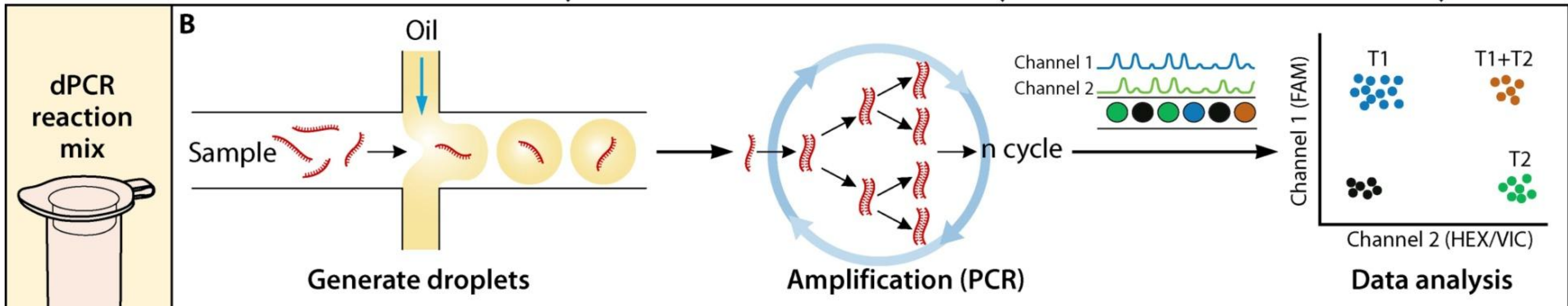
➢ Ideally, each droplet contains 1 molecule of the nucleic acid template. After the amplification is completed, the fluorescent signals of all droplets are identified and counted, and the number of negative and positive reactions is calculated.

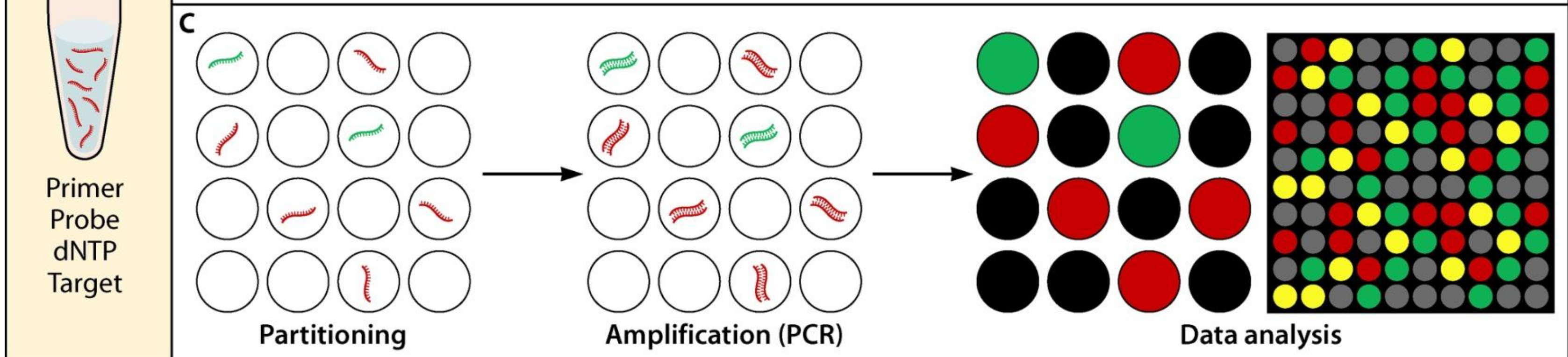
➢ Finally, the concentration of the target molecule is calculated by the Poisson distribution principle, thereby realizing the absolute quantification of the target molecule.

➢ Digital PCR does not rely on standard curve quantification and is not affected by PCR amplification efficiency, with higher sensitivity and accuracy. Thus it is used for example, rare sequence detection, copy number variation (CNV) analysis, and gene expression analysis of the rare targets.

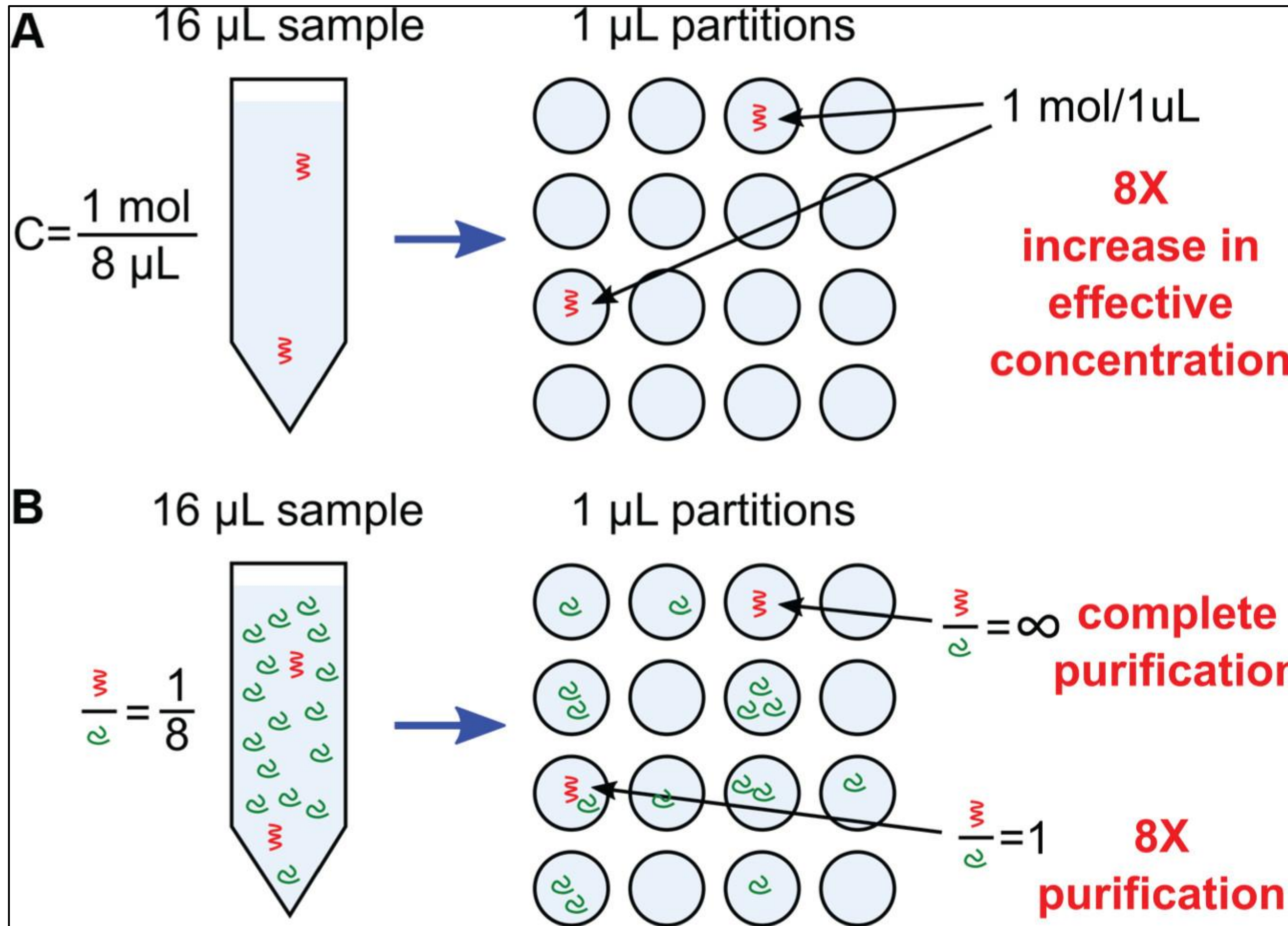
➢ Digital PCR







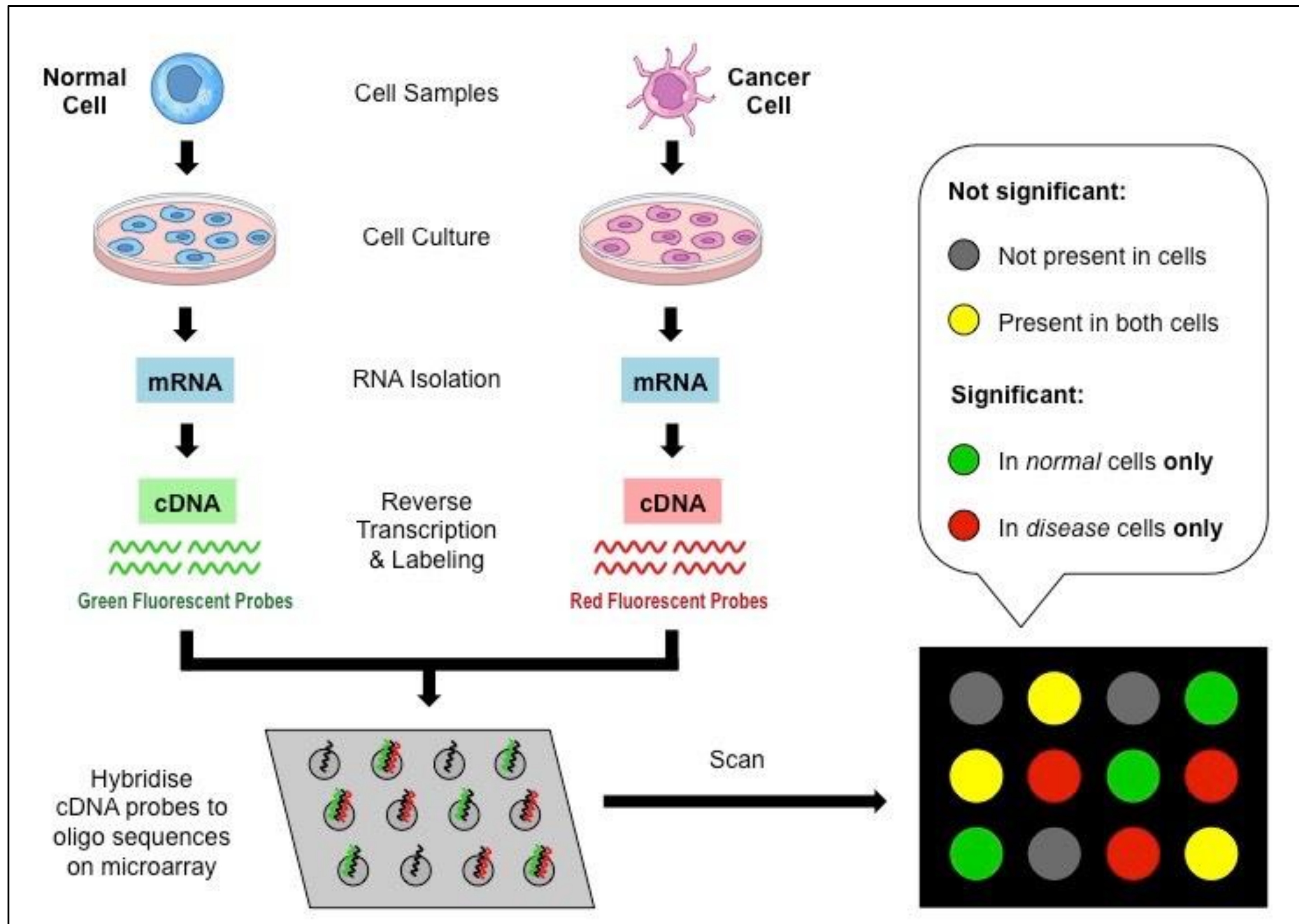
➢ Digital PCR



Digital PCR advantages include absolute quantification, No standard curve required, detection of rare samples and low concentration

➢ Microarray

➢ **A microarray is a laboratory tool used to detect the expression of thousands of genes at the same time. DNA microarrays are microscope slides that are printed with thousands of tiny spots in defined positions, with each spot containing a known DNA sequence or gene.**



➢Applications of PCR

Molecular Identification

- DNA fingerprinting
- Classification of organisms
- Genotyping
- Pre-natal diagnosis
- Mutation screening
- Drug discovery
- Genetic matching
- Detection of pathogens

Sequencing

- Bioinformatics
- Genomic cloning
- Human Genome Project

Genetic Engineering

- Site-directed mutagenesis
- Gene expression studies

➢ Next Generation Sequencing (NGS)

➢ Next-Generation Sequencing is a family of techniques that has accelerated the process of detecting nucleotides. Most techniques rely on **Sequencing by Synthesis**.

➢ In this process, the genome is excised into small 100-200 base pair fragments via mechanical or enzymatic means. The individual fragments can then be modified using RNA oligomers or adaptors (specific sequences that can be used as barcodes) via a ligation reaction.

➢ The fragments can then be immobilized to a solid surface or allowed to remain in solution. Critically, the fragments are hybridized to complementary sequences of known regions of interest (often called baits).

➢ The unbound DNA is removed, and the enriched DNA is eluted for NGS. Solution-based reactions often use baits with a moiety that can be used for purification.

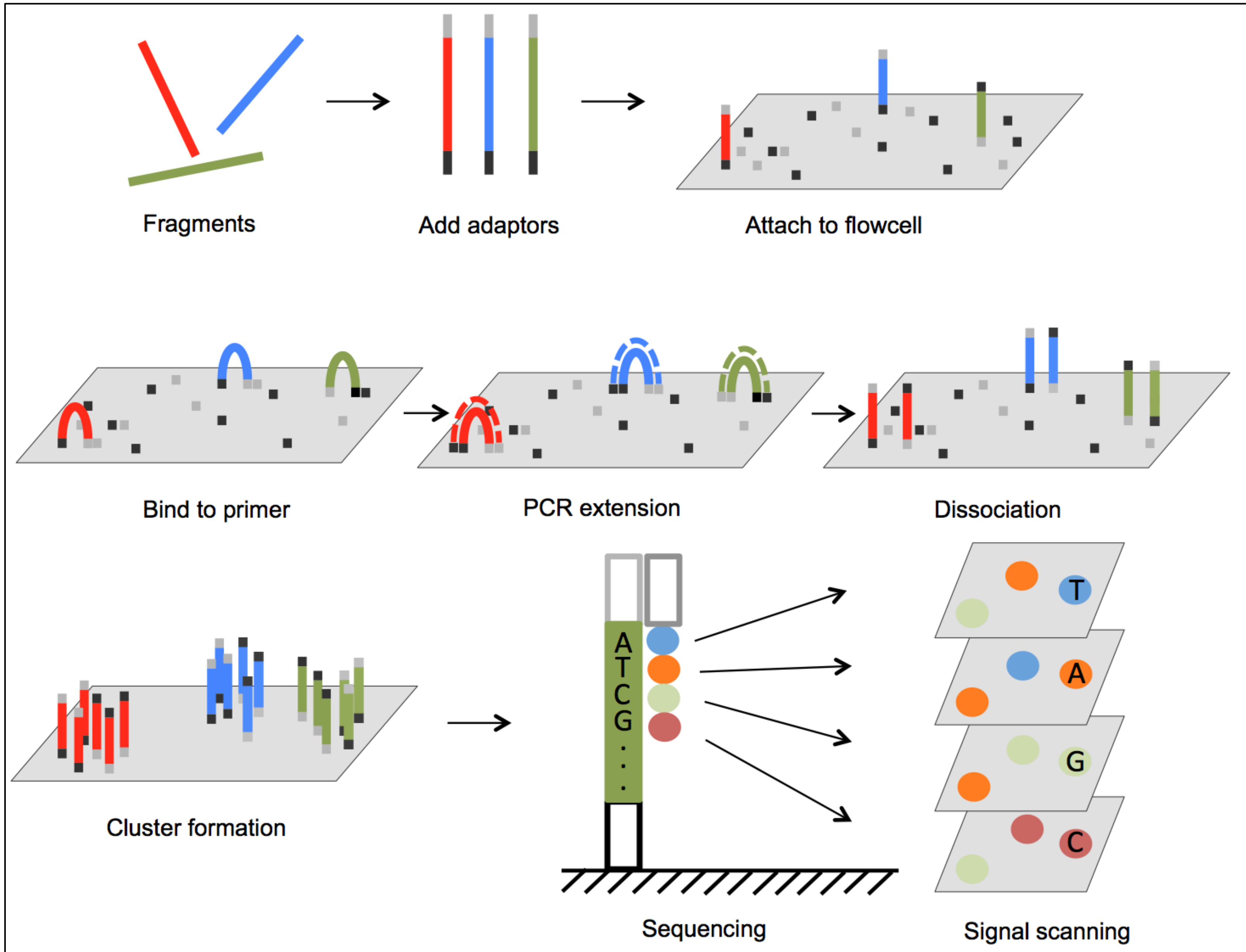
➢ Next Generation Sequencing (NGS)

➢ The dominant next-generation sequencing platform is Illumina. Illumina utilizes modified nucleotides that can only be added one at a time, preventing the addition of the next nucleotide due to a blocking moiety. The different nucleotides contain fluorophores that can be distinguished by their different spectra. Following detection, the blocking moiety and fluorophore are excised, allowing for an extension.

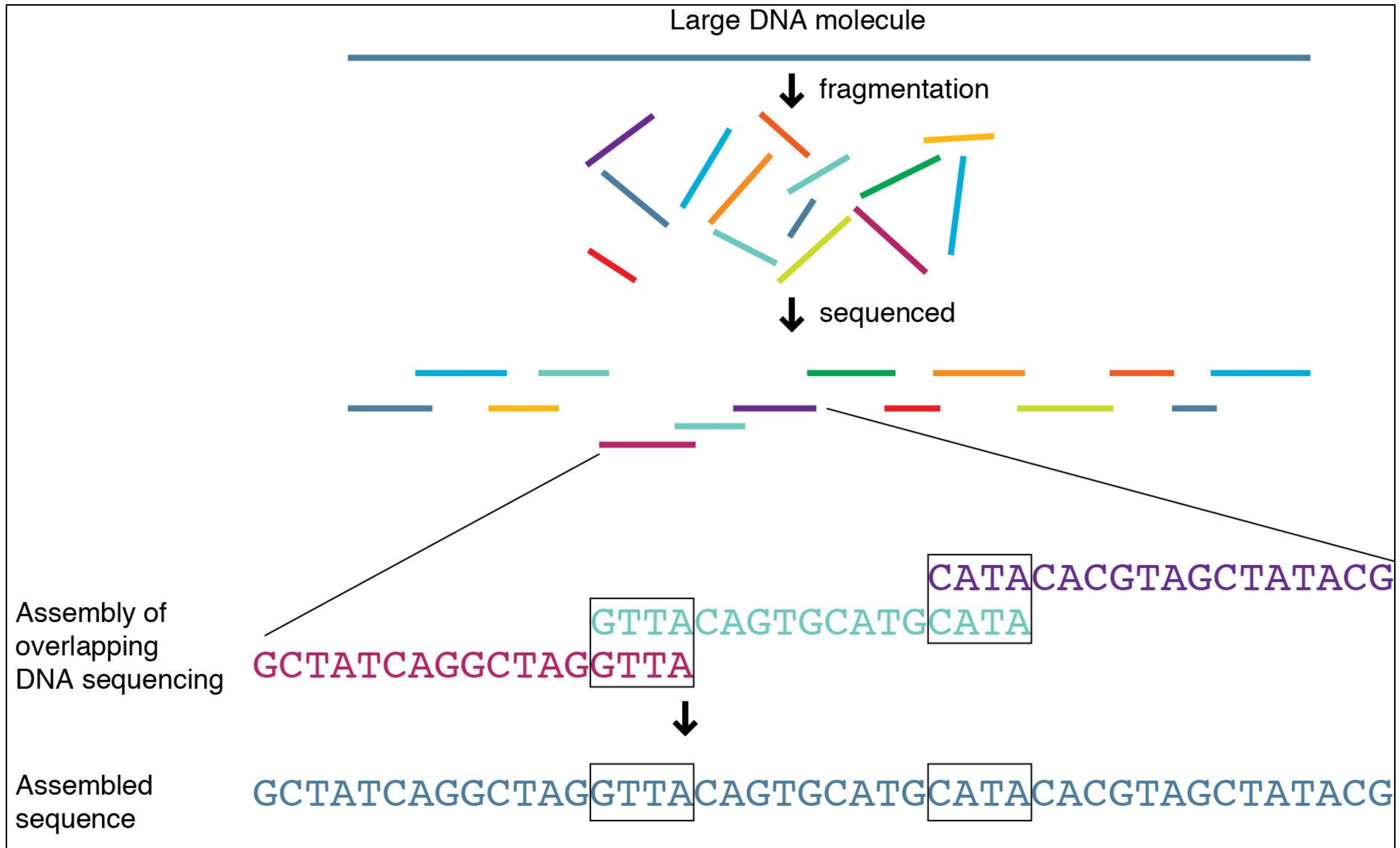
➢ Roche utilizes the by-product of the incorporation of the nucleotide, the pyrophosphate molecule. This reacts with firefly luciferase, which subsequently emits light corresponding to the number of nucleotides incorporated.

➢ Ion Torrent utilizes the pH change associated with nucleotide incorporation.

➢ Next Generation Sequencing

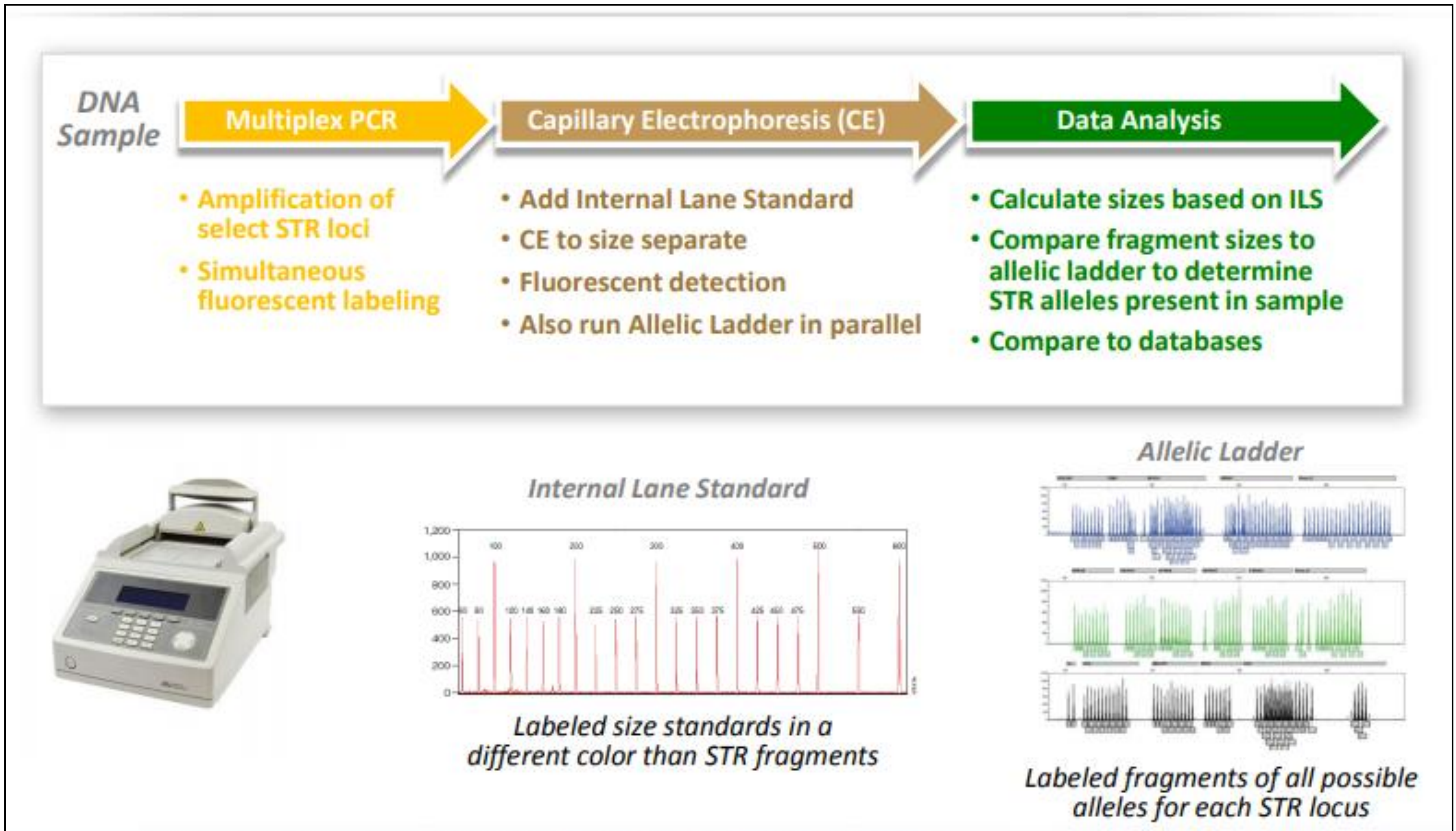


➢ Next Generation Sequencing



➢ DNA Fingerprinting OR Profiling

➢ **Short Tandem Repeats (STRs) are short repeated sequences (tetrameric) of DNA. The number of repeats varies between individuals, allowing for very high discrimination when used in combination. Thirteen STRs are used in forensics in the US under the Combined DNA Index System (CODIS).**



➢ DNA Fingerprinting OR Profiling

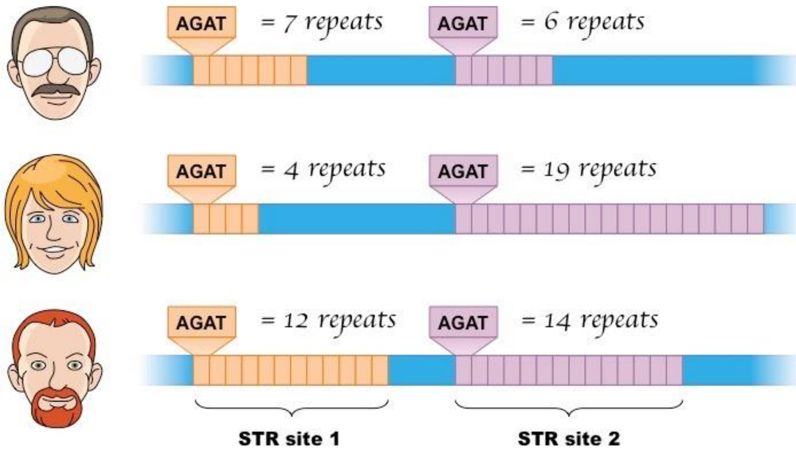
➢ **Currently allele classifications are identified by PCR-based fragment sizing by capillary electrophoresis (CE).**

➢ **Next-generation sequencing (NGS) adds ability to detect actual sequence context of the repeat motifs, the ability to multiplex even more markers, and the analysis of degraded samples (by purification and enrichments). The resulting STR profiles from NGS provide more information than just the allele number by analyzing the nucleotide sequence of the repeat motifs as well as nearby variations in the flanking regions.**

➢ **Additionally, NGS have generated thousands of intact genomes that can function as references.**

➢ **Moreover, NGS facilitate rapid, accurate detection of the entire mitochondrial genome. Autosomal DNA is limited to two copies per cell while mitochondrial DNA copy numbers can be in the thousands. It can be obtained from bones, teeth, hair shafts, feces, and other biological materials. Typically, detection of mitochondrial DNA focused on the hypervariable regions I and II. This process has been successful in achieving this in samples that were too damaged to undergo STR profiling.**

➢ DNA Fingerprinting OR Profiling



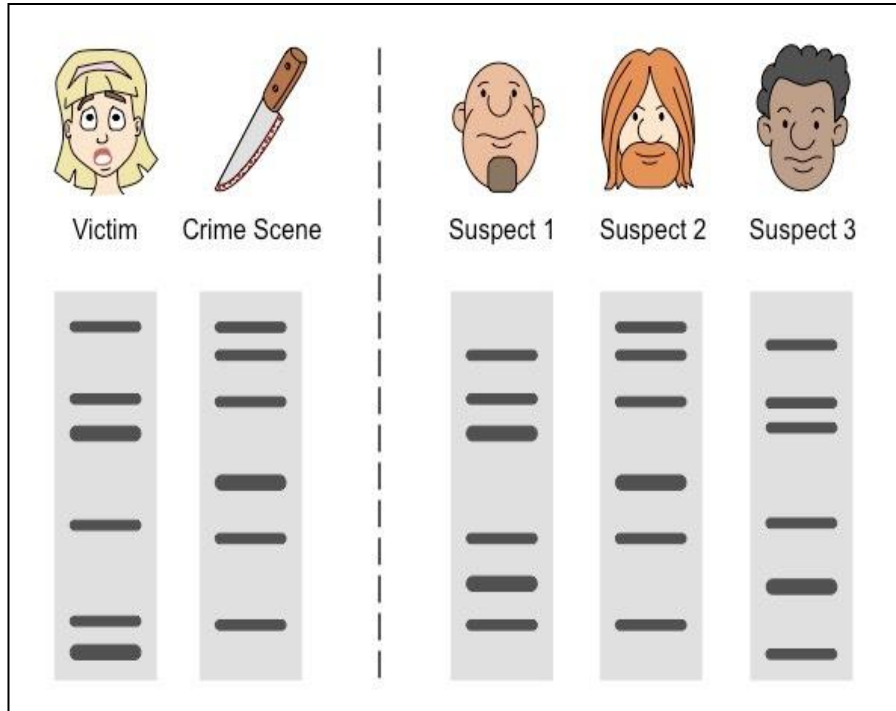
For example, at the genetic locus known as "D3S1358", This person has the genotype of "15, 18". This genotype is shared by about 8.2% of the population. By combining the frequency information for all 13 CODIS loci, Bob can calculate that the frequency of his profile would be 1 in 7.7 quadrillion Caucasians (1 in 7.7 times 10 to the 15th power!

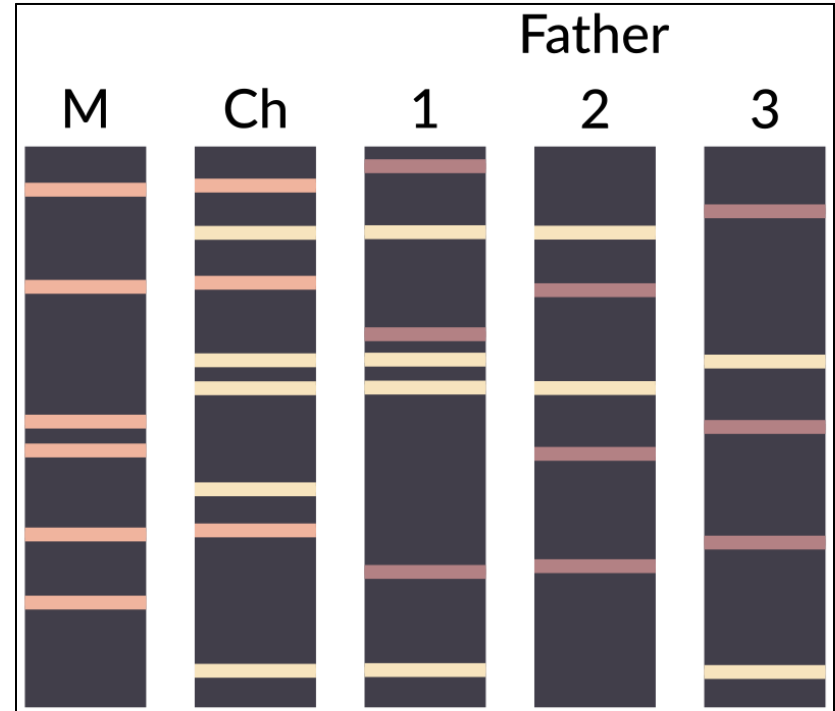
Genotype: represents no of repeats in each allele

Locus	D3S1358	vWA	FGA	D8S1179	D21S11	D18S51	D5S818
Genotype	15, 18	16, 16	19, 24	12, 13	29, 31	12, 13	11, 13
Frequency	8.2%	4.4%	1.7%	9.9%	2.3%	4.3%	13%

Locus	D13S317	D7S820	D16S539	THO1	TPOX	CSF1PO	AMEL
Genotype	11, 11	10, 10	11, 11	9, 9.3	8, 8	11, 11	X Y
Frequency	1.2%	6.3%	9.5%	9.6%	3.52%	7.2%	(Male)

➢ DNA Fingerprinting OR Profiling

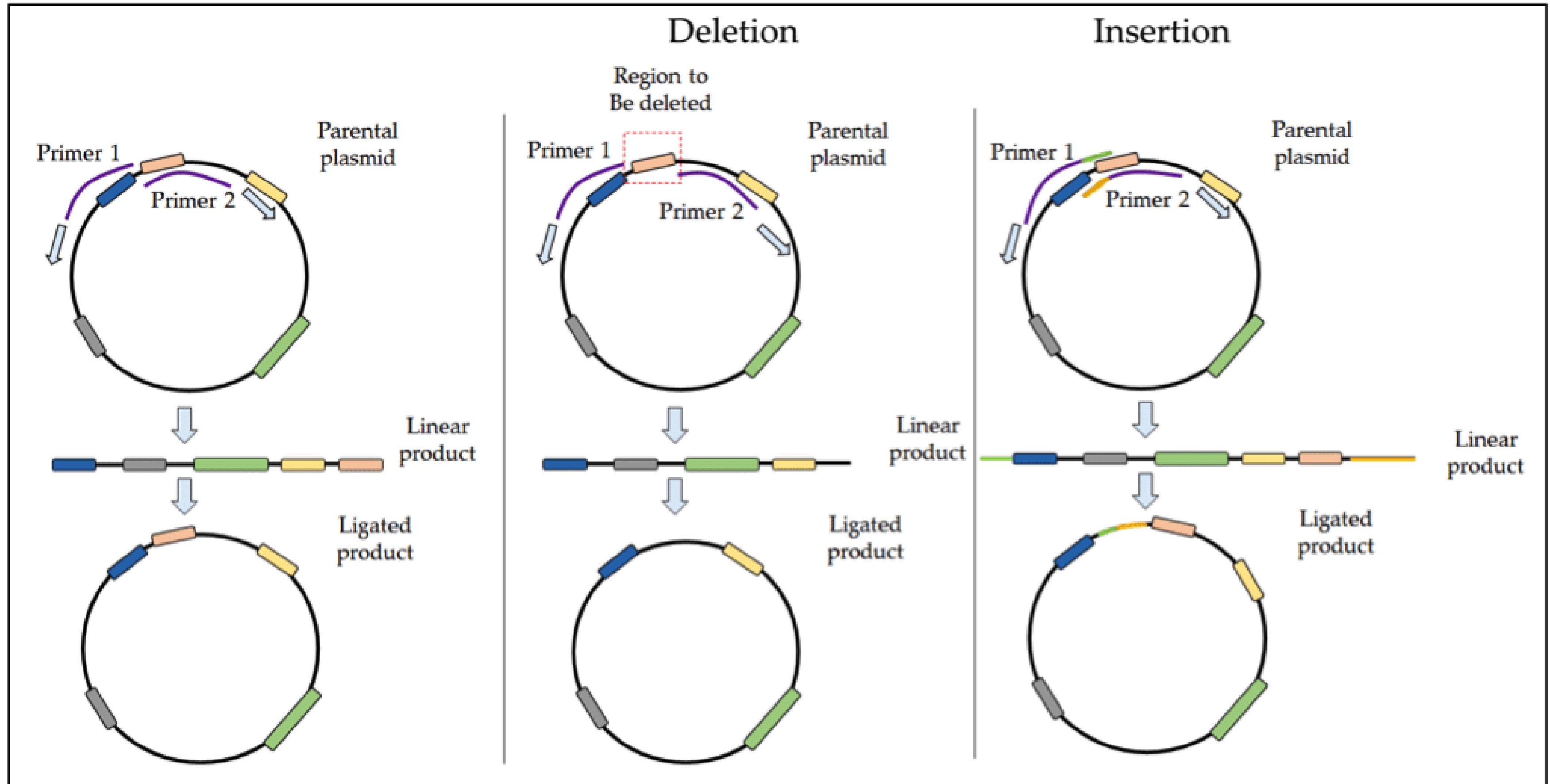




Forensics

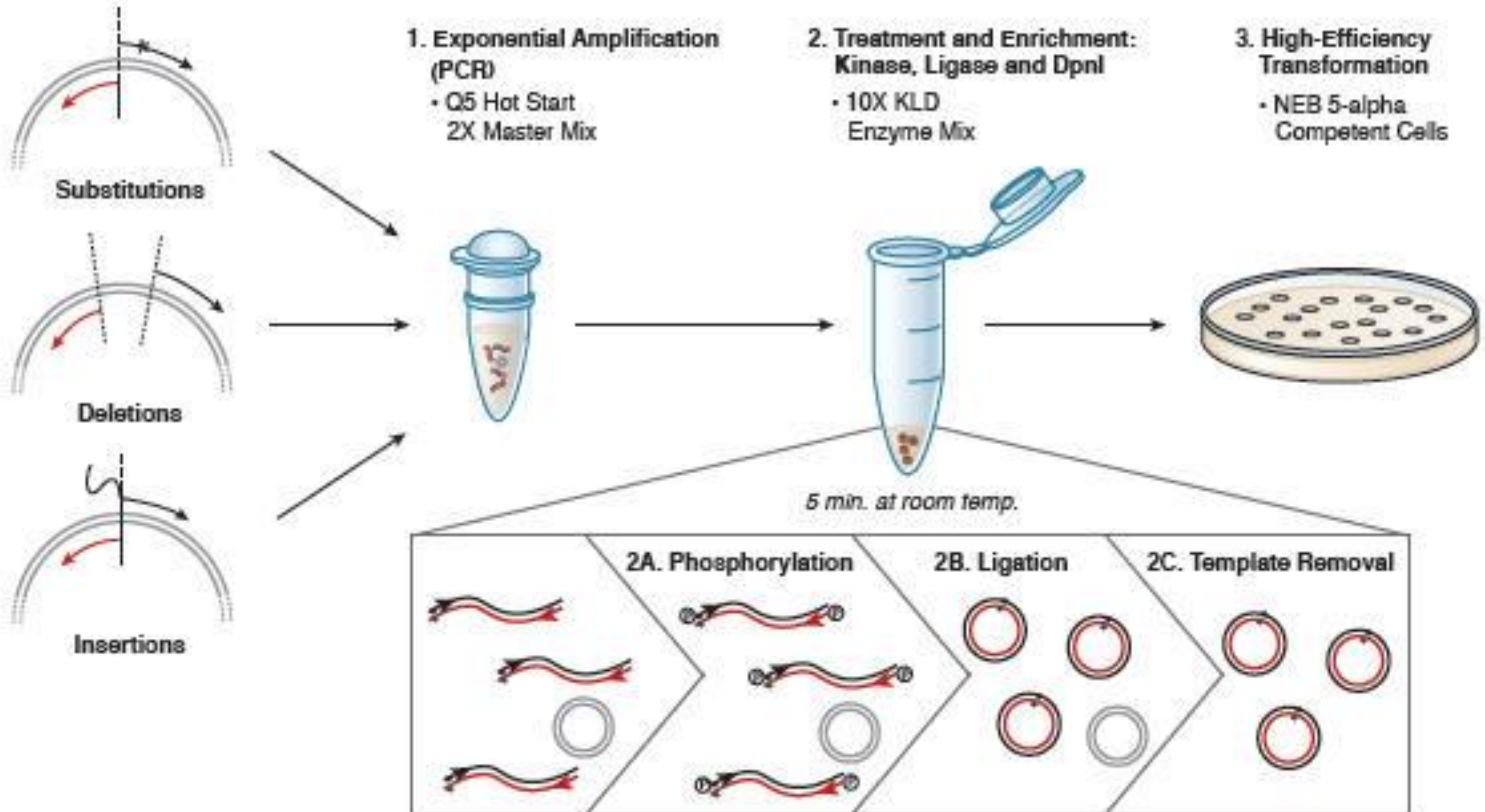
Paternity Testing

➢ Site-Directed Mutagenesis



Ex. Synthesis of Insulin Analogues

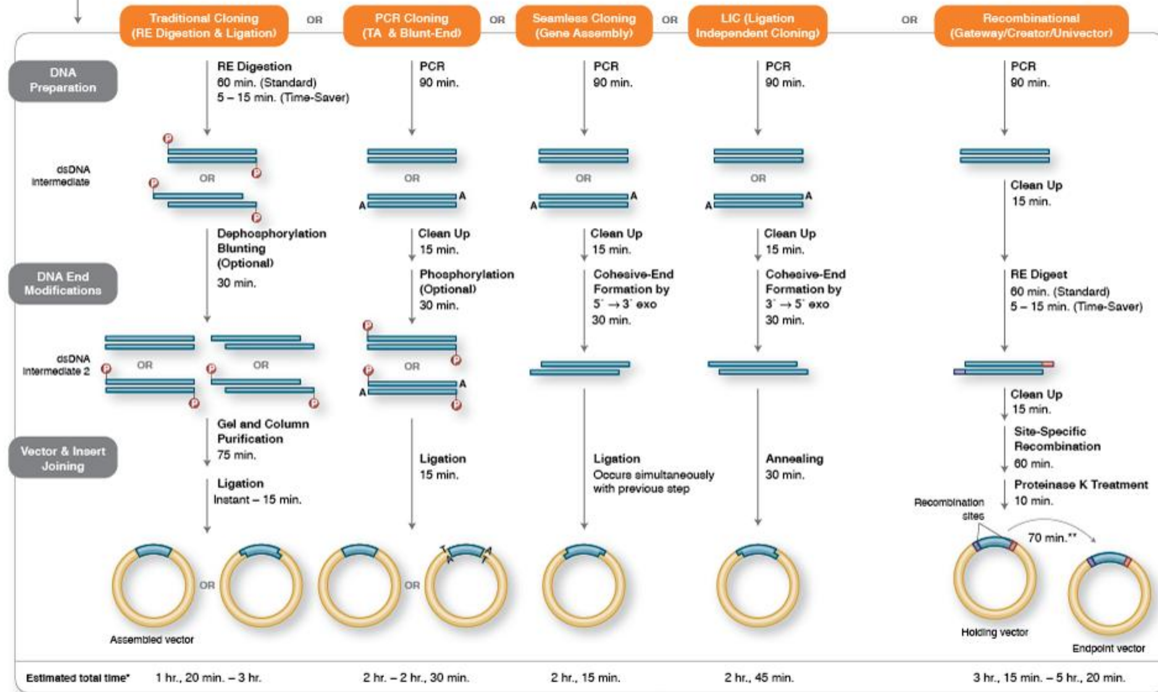
➢ Site-Directed Mutagenesis



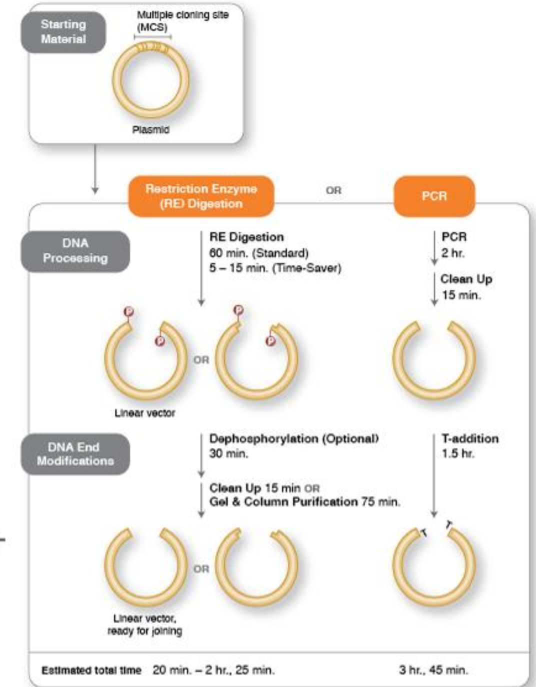
Ex. Synthesis of Insulin Analogues

➢ Cloning

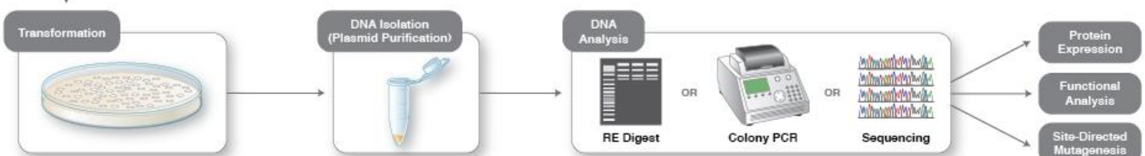
INSERT PREPARATION



VECTOR PREPARATION



* Note that times are based on estimates for moving a gene from one plasmid to another. If the source for gene transfer is gDNA, add 2 hours to calculation for the traditional cloning method. Total time does not include transformation, isolation or analysis.
** 70 minutes for recombination occurs on second day



Thank you