ISTITUTO ZOOPROFILATTICO SPERIMENTALE
DELLA LOMBARDIA E DELL'EMILIA ROMAGNA
"BRUNO UBERTINI"
(ENTE SANITARIO DI DIRITTO PUBBLICO)

Prova estratta [redacted]

CONCORSO PUBBLICO PER TITOLI ED ESAMI PER LA COPERTURA DI UN POSTO A TEMPO INDETERMINATO E TEMPO PIENO DI DIRIGENTE BIOLOGO DA ASSEGNARE ALLA STRUTTURA ANALISI DEL RISCHIO ED EPIDEMIOLOGIA GENOMICA (PRESSO LA SEDE DI PARMA).

PROVA ORALE N° 1

1. Come viene definita la precisione di un test diagnostico quantitativo?

2. Differenze tra PCR real time quantitativa e digital PCR

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NON ESTRATTA [redacted]

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PROVA ORALE N° 2

1. Qual è l'importanza della ISO17025 in un laboratorio di prova?

2. Ricerca di un agente virale non citopatico

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ESTRATTA [redacted]

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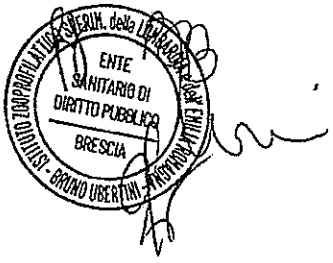
PROVA ORALE N° 3

1. Caratteristiche di un locale di biosicurezza BSL2
2. Differenze tra terreno colturale in batteriologia e virologia



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Prova [redacted]

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PROVA ORALE N° 4

1. Cosa sono e come si utilizzano le carte di controllo?
2. Diagnosi di laboratorio del virus della peste suina africana

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PROVA ESTRATTA [redacted]

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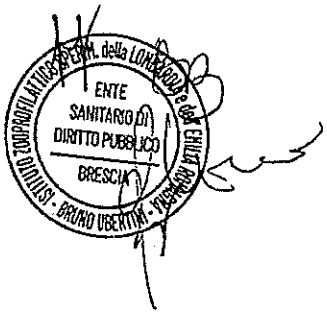
PROVA ORALE N° 5

1) Caratteristiche di un locale di biosicurezza BSL3
2) Gestione di una MTA (malattia trasmessa da alimenti)



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PROVA ESTRATTA [redacted]

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PROVA ORALE N° 6

1. Cosa sono ed a cosa servono i proficiency test?

2. Principali patogeni trasmessi dagli alimenti

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PROVA ESTRATTA [redacted]

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PROVA ORALE N° 7

1. Sensibilità diagnostica e sensibilità analitica

2. Principali patogeni trasmessi da vettori

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PROVA ESTRATTA [redacted]

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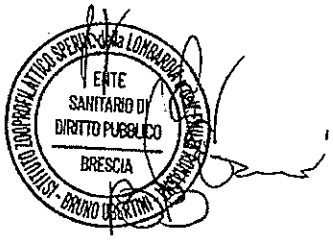
PROVA ORALE N° 8

1. Cosa si intende per limit of detection di un metodo molecolare?

2. Principali agenti zoonotici attualmente presenti in Italia



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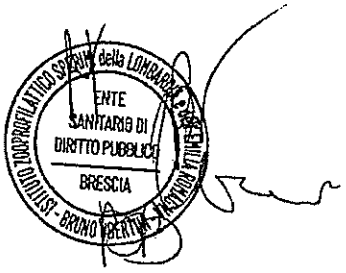
PROVA ORALE N° 9

1. Controlli di qualità di un'analisi molecolare
2. Cosa si intende per epidemiologia molecolare?



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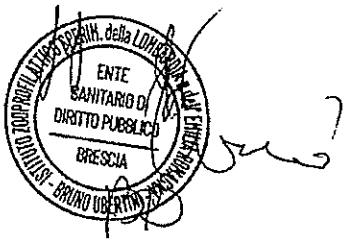
PROVA ORALE N° 10

1. Specificità diagnostica e specificità analitica

2. Tecniche di genotipizzazione batterica



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PROVA ESTRATTA [redacted] [redacted]

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PROVA ORALE N° 11

1. Validazione di un metodo home made e di un metodo normato

2. Finalità di un saggio sierologico rispetto ad un saggio molecolare

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Research article

Next-generation sequencing technologies: An overview

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ABSTRACT

Since the days of Sanger sequencing, next-generation sequencing technologies have significantly evolved to provide increased data output, efficiencies, and applications. These next generations of technologies can be categorized based on read length. This review provides an overview of these technologies as two paradigms: short-read, or "second-generation," technologies, and long-read, or "third-generation," technologies. Herein, short-read sequencing approaches are represented by the most prevalent technologies, Illumina and Ion Torrent, and long-read sequencing approaches are represented by Pacific Biosciences and Oxford Nanopore technologies. All technologies are reviewed along with reported advantages and disadvantages. Until recently, short-read sequencing was thought to provide high accuracy limited by read-length, while long-read technologies afforded much longer read-lengths at the expense of accuracy. Emerging developments for third-generation technologies hold promise for the next wave of sequencing evolution, with the co-existence of longer read lengths and high accuracy.

1. Introduction

Nearly 25 years after the structure of DNA was discovered, the first method for sequencing DNA was published [1,2]. This method involved the addition of chain-terminating and radioactively labeled (earlier approach) or fluorescently labeled (later approach) dideoxynucleotides to perform sequencing of a DNA strand complementary to the interrogated template strand. The fragments were then size separated and analyzed by gel electrophoresis to determine the sequence. Known as Sanger-sequencing, the method continued to improve with the introduction of capillary electrophoresis and gained wide acceptance as a "first-generation sequencing" method to sequence small and large genomes from bacteria and phages, to humans. Given that only one sequencing reaction could be analyzed at a time, the method was of limited throughput. Sequencing of diploid DNA also complicated the discernment of haploid sequences critical to many diagnostic and investigative purposes, necessitating subcloning, plating, and DNA preparation of individual subclones before sequencing. These labor-intensive processes contributed to the first human genome project, taking over a decade and costing $2.7 billion to complete [3]. Despite additional advancements permitting additional human genome sequencing for $10 million, the technology had reached its ceiling for time and cost [4]. It was evident that in order to expand our sequencing capabilities, new technologies had to be developed.

Between 2004 and 2006 "next-generation sequencing (NGS)" technologies were introduced, which transformed biomedical inquiry and resulted in a dramatic increase in sequencing data-output [5]. The significant increase in data output was due to the nanotechnology principles and innovations that allowed massively parallel sequencing of single DNA molecules. The combined features of high throughput and single-molecule DNA sequencing are hallmarks of NGS, irrespective of the sequencing platform. The technology's evolved procedures were better merged with data acquisition and analysis, freeing the community from more labor-intensive and low-efficiency historical Sanger sequencing approaches and facilitating an extraordinary increase in data output. Second-generation approaches, such as on the Illumina or Ion Torrent platforms, generally start with DNA fragmentation, DNA end-repair, adapter ligation, surface attachment, and in-situ amplification. These "short-read" sequencing technologies involve the massively parallel sequencing of short reads, whereby millions of individual sequencing reactions occur in parallel. However, by nature of being short-read technologies, sequencing data over long stretches of DNA must be reassembled, presenting challenges with structural variations or low-complexity regions.

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0198-8859/

Third-generation sequencing, such as on the Pacific Biosciences or Oxford Nanopore technology platforms, can achieve read lengths upwards of 10 kb, well beyond Sanger or short-read sequencing technologies. These "long-read" technologies can overcome issues encountered with short-reads, such as genome-wide repeats and structural variant detection. Compared to second-generation methods, process changes include minimal library preparation steps and direct targeting of unfragmented DNA molecules in real-time, where the limiting factor is the production of high molecular weight DNA for these purposes. An early limitation of these third-generation technologies compared to second-generation methods was the accuracy of the reads, which continues to improve over time, particularly with software analysis advancements.

Considering the currently available technologies and related advantages or disadvantages, the use of short-read or long-read sequencing depends on the research or clinical application. Thus far, research and clinical needs have dictated additional technological advancements. However, the limitations of these technologies have hindered additional scientific discoveries or clinical applications. The future direction of these technologies is an evolution from limiting factors to potentiating ones, giving rise to new research and clinical applications.

This review provides an overview of available NGS technologies categorized as short versus long reads, their benefits and limitations, remaining questions, and their future. While detailed clinical applications, data analysis software, and algorithms are beyond the scope of this review and are covered elsewhere in this Special Issue, human leukocyte antigen (HLA) genotyping by NGS is referenced to illustrate each technology.

2. Short-read sequencing

Short-read NGS, or second-generation sequencing, refers to the next advancement of sequencing technologies after traditional first-generation Sanger sequencing. The common feature of short-read technologies is massive sequencing of short (250–800 bp), clonally amplified DNA molecules sequenced in parallel [6]. NGS workflow includes library preparation, sequencing, and data analysis [7].

2.1. Library preparation

A pre-requisite for NGS is a good quality library. Library preparation includes first obtaining templates corresponding to molecules of interest for sequencing and subsequently prepare the fragments to make them compatible with the sequencing platform used [8]. NGS can be broadly divided into DNA sequencing (DNA-Seq) and RNA sequencing (RNA-Seq) [9–11].

2.1.1. DNA-seq library preparation

Depending on the sequencing template, DNA-Seq can include Whole Genome Sequencing (WGS), Whole Exome Sequencing (WES), Epigenome Sequencing, or Targeted Sequencing (TS) [9,12].

Two approaches for template preparation include polymerase chain reaction (PCR) and hybridization capture-based approaches. PCR-based methods are commonly used to prepare template for TS [8,13]. In its early stages, amplicon sequencing focused on a limited number of genes or exons (short-range PCR). Such was the case for early HLA genotyping, which focused on the amplification of exons encoding the antigen-recognition site (ARS), but this approach presented the same issues of typing ambiguities seen with Sanger-based typing [14]. The advent of long-range PCR (LR-PCR) lead to shotgun sequencing (sequencing of randomly broken, shorter than the amplicon, fragments) as a dominant approach, and entire genes, including intronic, untranslated, upstream, and downstream regions, could be sequenced. Thus, LR-PCR improved issues of sequence ambiguities seen with short amplicon sequencing [15,16]. It should be noted, however, that the LR-PCR-based approach, especially for HLA genotyping, is occasionally characterized by allele dropouts.

Hybridization capture-based preparation of templates is utilized for WES and TS [17,18], whereby complementary biotinylated probes are hybridized with regions of interest, which are then isolated using streptavidin-coated magnetic beads. Compared to PCR-based approaches, this target enrichment approach is a cost-effective method for TS of larger genomic regions and a greater number of genes with decreased instances of allele dropout. That said, these two methods are not mutually exclusive. A combined approach, Regional-specific extraction (RSE), has been reported to capture the large genomic region of the major histocompatibility complex (MHC) [19] and other genomic regions [20,21]. The RSE methodology involves enzymatic extension of a non-biotinylated oligonucleotide hybridized to a particular sequence and incorporating biotinylated nucleotides as it extends. The extended DNA is then captured with streptavidin-coated magnetic beads, enriched with whole-genome amplification techniques, and prepared for sequencing.

The most common tools for studying epigenetic modifications and their impact on gene regulation are Whole-genome bisulfite sequencing (WGBS) and chromatin immunoprecipitation, followed by NGS (ChIP-Seq) [8,22,23]. WGBS enables a genome-wide analysis of DNA methylation (5mC) at base-pair resolution. Preparation of genomic samples for WGBS is commonly performed through the post-bisulfite treatment of DNA and de-tagging before index adaptor ligation for NGS sequencing [8]. ChIP-Seq allows for genome-wide mapping of DNA-binding proteins and histone modifications at base-pair resolution. To prepare samples for ChIP-Seq, formaldehyde-fixed or natural chromatin is fragmented by micrococcal nuclease (MNase) or sonication, which is further immunoprecipitated with target-specific antibody conjugated to magnetic beads. Isolated DNA from the precipitated protein-DNA complexes is used to generate libraries [8].

DNA-Seq library construction involves the core steps of fragmentation, end-repair, adaptor ligation, and size selection [24]. Fragmentation shears DNA into the optimal platform-specific size range. Three approaches for fragmentation include physical (i.e., acoustic shearing or sonication), enzymatic (i.e., fragmentase or transposase tagmentation), or chemical (heat with divalent metal cation) methods [8]. The integration of fragmentation into template preparation for WGBS and ChIP-Seq obviates additional fragmentation steps. Fragmentation, which permits representation of both small and large fragments, is a key consideration with respect to HLA genotyping on the Illumina MiSeq, a short-read sequencer. Smaller fragments contribute to high-quality sequencing data, whereas longer fragments provide distal phase information [25]. Subsequent end-repair prepares libraries for adaptor ligation by ensuring DNA fragment ends are free of overhangs that contain 5′ phosphate and 3′ hydroxyl groups, by blunt ending and dA tailing (addition of non-template deoxyadenosine 5′-monophosphate to blunted ends for ligation to adaptors with complementary dT-overhangs) for Illumina, or blunt ending only for Ion Torrent.

Adaptors include platform-specific sequences for fragment recognition by the sequencing instrument, such as DNA fragment binding to the flow cells of Illumina platforms. Adaptors also increasingly include a short, unique sequence, known as a barcode or index, to identify individual samples, allowing large numbers of samples to be pooled and sequenced simultaneously in a single run (multiplex sequencing). These barcodes can be identified and used to assign reads to individual samples during the data analysis. In the context of HLA genotyping, multiple HLA loci from a single