DEPARTAMENTO DE MICroBiologia UNIVERSIDADE DE SÃO PAULO

Aplicação de técnicas moleculares no diagnóstico de infecções causadas por anaeróbios

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Diagnóstico clínico

SINAIS + **SINTOMAS**

SINAIS → Mensuráveis

SINTOMAS → Subjetivos

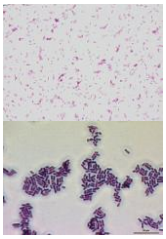
Origem	Etiologia	Natureza
Microbiota endógena	Monomicrobiana	Infecciosa
Microbiota exógena	Polimicrobiana	Não - Infecciosa

Diagnóstico laboratorial

- ✓ Identificação agentes envolvidos em patologias;
- ✓ Suporte para diagnóstico/tratamento clínico;
- ✓ Investigação epidemiológica;
- ✓ Investigação científica.

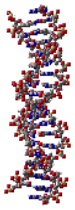
Diagnóstico microbiológico

Microscopia



Cultivo

Biologia Molecular



Técnicas de Biologia Molecular

Identificação/diagnóstico de doenças infecciosas

- ✓ Técnicas de hibridização (sondas genéticas);
- ✓ PCR (Polimerase Chain Reaction) e suas variações;
- ✓ Sequenciamento (gerações).

Epidemiologia

- ✓ PFGE (Pulsed Field Gel Electrophoresis);
- ✓ RAPD-PCR (Random Amplified Polymorphic DNA);
- ✓ MLST (Multilocus Sequence Typing).

Técnicas de Hibridização (Sondas Genéticas)

Técnica de Hibridização (Sonda Genética)

❄ Definição: técnica na qual uma sonda de DNA ou RNA de fita simples, é utilizada para localizar um gene ou molécula em uma célula ou tecido.

❄ Detecção de sequências de nucleotídeos específicas.

Técnica de Hibridização (Sonda Genética)

✓ Southern-blot:
Análise do DNA transferido para a membrana.

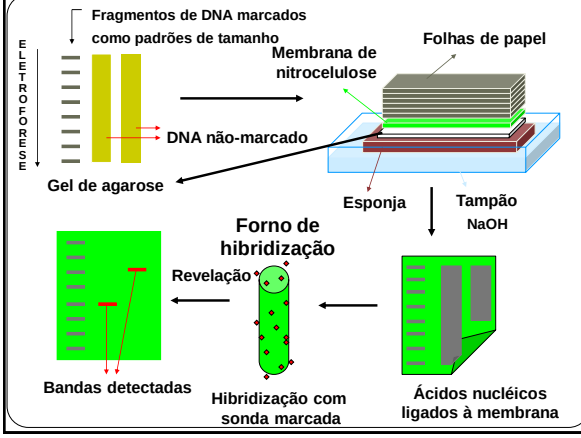
✓ Northern-blot:
Análise do RNA transferido para a membrana.

✓ Dot-blot:
Análise de colônias transferidas para a membrana.

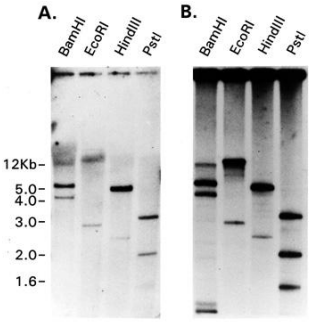
Southern-blot

→ Técnica na qual fragmentos de DNA (enzimas), separados por eletroforese, são imobilizados em uma membrana.

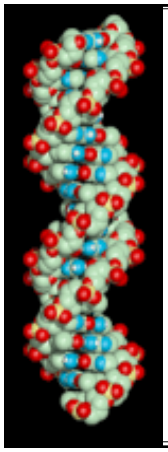
→ Moléculas específicas são então detectadas com uma sonda de ácido nucléico marcada (Radioativo/Fluorescente).



Southern-blot



PCR – Polimerase Chain Reaction



Diversidade da PCR

Qualitativa

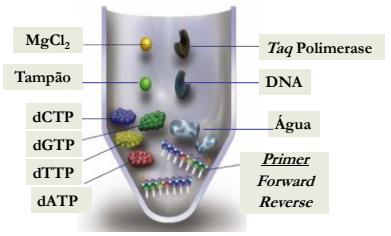
- ✓ PCR
- ✓ Multiplex
- ✓ Nested-PCR
- ✓ Transcriptase Reversa
- ✓ RAPD/AP/RFLP
- ✓ MLST (Multilocus Sequence Typing)
- ✓ HRM (High Resolution Melting)

Qualitativa e Quantitativa

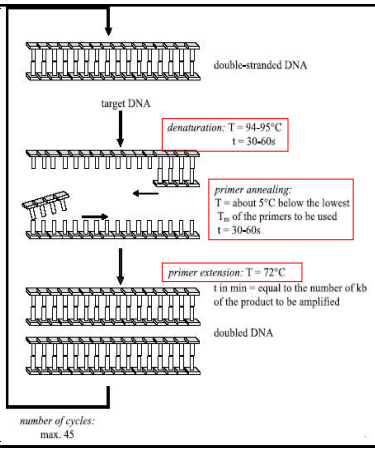
- ✓ qPCR
- ✓ Multiplex
- ✓ Transcriptase Reversa

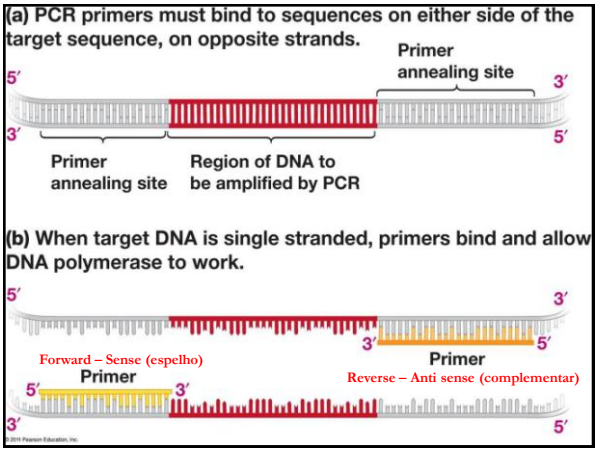
Reação em Cadeia da Polimerase

Método de amplificação de DNA sem uso de um organismo vivo



A técnica da PCR possui 3 etapas

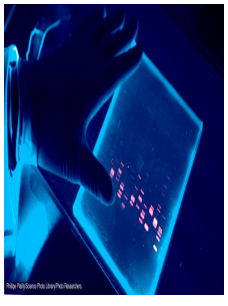




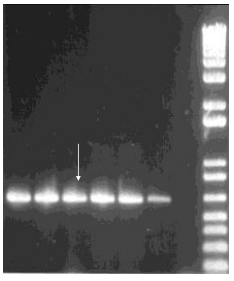
PCR convencional tem 3 processos

Ciclagem no termociclador

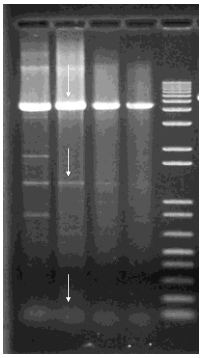
Aplicação no gel de agarose



Visualização no transiluminador



Bandas específicas e intensas



Bandas específicas e inespecíficas juntas

Mem Inst Oswaldo Cruz, Rio de Janeiro, Vol. 98(4): 451-454, June 2003 451

Prevalence of *Clostridium* spp. and *Clostridium difficile* in Children with Acute Diarrhea in São Paulo City, Brazil

Claudia EA Ferreira++, Viviane Nakano, Edison L Durigon, Mario J Avila-Campos+

Current Microbiology Vol. 53 (2006), pp. 113–117
DOI: 10.1007/s00284-005-0321-6

Current Microbiology
An International Journal

Evaluation of the Pathogenicity of the *Bacteroides fragilis* Toxin Gene Subtypes in Gnotobiotic Mice

Viviane Nakano,[1] Danielle A. Gomes,[2] Rosa M. E. Arantes,[3] Jacques R. Nicoli,[2] Mario J. Avila-Campos[1]

CLINICS 2011;66(4):543-547 DOI:10.1590/S1807-59322011000400004

CLINICAL SCIENCE

Antimicrobial resistance and prevalence of resistance genes in intestinal *Bacteroidales* strains

Viviane Nakano,[I] Amanda do Nascimento e Silva,[I] Victor Rafael Castillo Merino,[I] Hannah M. Wexler,[II,III] Mario Julio Avila-Campos[I]

Brazilian Journal of Microbiology 46, 4, 1141-1145 (2015)
ISSN 1678-4405
DOI: http://dx.doi.org/10.1590/S1517-838246420140728

Research Paper

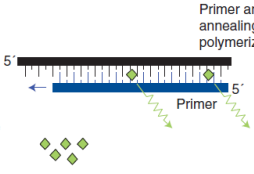
Enterotoxigenic and non-enterotoxigenic *Bacteroides fragilis* from fecal microbiota of children

Aline Ignacio, Miriam Rodriguez Fernandes, Mario Julio Avila-Campos, Viviane Nakano

PCR em tempo real (qPCR)

SYBR - Green

Unbound SYBR Green 1 DNA-binding dye in solution exhibits very little fluorescence. During primer extension and polymerization, SYBR Green 1 molecules become intercalated within the double-stranded DNA product, resulting in an increase in detected fluorescence



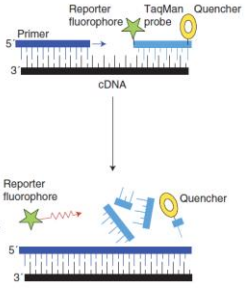
(Arya et al., 2005)

PCR em tempo real (qPCR)

Sonda de hidrólise

When the TaqMan probe is intact, the reporter and quencher stay close to each other, which prevents the emission of any fluorescence

The primer and TaqMan probe anneal to the complementary DNA strand following denaturation



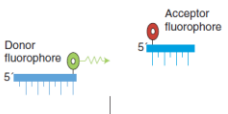
After hybridization and during the extension phase, the 5´ endonuclease activity of the Taq DNA polymerase cleaves the probe which separates reporter and quencher dyes and fluorescence is detected.

(Arya et al., 2005)

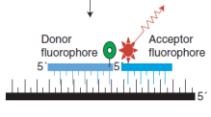
PCR em tempo real (qPCR)

Sonda de hibridização

One hybridization probe carries a donor fluorophore at its 3´ end and the other is labeled with an acceptor fluorophore at its 5´ end. During denaturation both hybridization probes remain separate in solution and any fluorescent emission from the donor fluorophore (e.g., green fluorescence, which occurs when excited by the LightCycler's light source) is disregarded by the detector.



During annealing the probes hybridize in a head-to-tail conformation, bringing the two dyes next to each other. Excitation of the donor leads to fluorescence resonance energy transfer to the acceptor resulting in a change of the fluorescent signal and emission of fluorescent light at a longer wavelength (red).



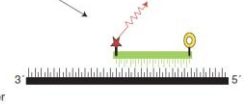
(Arya et al., 2005)

PCR em tempo real (qPCR)

Sonda de hibridização

Molecular beacons adopt a hairpin structure whilst free in solution. The hairpin structure consists of a stem built of two complementary arms and a loop that is complementary to the target sequence. This configuration helps the reporter fluorescent dye and the quencher to remain extremely close and therefore no fluorescence is detected.

During annealing, beacons hybridize to the target sequence, which changes their conformation and separates reporter and quencher dyes resulting in fluorescence being emitted.

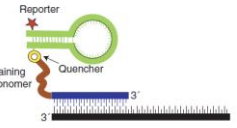


(Arya et al., 2005)

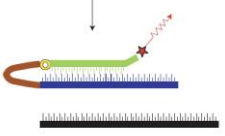
PCR em tempo real (qPCR)

Sonda de hibridização

The Scorpion probe adopts a hairpin loop structure that is linked to the 5′ end of a specific primer through a PCR stopper that prevents readthrough of the hairpin loop. During PCR, the Scorpion primers are extended to become part of the amplicon



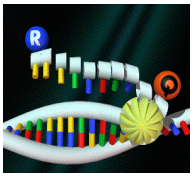
During annealing, the probe sequence in the Scorpion hairpin loop hybridizes to the newly formed complementary target sequence in the PCR product, separating the fluorophore and quencher dyes and leading to emission of a fluorescent signal. As the tail of the Scorpion and the PCR product are now part of the same DNA strand, the interaction is intramolecular



(Arya et al., 2005)

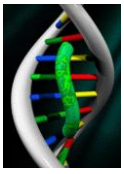
PCR em tempo real (qPCR)

TaqMan

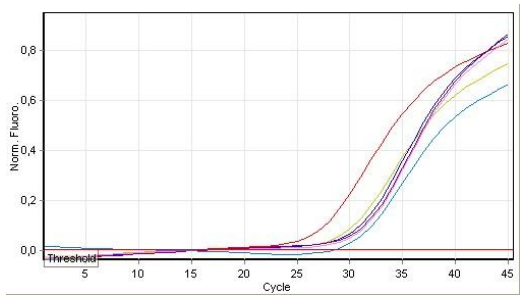


Termociclador

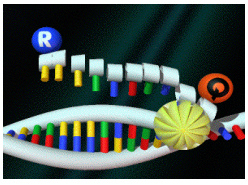
SYBR Green



Resultado

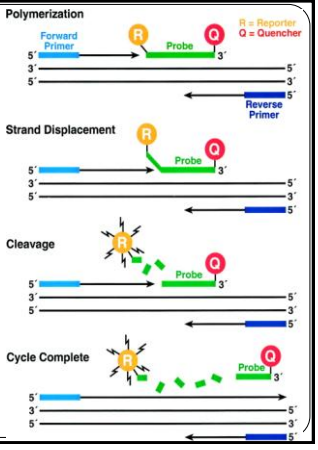


Sistema TaqMan



Sonda com fluoróforos
Q – silenciador
R – emite a fluorescência

Mais específico
Custo elevado



Característica da Sonda

> 10° C ΔTm dos *primer*

GC deve ser 20-80% (regiões ricas de GC)

Comprimento deve ser entre 23-30 bases

O sentido da sonda deve ser sempre *Forward* (preferencialmente)

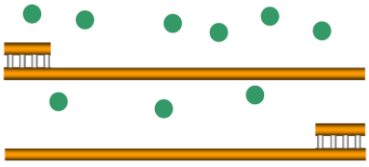
A sonda deve estar sempre próxima dos *primer*

O tamanho do *amplicon* deve ser de 150 pb

Exemplo de Sonda

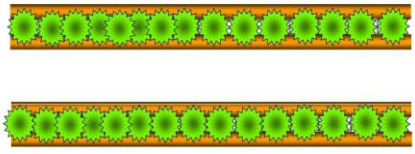
```
1 GTGATTTTTGTGGAAATTCTTTNGGGAATNGAAATNGAATCNAAGTGGCGAACGGGTGAG
61                                                AAACGACGATTAATACC
61 TAACACGTGAGCAACCTACCTTACACAGGGGGATAGCCGTTGGAAACGACGATTAATACC
121 ACATGAGAC 3' Forward
121                                               TCAAAGATTTATCGGTGTAAGAAGGGCT
121 ACATGAGACCACAGAATCGCATGNTATAGGGGTCAAAGATTTATCGGTGTAAGAAGGGCT
181 CGC 3' Probe
181 CGCGTCTGATTAGCTAGTTGGAAGGGTAAAGGCCTACCAAGGCGACGATCAGTAGCCGGT
241                                               GAGATGCCCTCCGTCG
241 CTGAGAGGATGAACGGCCACATTGGGACTGAGACACGGTCCAAACTCTACGGGAGGCAGC
301 TCA 5' Reverse
301 AGTGGGGAATATTGCACAATGGGGGGAA
```

Sistema SYBR Green

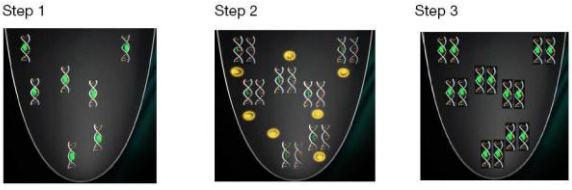


O corante fluorescente está na solução mãe (MasterMix)

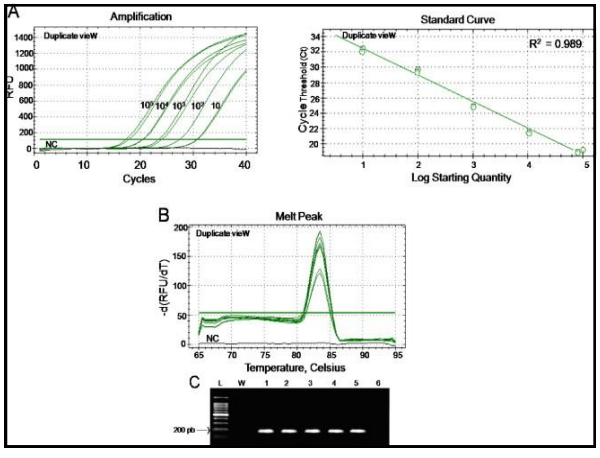
Menos específico
Custo mais baixo



Sistema SYBR Green



O corante liga-se imediatamente a todos os dsDNA
Amplificação dos genes alvos
O corante liga-se a cada nova cópia de dsDNA alvo



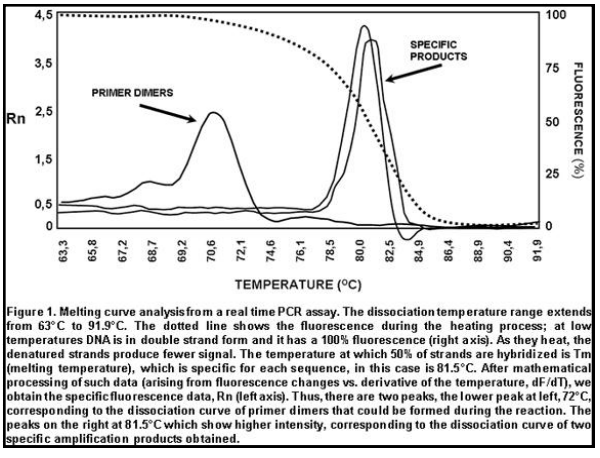
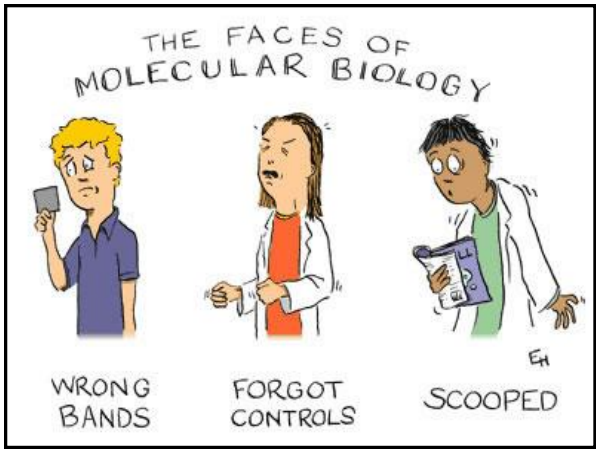


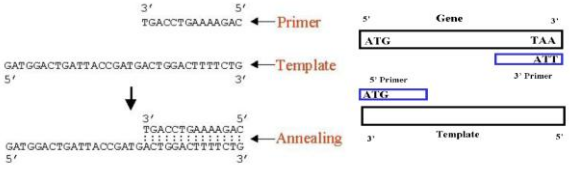
Figure 1. Melting curve analysis from a real time PCR assay. The dissociation temperature range extends from 63°C to 91.9°C. The dotted line shows the fluorescence during the heating process; at low temperatures DNA is in double strand form and it has a 100% fluorescence (right axis). As they heat, the denatured strands produce fewer signal. The temperature at which 50% of strands are hybridized is Tm (melting temperature), which is specific for each sequence, in this case is 81.5°C. After mathematical processing of such data (arising from fluorescence changes vs. derivative of the temperature, dF/dT), we obtain the specific fluorescence data, Rn (left axis). Thus, there are two peaks, the lower peak at left, 72°C, corresponding to the dissociation curve of primer dimers that could be formed during the reaction. The peaks on the right at 81.5°C which show higher intensity, corresponding to the dissociation curve of two specific amplification products obtained.



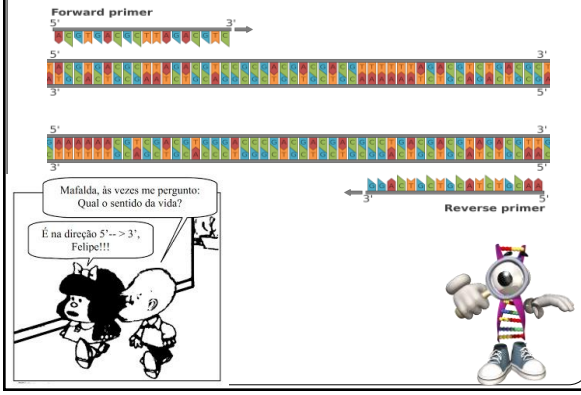
O que é um *primer*?

Sequencia de DNA que serve como ponto de início para extensão

DNA polimerase só pode estender uma fita pré existente



Lembre-se sempre da direção!



Considerações no desenho de *primer*

- ✓ Comprimento do *amplicon* (produto)
- ✓ Temperatura de *Melting* (Tm)
- ✓ Temperatura de Anelamento (TA)
- ✓ % GC
- ✓ Problemas na estrutura dos *primer*
- ✓ Comprimento dos *primer*
- ✓ Tipos de *primer*

Comprimento do *amplicon*

Recomendações:

PCR convencional – variado (≤ 2000 pb)

qPCR TaqMan – 150 pb (≥ 300 pb ↓)

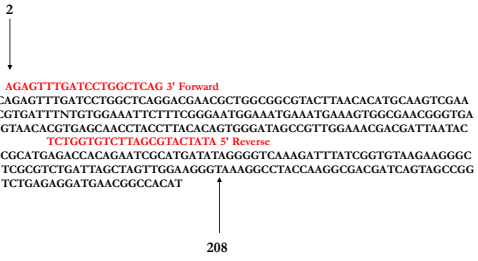
qPCR SYBR Green – 150/200 pb

Cálculo do comprimento:

(Posição do *primer reverse* – posição do *primer forward*) + 1 =

(última base) (primeira base)

Comprimento do *amplicon*



Cálculo do comprimento:

(Posição do *primer reverse* – posição do *primer forward*) + 1 = 207 pb

(208) (2)

Temperatura de *Melting*

T° na qual metade das fitas estão na forma simples e a outra na forma de dupla hélice.

Tm → % GC + comprimento *primer*

Diferença entre Tm dos *primer* → < de 3°

Cálculo básico: Tm = 4(G+C)+ 2(A+T)

AGAGTTTGATCCTGGCTCAG 3' Forward

Tm = 4(6+4) + 2(4+7) → 62° C

TCTGGTGTCTTAGCGTACTATA 5' Reverse

Tm = 4(5+4) + 2(4+9) → 62° C

Temperatura de Anelamento

T° na qual os *primer* se pareiam ao DNA molde. É calculada a partir da Tm.

Cálculo básico: Tanel = Tm – 4/5° C

↑ Tanel: pouco produto ou ausência de anelamento

↓ Tanel: anelamento inespecífico

Recomendável: teste com PCR gradiente

Conteúdo GC

40-60% GC → > Estabilidade

Ligações GC → > Estabilidade (>Tm)

Problemas Estruturais

Self-Dimer

```
4 bp, delta G = -6.6 kc/m (bad!) (worst= -36.6)
      5' GGGAAAATTCCAGGATCTAT 3'
         ||||  ||||
3' TATCTAGGACCTTAAAAGGG 5'

4 bp, delta G = -5.4 kc/m (bad!) (worst= -36.6)
      5' GGGAAAATTCCAGGATCTAT 3'
         ||||
3' TATCTAGGACCTTAAAAGGG 5'
```

Hairpin

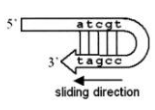
```
Oligo, 3 bp (Loop=4), delta G = -0.1 kc/m
      5'  GGGAAA
          |||
3'  TATCTAGGACCTTA

Oligo, 2 bp (Loop=3), delta G = 2.1 kc/m
      5'  GGGAA
          || |  A
3'  TATCTAGGACCTTA
```

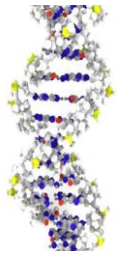
Cross-Dimer

```
forward primer
5' TATCTAGGACCTTAAAAGGG 3'
      |||||
   3' CATGGAAACGTAGGAGAC 5'
reverse primer
```

Hairpin structure



Comprimento do *primer*



Recomendado: 15-30 bases

> Comprimento do *primer* > possibilidade de ser bem específico e > Tm e Tanel.

Primer longos: > probabilidade de estruturas secundárias (*dimer, hairpin*)

Tipos de *primer*

Específicos

(5' AGAGTTTGATCCTGGCTCAG 3')

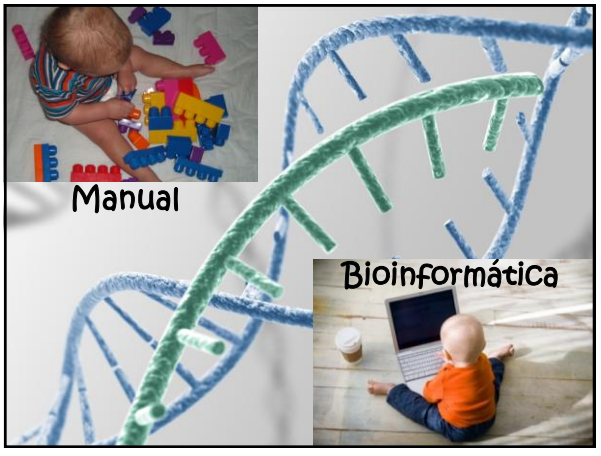
Degenerados

(5' ARYGTVTGSTNCTGWCTCAG 3')

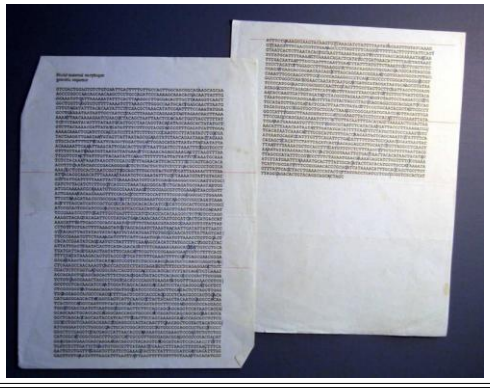
Código das bases degeneradas

Símbolo	Descrição	Bases representadas			
A	adenosine	A			
C	cytidine		C		
G	guanosine			G	
T	thymidine				T
U	uridine				U
W	weak	A			T
S	strong		C	G	
M	amino	A	C		
K	keto			G	T
R	purine	A		G	
Y	pyrimidine		C		T
B	not A		C	G	T
D	not C	A		G	T
H	not G	A	C		T
V	not T	A	C	G	
N	any base (not a gap)	A	C	G	T

(5' ARYGTVTGSTNCTGWCTCAG 3')



Desenhar manualmente



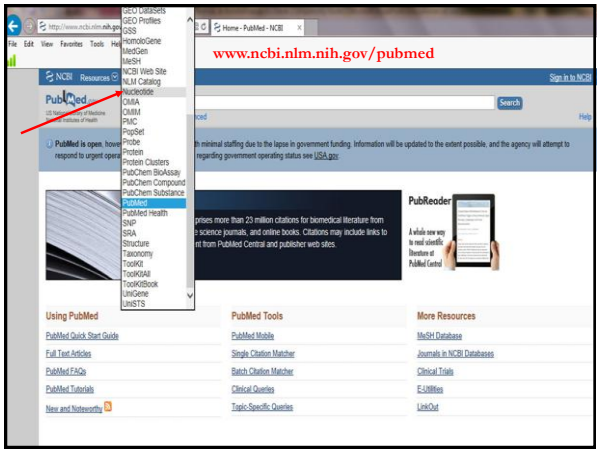
Bioinformática

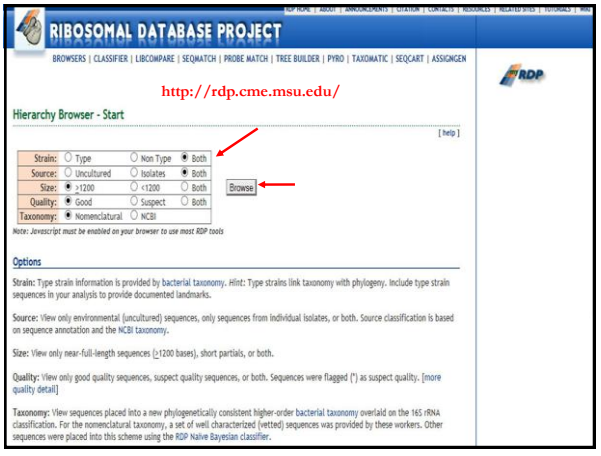
Sequencia do gene alvo
(NCBI, RDP entre outros banco de dados)

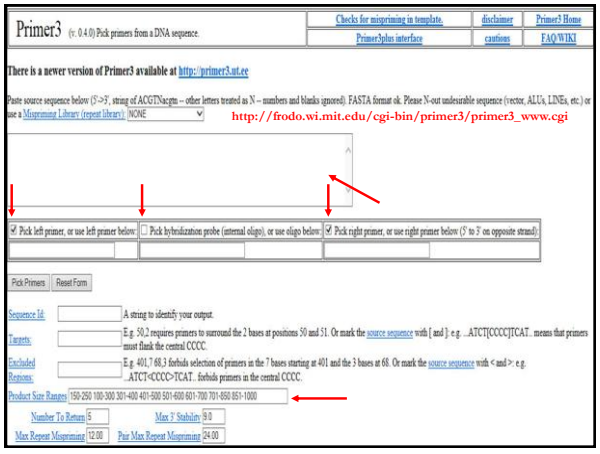
Primer Designer
(Primer3, BLAST tool)

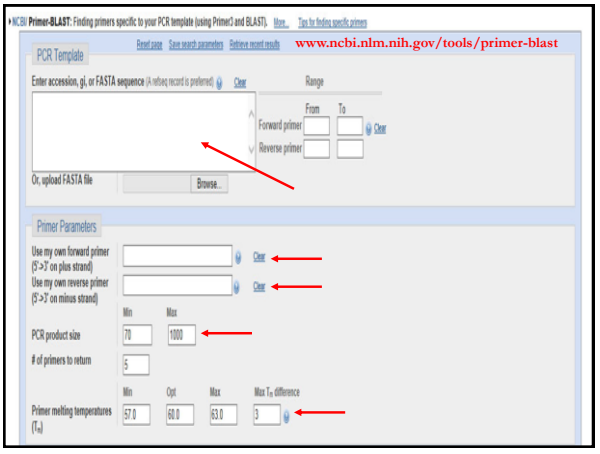
Análise *primer*
(NetPrimer, Sequence Manipulation Suite, IDT)

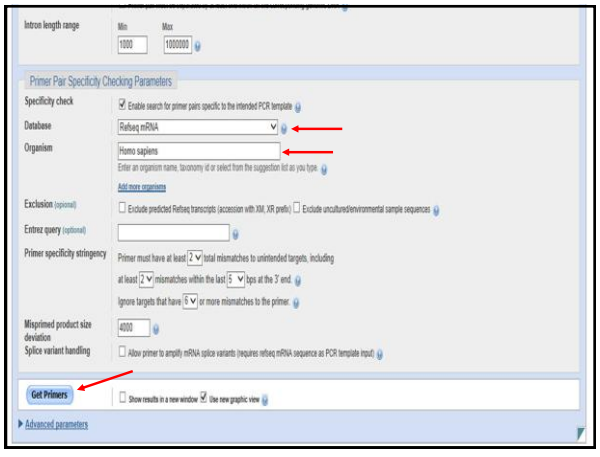
Especificidade *primer*
(BLAST tool)

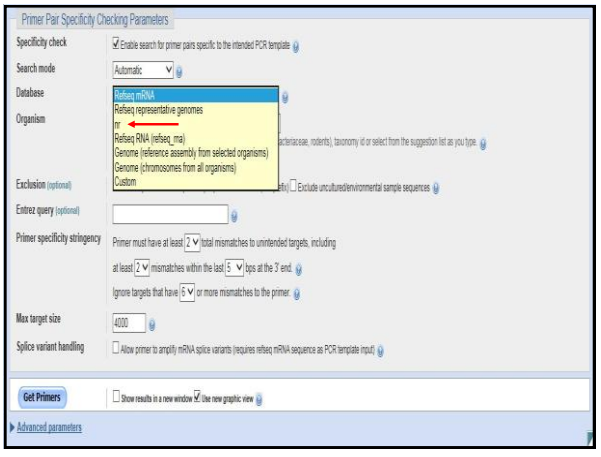


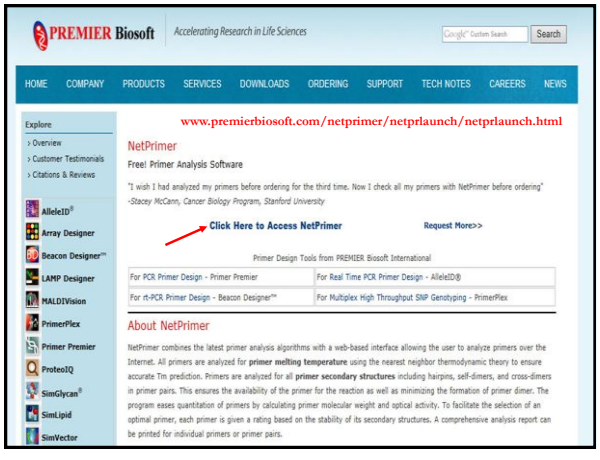



Primer3
There is a newer version of Primer3 available at http://primer3.ut.ee
http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi
Pick Primers
Reset Form


Primer-BLAST
www.ncbi.nlm.nih.gov/tools/primer-blast
PCR Template
Primer Parameters
PCR product size
# of primers to return


Primer Pair Specificity Checking Parameters
Specificity check
Database
Organism
Homo sapiens
Primer specificity stringency
Get Primers
Advanced parameters


Primer Pair Specificity Checking Parameters
Specificity check
Search mode
Database
Refseq mRNA
Refseq representative genomes
nr
Refseq RNA (refseq_rna)
Genome (reference assembly from selected organisms)
Genome (chromosomes from all organisms)
Custom
Organism
Exclusion (optional)
Entrez query (optional)
Primer specificity stringency
Max target size
Splice variant handling
Get Primers
Advanced parameters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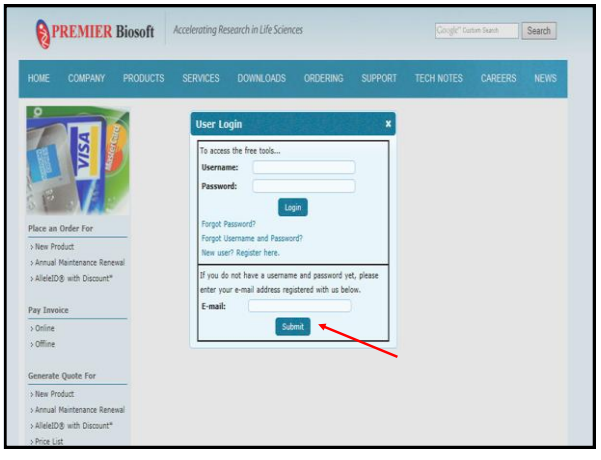
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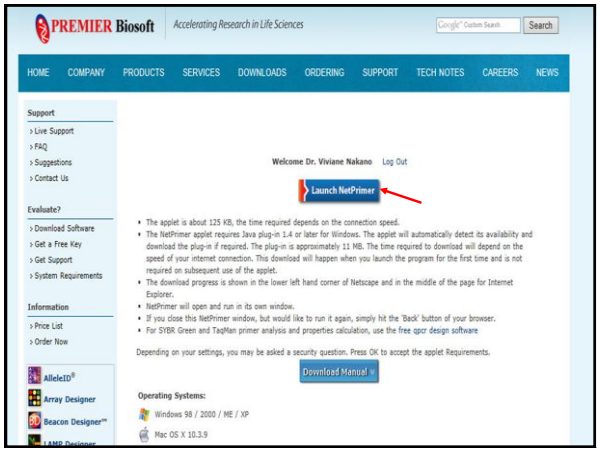
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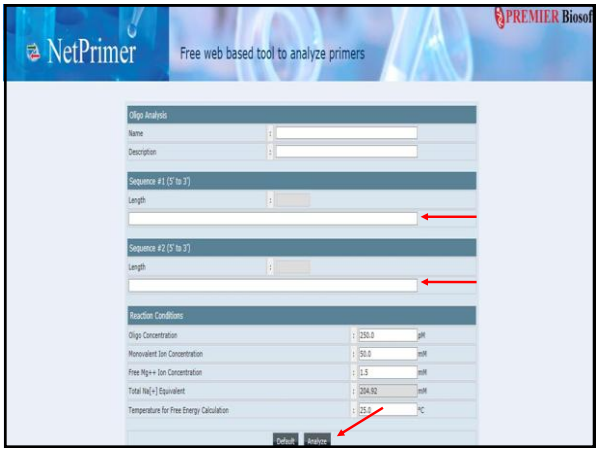
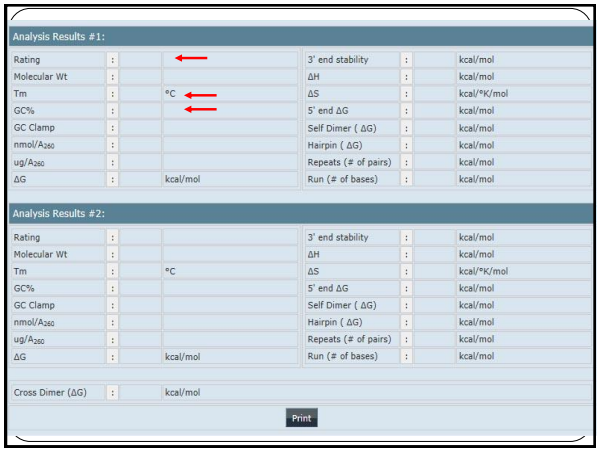
Welcome Dr. Viviane Nakano Log Out
Launch NetPrimer
The applet is about 125 KB, the time required depends on the connection speed.
The NetPrimer applet requires Java plug-in 1.4 or later for Windows. The applet will automatically detect its availability and download the plug-in if required. The plug-in is approximately 11 MB. The time required to download will depend on the speed of your internet connection. This download will happen when you launch the program for the first time and is not required on subsequent use of the applet.
The download progress is shown in the lower left hand corner of Netscape and in the middle of the page for Internet Explorer.
NetPrimer will open and run in its own window.
If you close this NetPrimer window, but would like to run it again, simply hit the 'Back' button of your browser.
For SYBR Green and TaqMan primer analysis and properties calculation, use the free qpcr design software
Depending on your settings, you may be asked a security question. Press OK to accept the applet Requirements.
Download Manual
Operating Systems:
Windows 98 / 2000 / ME / XP
Mac OS X 10.3.9


NetPrimer
Free web based tool to analyze primers
PREMIER Biosoft
Oligo Analysis
Name
Description
Sequence #1 (5' to 3')
Length
Sequence #2 (5' to 3')
Length
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
Default
Analyze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Tm °C
GC%
GC Clamp
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ug/A260
ΔG kcal/mol
3' end stability kcal/mol
ΔH kcal/mol
ΔS kcal/°K/mol
5' end ΔG kcal/mol
Self Dimer (ΔG) kcal/mol
Hairpin (ΔG) kcal/mol
Repeats (# of pairs) kcal/mol
Run (# of bases) kcal/mol
Analysis Results #2:
Rating
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Tm °C
GC%
GC Clamp
nmol/A260
ug/A260
ΔG kcal/mol
3' end stability kcal/mol
ΔH kcal/mol
ΔS kcal/°K/mol
5' end ΔG kcal/mol
Self Dimer (ΔG) kcal/mol
Hairpin (ΔG) kcal/mol
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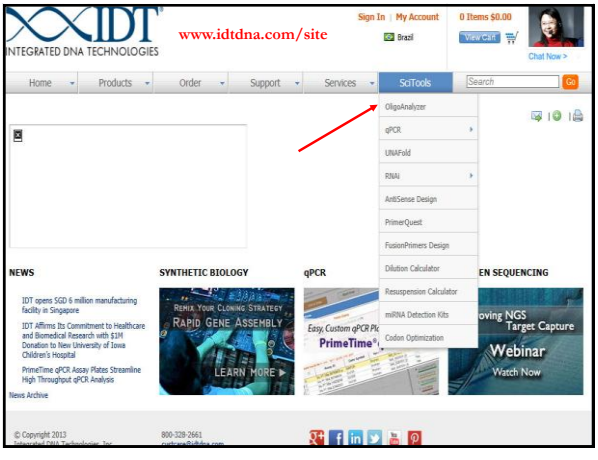
Run (# of bases) kcal/mol
Cross Dimer (ΔG) kcal/mol
Print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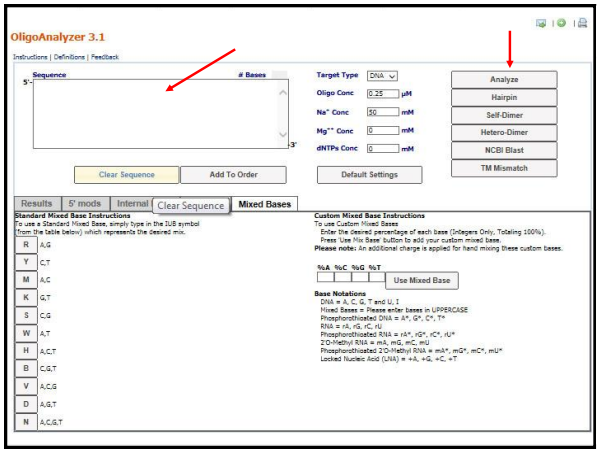
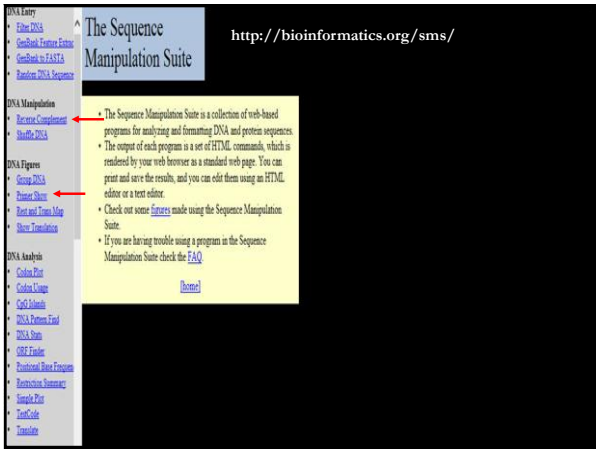

OligoAnalyzer 3.1
Instructions | Definitions | Feedback
Sequence
Bases
5'
3'
Target Type DNA
Oligo Conc 0.25 µM
Na+ Conc 50 mM
Mg++ Conc 0 mM
dNTPs Conc 0 mM
Analyze
Hairpin
Self-Dimer
Hetero-Dimer
NCBI Blast
TM Mismatch
Clear Sequence
Add To Order
Default Settings
Results
5' mods
Internal
Clear Sequence
Mixed Bases
Standard Mixed Base Instructions
To use a Standard Mixed Base, simply type in the IUB symbol from the table below) which represents the desired mix.
R A,G
Y C,T
M A,C
K G,T
S C,G
W A,T
H A,C,T
B C,G,T
V A,C,G
D A,G,T
N A,C,G,T
Custom Mixed Base Instructions
To use Custom Mixed Bases
Enter the desired percentage of each base (Integers Only, Totaling 100%).
Press 'Use Mix Base' button to add your custom mixed base.
Please note: An additional charge is applied for hand mixing these custom bases.
%A %C %G %T
Use Mixed Base
Base Notations
DNA = A, C, G, T and U, I
Mixed Bases = Please enter bases in UPPERCASE
Phosphorothioated DNA = A*, G*, C*, T*
RNA = rA, rG, rC, rU
Phosphorothioated RNA = rA*, rG*, rC*, rU*
2O-Methyl RNA = mA, mG, mC, mU
Phosphorothioated 2O-Methyl RNA = mA*, mG*, mC*, mU*
Locked Nucleic Acid (LNA) = +A, +G, +C, +T


DNA Entry
Fetch DNA
GenBank Feature Extract
GenBank to FASTA
Random DNA Sequence
DNA Manipulation
Reverse Complement
Shuffle DNA
DNA Figures
Group DNA
Primer Show
Restriction Map
Show Translation
DNA Analysis
Codon Plot
Codon Usage
CpG Islands
DNA Pattern Find
DNA Stats
ORF Finder
Positional Base Frequency
Restriction Summary
Simple Plot
TestCode
Translate
The Sequence Manipulation Suite
http://bioinformatics.org/sms/
The Sequence Manipulation Suite is a collection of web-based programs for analyzing and formatting DNA and protein sequences.
The output of each program is a set of HTML commands, which is rendered by your web browser as a standard web page. You can print and save the results, and you can edit them using an HTML editor or a text editor.
Check out some figures made using the Sequence Manipulation Suite.
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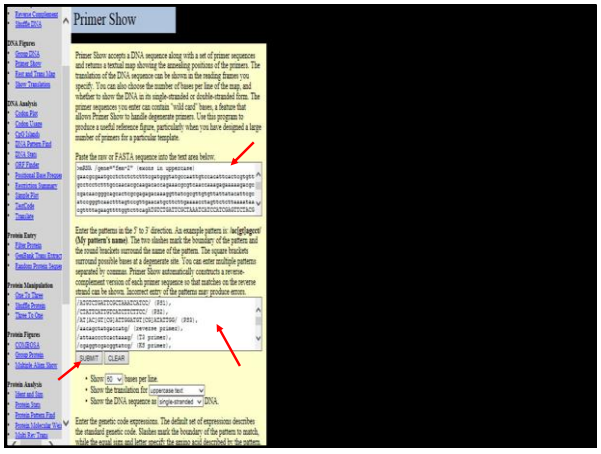
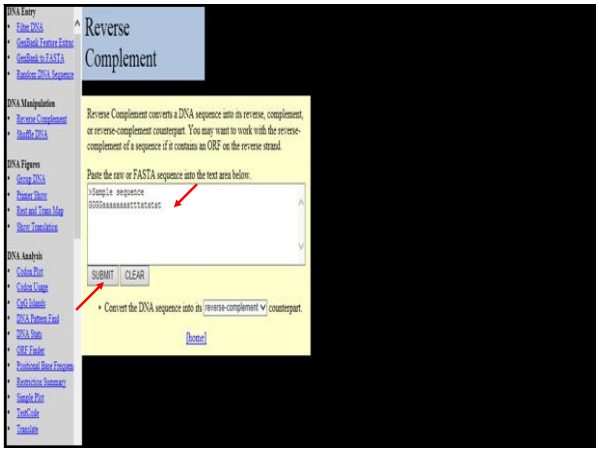
[home]





JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 2011, p. 416–418 Vol. 49, No. 1
0095-1137/11/$12.00 doi:10.1128/JCM.01556-10

Quantitative Detection of Enterotoxigenic *Bacteroides fragilis* Subtypes Isolated from Children with and without Diarrhea[▽]

V. R. C. Merino,[1] V. Nakano,[1] C. Liu,[2] Y. Song,[2] S. M. Finegold,[2,3,4] and M. J. Avila-Campos[1]*

Anaerobe Laboratory, Department of Microbiology, Institute of Biomedical Science, University of São Paulo, Av. Prof. Lineu Prestes 1374, 05508-900 São Paulo, SP, Brazil[1]; *VA Medical Center West Los Angeles, Los Angeles, California*[2]; *and Department of Medicine*[3] *and Department of Microbiology, Immunology and Molecular Genetics,*[4] *UCLA School of Medicine, Los Angeles, California*

110 crianças com diarreia e 150 crianças sem diarreia.

TaqMan – gene da toxina de *B. fragilis* (subtipos).

TABLE 2. Quantitative analysis of the different *bft* genes among ETBF strains from diarrhea and nondiarrhea fecal samples

Target gene	No. of positive samples (range of $\log_{10}$ no. of copies [mean ±SD])		*P* value
	Diarrhea	Nondiarrhea	
bft	10 (2.4 to 7.5 [4.5 ± 1.8])	7 (2.2 to 6.1 [3.9 ± 1.3])	0.46
bft-1	9 (1.1 to 7.3 [3.7 ± 1.8])	7 (2.2 to 5.6 [3.8 ± 1.2])	0.91
bft-3	1 (0.34)	0	

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Volume 2014, Article ID 594014, 4 pages
http://dx.doi.org/10.1155/2014/594014

Hindawi

Research Article

Genes Encoding Toxin of *Clostridium difficile* in Children with and without Diarrhea

Victor R. C. Merino,[1] Viviane Nakano,[1] Sydney M. Finegold,[2,3,4] and Mario J. Avila-Campos[1]

110 crianças com diarreia e 150 crianças sem diarreia.

TaqMan – genes 16S rRNA e das toxinas de *C. difficile*.

TABLE 2: Quantitative detection of *Clostridium difficile* and target genes in diarrhea and nondiarrhea samples.

Target gene	Diarrhea (n = 110)		Nondiarrhea (n = 150)		P
	Number (range of $\log_{10}$)	Mean ± SD	Number (range of $\log_{10}$)	Mean ± SD	
16S rRNA*	20 (1.2–6.9)	4.0 ± 1.7	37 (1.3–8.0)	4.8 ± 1.5	0.063
tcdA	1 (2.1)	ND	3 (1.3–7.6)	2.9 ± 1.3	ND
tcdB	0 (0)	ND	3 (3.2–5.3)	4.6 ± 1.2	ND
cdtA	1 (2.7)	ND	0 (0)	ND	ND
cdtB	1 (3.7)	ND	0 (0)	ND	ND

*Used to detect *C. difficile*.
ND: not determined.

Brazilian Journal of Microbiology 46, 4, 1135-1140 (2015)
ISSN 1678-4405
DOI: http://dx.doi.org/10.1590/S1517-838246420140665

Research Paper

High occurrence of *Fusobacterium nucleatum* and *Clostridium difficile* in the intestinal microbiota of colorectal carcinoma patients

Márcia H. Fukugaiti[1], Aline Ignacio[1], Miriam R. Fernandes[1], Ulysses Ribeiro Júnior[2], Viviane Nakano[1], Mario J. Avila-Campos[1]

[1]Departamento de Microbiologia, Universidade de São Paulo, São Paulo, SP, Brazil.
[2]Instituto do Câncer do Estado de São Paulo, São Paulo, SP, Brazil.

7 pacientes com carcinoma cólon retal e 10 pacientes saudáveis (controle).

SYBR – genes 16S rRNA (11 microrganismos diferentes).

Table 2 - Qualitative and quantitative analysis of oral and intestinal microorganisms from fecal samples.

Microorganisms	No. of positive samples (range of $\log_{10}$ no. of copies [mean ± SD])		p value
	Patients with carcinoma	Healthy patients	
Fusobacterium nucleatum	7 (3.5 to 8.0 [6.2 ± 1.5])	9 (1.0 to 6.4 [4.0 ± 1.5])	0.01*
Porphyromonas gingivalis	7 (3.1 to 8.1 [4.5 ± 1.7])	10 (2.1 to 8.7 [3.9 ± 1.9])	0.50
Prevotella intermedia	7 (3.9 to 9.6 [7.5 ± 2.2])	10 (3.2 to 10.4 [8.0 ± 2.3])	0.66
Clostridium difficile	7 (1.5 to 3.5 [2.5 ± 0.6])	8 (0.4 to 3.4 [1.6 ± 0.8])	0.04*
Clostridium perfringens	4 (3.7 to 4.8 [4.3 ± 0.4])	8 (2.1 to 4.7 [3.5 ± 1.1])	0.26
Bacteroides fragilis	6 (1.4 to 8.0 [4.8 ± 2.6])	6 (0.9 to 7.6 [4.4 ± 2.5])	0.78
Bacteroides vulgatus	7 (3.5 to 7.6 [5.9 ± 1.6])	10 (0.9 to 8.6 [5.3 ± 2.3])	0.57
Parabacteroides distasonis	7 (2.1 to 6.6 [4.9 ± 1.5])	9 (3.2 to 7.3 [5.0 ± 1.3])	0.98
Lactobacillus spp.	7 (5.3 to 7.8 [6.4 ± 1.0])	10 (3.5 to 7.4 [5.5 ± 1.3])	0.17
Bifidobacterium spp.	7 (4.3 to 10.2 [8.0 ± 1.8])	9 (3.9 to 9.3 [7.1 ± 1.6])	0.32
Escherichia coli	7 (2.8 to 9.5 [6.7 ± 2.4])	10 (1.7 to 9.5 [6.4 ± 2.5])	0.78

*Statistically significant values ($p < 0.05$).

Clin Microbiol Infect 2016; 22: 258.e1–258.e8

http://dx.doi.org/10.1016/j.cmi.2015.10.031

Correlation between body mass index and faecal microbiota from children

A. Ignacio[1], M. R. Fernandes[1], V. A. A. Rodrigues[1], F. C. Groppo[2], A. L. Cardoso[3], M. J. Avila-Campos[1] and V. Nakano[1]

1) Anaerobe Laboratory, Department of Microbiology, Institute of Biomedical Science, University of Sao Paulo, Sao Paulo, 2) Campinas State University, Campinas and 3) Institute of Children, Faculty of Medicine, University of Sao Paulo, Sao Paulo, SP, Brazil

Três grupos crianças: 30 obesos, 24 sobrepeso e 30 eutróficos.

SYBR – genes 16S rRNA (6 microrganismos diferentes).

TABLE 4. Bacterial prevalence and quantification verified in faeces of obese, overweight and lean children by quantitative PCR

	Obese (n = 30)	Overweight (n = 24)	Lean (n = 30)	Total (n = 84)	p value
Presence of genus or species[a]					
Bacteroides fragilis group	30 (100%)	24 (100%)	30 (100%)	84 (100%)	ND
Clostridium Cluster I	29 (96.6%)	24 (100%)	30 (100%)	83 (98.8%)	ND
Bifidobacterium spp.	28 (93.3%)	24 (100%)	30 (100%)	82 (97.6%)	ND
Lactobacillus spp.	29 (96.6%)	24 (100%)	27 (90%)	80 (95.2%)	ND
Escherichia coli	30 (100%)	23 (95.8%)	30 (100%)	83 (98.8%)	ND
Methanobrevibacter smithii	20 (66.6%)	21 (87.5%)	27 (90%)	68 (80.9%)	0.044
Quantitative determination ($\log_{10}$ copies/g faeces)[b]					
Bacteroides fragilis group	9.2 (9–9.6)[c]	9.1 (8.9–9.5)[c]	8.9 (8.7–9.7)	9.1 (8.9–9.7)	0.015
Clostridium Cluster I	7 (2.8–8.4)	6.9 (5–8.4)	8.4 (8.1–8.8)	8.1 (6.1–8.5)	0.010
Bifidobacterium spp.	7 (6.6–8.6)	6.8 (6.2–9.8)	7.6 (7.2–8.9)[c]	7.3 (6.6–9.8)	0.042
Lactobacillus spp.	5.7 (5.2–7.8)[c]	5.5 (5.3–7.5)[c]	5.2 (4.8–6.2)	5.5 (5–7.8)	0.022
Escherichia coli	7.3 (6.8–8.5)	7.7 (6.6–9.2)	7.3 (6.9–9.4)	7.3 (6.9–9.4)	0.672
Methanobrevibacter smithii	4.1 (0–8.4)	4.5 (3.9–8.6)	4.5 (3.8–8.8)	4.4 (3.7–8.8)	0.262

ND, without sufficient positive samples to perform the chi-square test.
[a]Values noted as number (percentage), chi-square test.
[b]Data were presented as median (interquartile range; IQR); differences among three groups were compared using Kruskal–Wallis test (Dunn post-test).
[c]$p < 0.05$ indicated significant differences as compared with the lean group.
[d]$p < 0.05$ indicated significant differences compared with the obese and overweight groups.

MDR-2015-0320-ver9-Fernandes_1P
Type: research-article
EPIDEMIOLOGY

MICROBIAL DRUG RESISTANCE
Volume 00, Number 00, 2016

DOI: 10.1089/mdr.2015.0320

Alterations of Intestinal Microbiome by Antibiotic Therapy in Hospitalized Children

Miriam R. Fernandes,[1,*] Aline Ignacio,[1,*] Viviane A.A. Rodrigues,[1] Franciso C. Groppo,[2] Ary L Cardoso,[3] Mario J. Avila-Campos,[1] and Viviane Nakano[1]

Dois grupos crianças: 30 antibioticoterapia e 30 controles (sem antibióticos.

SYBR – genes 16S rRNA (12 microrganismos diferentes).

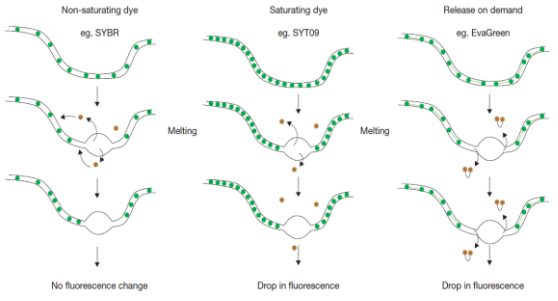
TABLE 3. BACTERIAL PREVALENCE AND QUANTIFICATION VERIFIED IN FECES OF ANTIBIOTIC-TREATED AND CONTROL CHILDREN BY qPCR

	Antibiotic-treated ($n=31$)	*Control* ($n=30$)	*Total* ($n=61$)	p
Presence of genus, species, or phylum[a], n (%)				
Bifidobacterium spp.	29 (93.5)	30 (100)	59 (96.7)	0.4918
Lactobacillus spp.	31 (100)	27 (90)	58 (95)	0.1128
B. vulgatus	31 (100)	30 (100)	61 (100)	ND[b]
B. fragilis	29 (93.5)	30 (100)	59 (96.7)	0.4918
P. distasonis	30 (96.7)	29 (96.6)	59 (96.7)	1
P. merdae	31 (100)	29 (96.6)	60 (98.3)	0.4918
C. perfringens	31 (100)	29 (96.6)	60 (98.3)	0.4918
C. difficile	26 (83.8)	29 (96.6)	55 (90.1)	0.1953
E. coli	31 (100)	30 (100)	61 (100)	ND[b]
M. smithii	19 (61.2)	28 (93.3)	47 (77)	**0.0050**
Bacteroidetes	31 (100)	30 (100)	61 (100)	ND[b]
Firmicutes	31 (100)	30 (100)	61 (100)	ND[b]
Quantitative determination ($\log_{10}$ copies/g feces)[c]				
Bifidobacterium spp.	4.62 (4.0–9.59)[d]	7.29 (6.51–8.95)	6.52 (4.43–9.59)	**0.0002**
Lactobacillus spp.	5.91 (5.53–8.86)[d]	5.52 (4.87–6.95)	5.71 (5.17–8.86)	**0.0092**
B. vulgatus	4.29 (3.08–8.74)	7.12 (5.91–8.88)	6.81 (4.10–8.88)	0.0901
B. fragilis	4.69 (3.18–8.11)	6.6 (5.29–8.53)	5.83 (3.96–8.53)	0.0055
P. distasonis	4.12 (3.02–8.28)	3.82 (2.96–7.86)	3.98 (3–8.28)	1
P. merdae	4.58 (3.53–8.74)	7.27 (5.52–8.38)	6.43 (3.9–8.74)	0.1700
C. perfringens	4.37 (3.6–6.35)[d]	5.84 (5.47–6.76)	5.45 (3.88–6.76)	**<0.0001**
C. difficile	1.67 (0.75–5.51)	5 (2–5.85)	2.75 (0.95–5.85)	0.0700
E. coli	5.89 (4.85–9.61)[d]	7.68 (7.11–9.28)	7.54 (5.47–9.61)	**0.0268**
M. smithii	4.29 (0–8.22)[d]	4.51 (4.05–8.98)	4.44 (3.56–8.98)	**0.0444**
Bacteroidetes	8.9 (7.72–10.54)	8.92 (8.37–9.78)	8.92 (8.42–10.53)	0.7613
Firmicutes	7.86 (7.44–8.84)[d]	8.42 (8.11–9.14)	8.13 (7.77–9.14)	**0.0009**

Significance levels in bold ($p<0.05$).
[a]Values noted as number (percentage), Fisher's exact test.
[b]ND: Without sufficient positive samples to perform the Fisher's exact test.
[c]Data are presented as median (interquartile range); differences among two groups are compared using Mann–Whitney test.
[d]$p<0.05$ indicated significant differences as compared with the control group.
qPCR, quantitative polymerase chain reaction.

High Resolution Melting (HRM)

✓ **Técnica – corantes saturados intercalantes**



High Resolution Melting (HRM)

✓ **Corantes saturados utilizados:**

LCG Green

SYTO Green

Eva Green

Chromofly

✓ **Vantagens:**

Custo efetivo – baixo consumo de reagentes

Simples e rápido

Alta acurácia - genotipagem

High Resolution Melting (HRM)

✓ **Aplicação:**

SNP Genotipagem

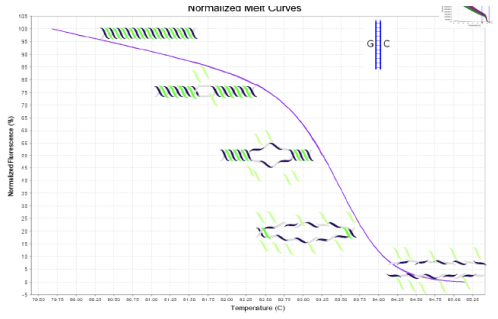
Mapeamento de DNA

Identificação de espécies microbianas

✓ **Desenho dos primers:**

Amplicon – 150 bp a 300 bp (< melhor)

High Resolution Melting (HRM)



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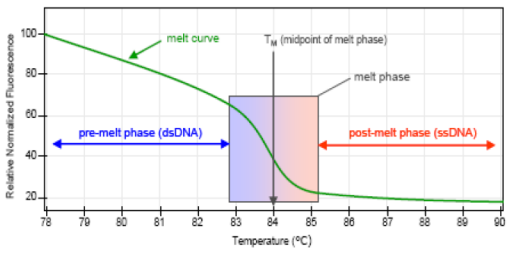

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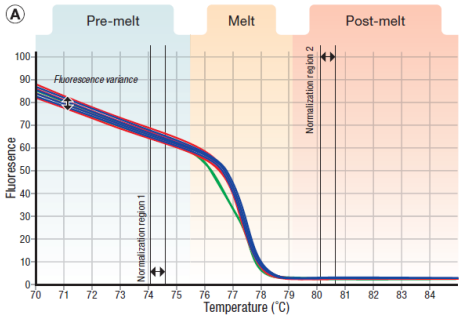

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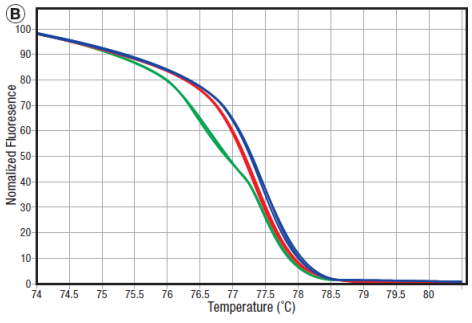
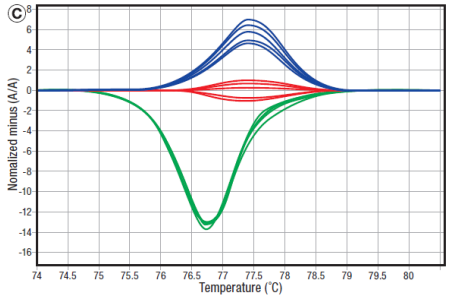


High Resolution Melting (HRM)



Journal of Microbiological Methods 89 (2012) 87–94

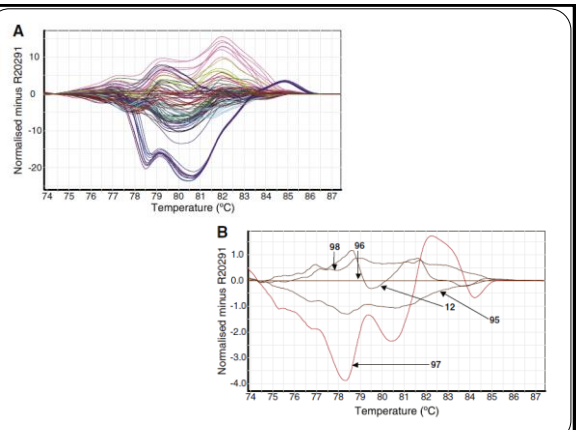
Contents lists available at SciVerse ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

High resolution melt analysis to track infections due to ribotype 027 *Clostridium difficile*

Danilla Grando [a,*], Mohamed M. Said [a], Barrie C. Mayall [b], Volker Gurtler [a,b]

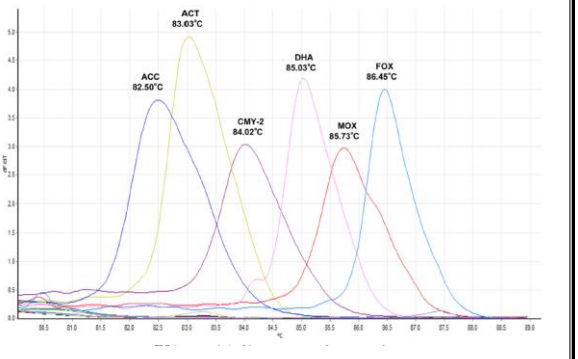


JCM
Journals.ASM.org

Multiplex High-Resolution Melting Analysis as a Diagnostic Tool for Detection of Plasmid-Mediated AmpC β-Lactamase Genes

Chelsie N. Geyer, Nancy D. Hanson

Creighton University School of Medicine, Department of Medical Microbiology and Immunology, Center for Research in Anti-Infectives and Biotechnology, Omaha, Nebraska, USA

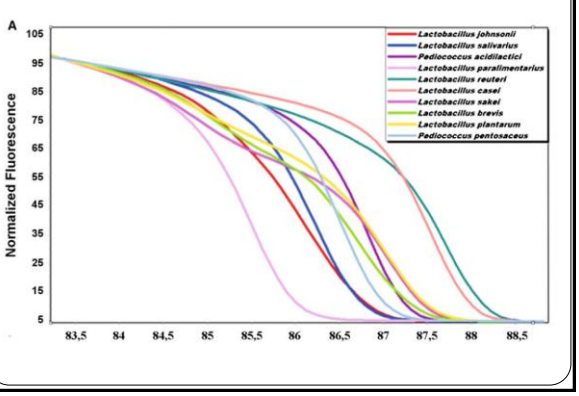


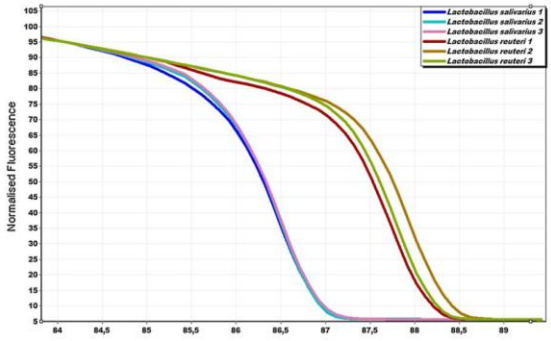
Eur Food Res Technol
DOI 10.1007/s00217-013-2134-3

ORIGINAL PAPER

Identification of lactic acid bacteria isolated from poultry carcasses by high-resolution melting (HRM) analysis

Ioannis Sakaridis · Ioannis Ganopoulos · Nikolaos Soultos · Panagiotis Madesis · Athanasios Tsaftaris · Anagnostis Argiriou





Sequenciamento

Sequenciamento

- ✓ **Primeria – Sanger (método eletroforese)**
- ✓ **Segunda – Pirosequencimento**
- ✓ **Terceira – Sequenciamento por síntese**
- ✓ **Quarta – Sequenciamento enzimático**

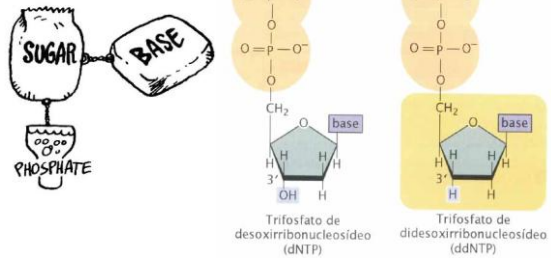
Sequenciamento de Nova Geração (NGS)

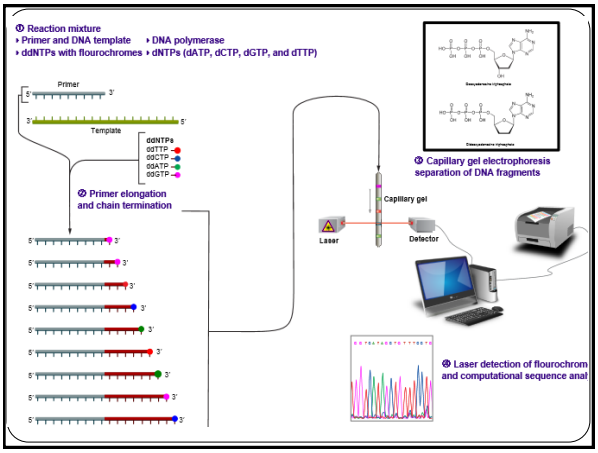
Next Generation Sequencing

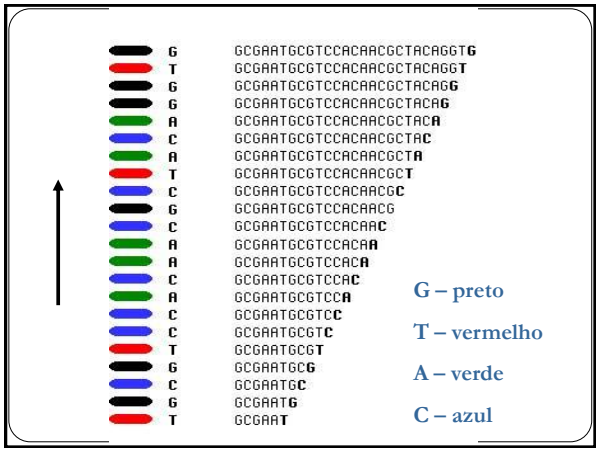
Sequenciamento Sanger

Método enzimático, dideoxi ou de término de cadeia

Síntese enzimática de uma fita complementar, cujo crescimento é interrompido pela adição de um dideoxinucleotídeo



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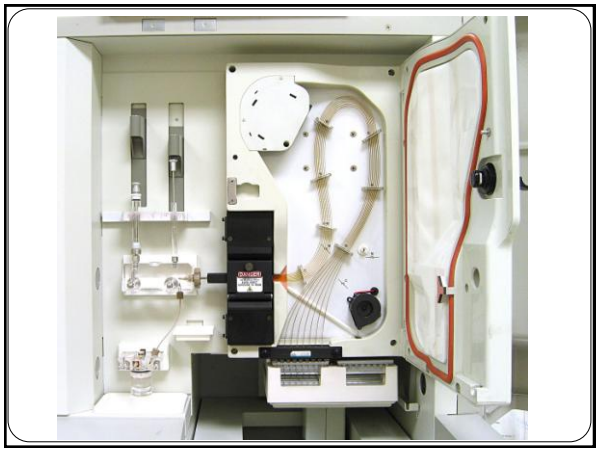
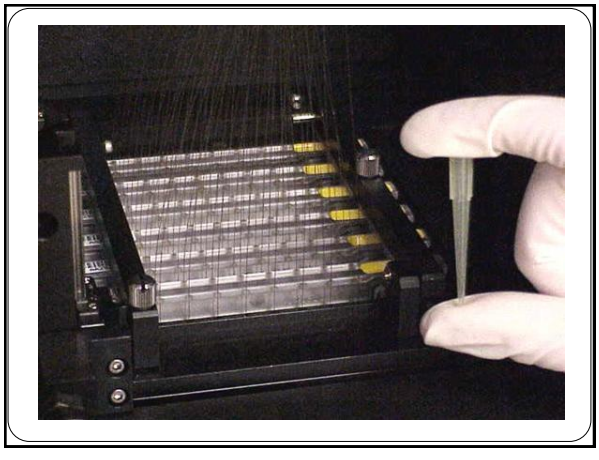
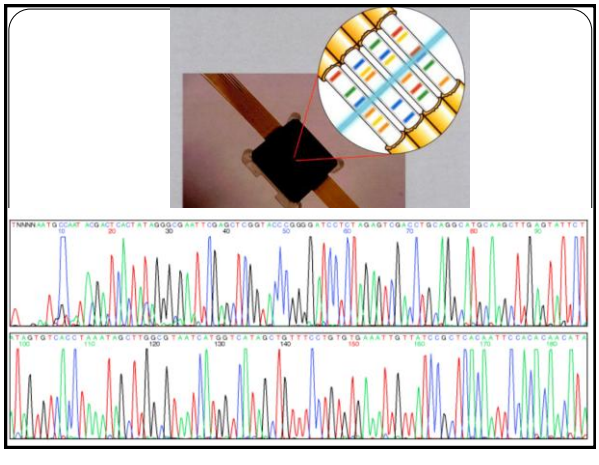
T GCGAATGCGTCCACAACGCTACAGGT
G GCGAATGCGTCCACAACGCTACAGG
G GCGAATGCGTCCACAACGCTACAG
A GCGAATGCGTCCACAACGCTACA
C GCGAATGCGTCCACAACGCTAC
A GCGAATGCGTCCACAACGCTA
T GCGAATGCGTCCACAACGCT
C GCGAATGCGTCCACAACGC
G GCGAATGCGTCCACAACG
C GCGAATGCGTCCACAAC
A GCGAATGCGTCCACAA
A GCGAATGCGTCCACA
C GCGAATGCGTCCAC
A GCGAATGCGTCCA
C GCGAATGCGTCC
C GCGAATGCGTC
T GCGAATGCGT
G GCGAATGCG
C GCGAATGC
G GCGAATG
T GCGAAT
G – preto
T – vermelho
A – verde
C – azul





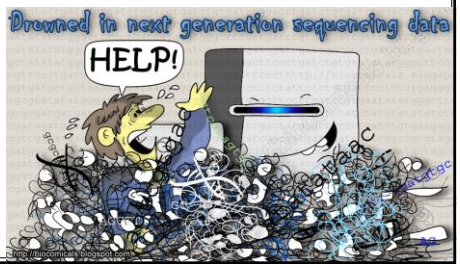



Yeah! How long are we gonna wait in the line?
Be patient! you should have seen this line 10 years ago!
How long?!
Calm down man!
Next Generation Sequencing Facility

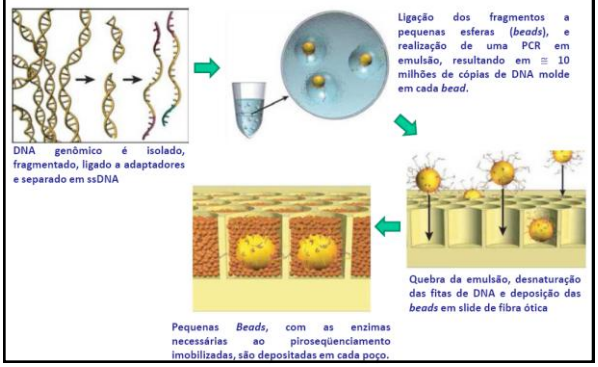
Pirosequenciamento

Método enzimático com 4 enzimas:

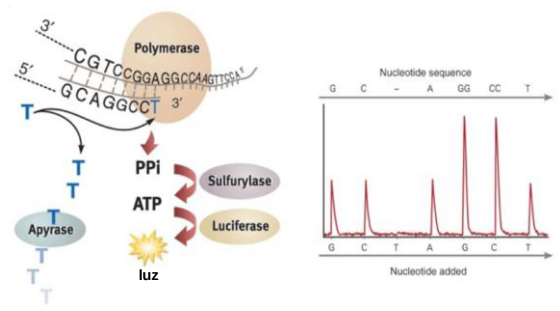
- DNA polimerase
- Sulfurilase
- Luciferase
- Apirase



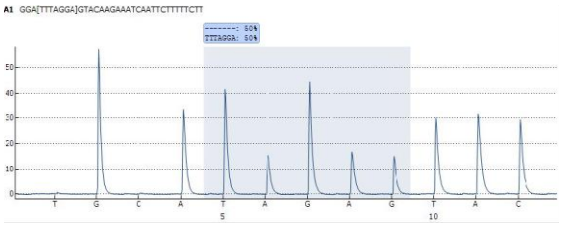
Pirosequenciamento



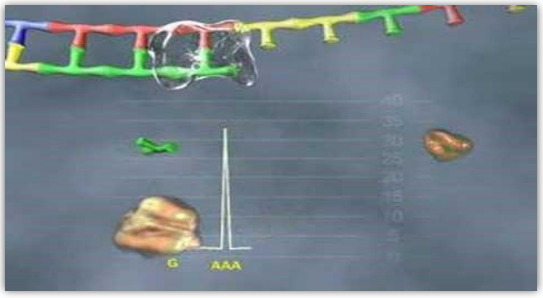
Pirosequenciamento



Pirograma



Pirosequenciamento



https://www.youtube.com/watch?v=nFfgWGFe0aA

Diferenças

Sanger

- 1 milhão de pb em 24 h;
- Reads de ≅ 700 bp;
- 6 meses de sequenciamento, 24 h por dia para sequenciar o genoma de um fungo.

Pirosequenciamento

- 25 milhão de pb em 24 h (100 x);
- Reads de ≅ 100 bp;
- 24 h para sequenciar o genoma de um fungo.

Sequenciamento por Síntese (NGS)

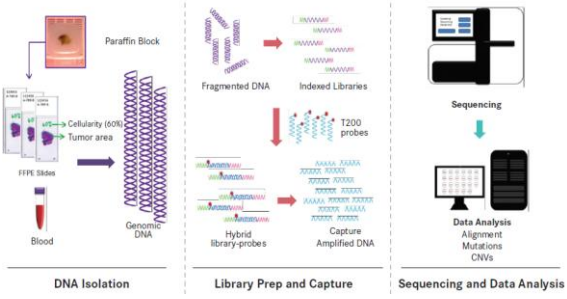
✓ **Acúmulo enorme de dados**

	PGM	MiSeq
Output	10 MB–100 MB	120 MB–1.5 GB
Read length	~200 bp	Up to 2 × 150 bp
Sequencing time	2 hours for 1 × 200 bp	3 hours for 1 × 36 single read 27 hours for 2 × 150 bp pair end read
Sample preparation time	8 samples in parallel, less than 6 hrs	As fast as 2 hrs, with 15 minutes hand on time
Sequencing method	semiconductor technology with a simple sequencing chemistry	Sequencing by synthesis (SBS)
Potential for development	Various parameters (read length, cycle time, accuracy, etc.)	Limited factors, major concentrate in flowcell surface size, insert sizes, and how to pack cluster in tighter
Input amount	μg	Ng (Nextera)
Data analysis	Off instrument	On instrument

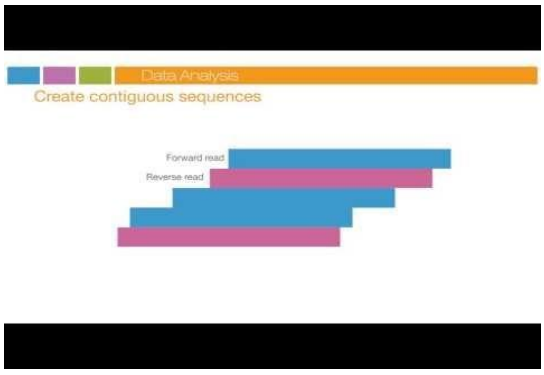
Liu et al., 2015

Sequenciamento por Síntese (NGS)

✓ **Workflow**



Basho et al., 2015



https://www.youtube.com/watch?v=womKfikWlxM

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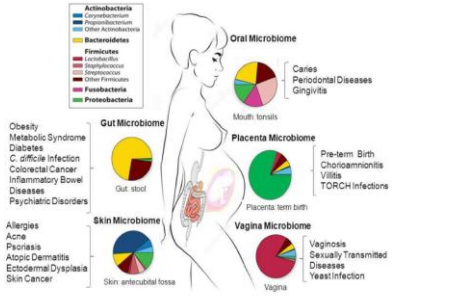


FIGURE 1 | Taxonomic distribution, prevalence and abundance of microbial taxa that inhabit healthy human body sites as defined in the human microbiome projects (HMP). The colored rectangles denote phylum/class and genera. Clinical studies of the microbiome will help to elucidate the link between microbes and the promotion of a large number of diseases and pathological conditions as shown in the figure. The images were adapted from NIH HMP (http://www.hmpdacc.org/) and National Human Genome Research Institute (https://www.genome.gov/). TORCH, Toxoplasmosis, Other infections (coxsackievirus, HIV, syphilis, etc), Rubella, Cytomegalovirus, Herpes simplex.

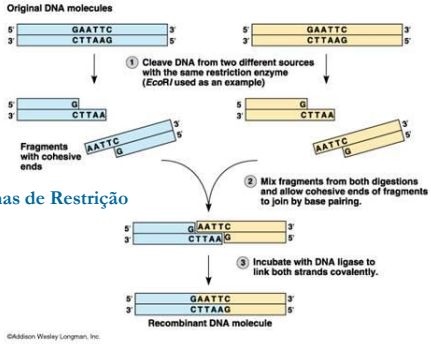
Belizário and Napolitano, 2015

PFGE (Pulsed-field gel electrophoresis)

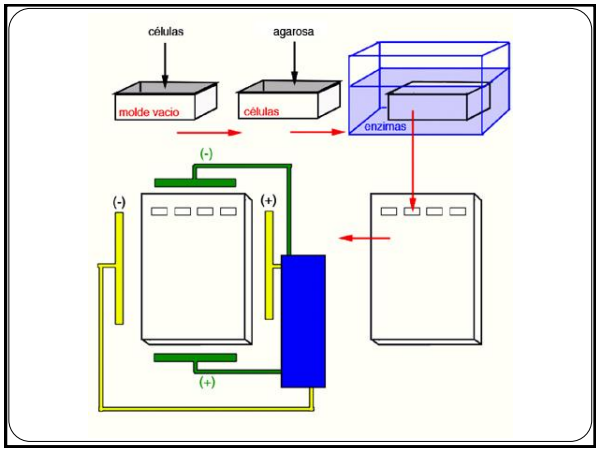
PFGE (Pulsed-field gel electrophoresis)

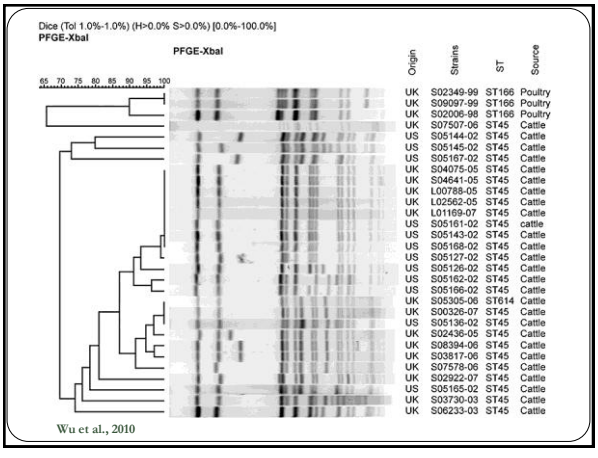
- Padrão "ouro";
- Campo elétrico (múltiplos eletrodos) ao redor do gel;
- Molécula íntegra tratada com endonucleases de restrição;
- Permite a separação de fragmentos grandes (10 a 800 kb);
- Superior a muitos outros métodos para tipagem.

Tratamento com endonucleases



Enzimas de Restrição





Wu et al., 2010

Random Amplified Polymorphic DNA (RAPD-PCR)

Random amplified polymorphic DNA

- Primers – arbitários, pequenos até 10 bases;
- 37 ºC a 42 ºC – ocorre hibridização;
- Amplicons são separados por eletroforese;
- Visualização após coloração com brometo de etídio (EtBr) → luz UV;
- Problemas relacionados à reprodutibilidade e padronização;

GTCCGTAATG

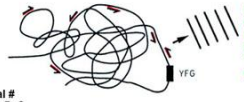
Mutações de ponto

CAGGCATTAC AATGGCTACCCAAT CAGGCATTAC CCGTA CACCCATTAC
GTCCGTAATG GTCCGTAATG GT

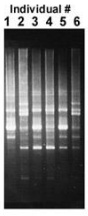
RAPD (Randomly Amplified Polymorphic DNA) marker

Basic technique

Half arrows: 10-nucleotide primer that will find an identical matching site at many different locations in the whole genome (black blob). Only primers that point towards eachother AND are in close enough proximity will result in a product during PCR-amplification reactions.



PCR amplified fragment (RAPD marker linked to YFG, Your Favorite Gene)



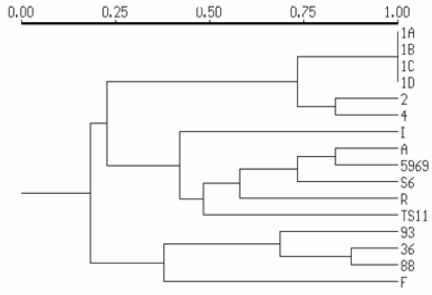
Example of a RAPD agarose gel. A mixture of many different PCR-amplified fragments has been separated in size by electrophoresis.

Planilha

PESO MOLECULAR	CEPA 1A	CEPA 2A	CEPA 1B	CEPA 4D	CEPA 1C	CEPA 6D	CEPA 1D
1000 pb	1	1	1	0	1	0	1
900 pb	0	1	0	0	0	0	0
800 pb	0	0	0	0	0	0	0
700 pb	1	0	1	0	1	1	1
600 pb	0	0	0	1	0	1	0
500 pb	0	0	0	0	0	1	0
400 pb	0	0	0	0	0	0	0
300 pb	1	0	1	1	1	1	1
200 pb	0	0	0	0	0	0	0

Programa NTSys – coeficiente de similaridade de Jaccard.

Filogenia - Dendrograma



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Anaerobe 13 (2007) 1–5

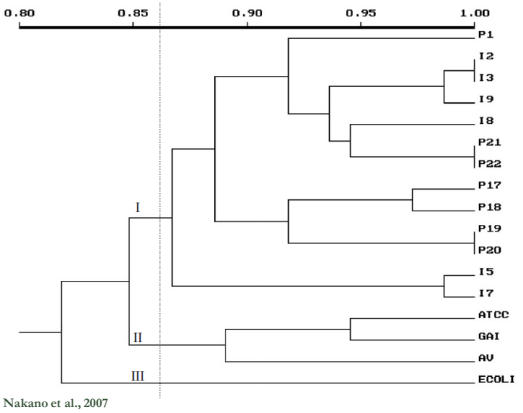
Anaerobe

www.elsevier.com/locate/anaerobe

Clinical Microbiology

bft gene subtyping in enterotoxigenic *Bacteroides fragilis* isolated from children with acute diarrhea

Viviane Nakano[a], Tânia A.T. Gomes[b], Mônica A.M. Vieira[b], Rita de Cássia Ferreira[c], Mario Julio Avila-Campos[a,*]

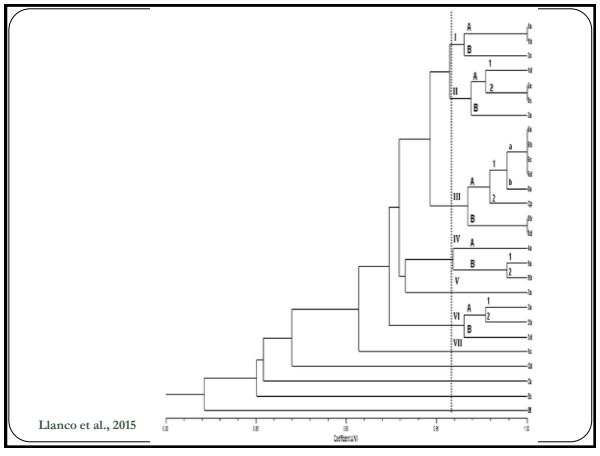


Nakano et al., 2007

Curr Microbiol (2015) 70:330–337
DOI 10.1007/s00284-014-0722-5

Sialidase Production and Genetic Diversity in *Clostridium perfringens* Type A Isolated from Chicken with Necrotic Enteritis in Brazil

Luis A. Llanco · Viviane Nakano · Mario J. Avila-Campos

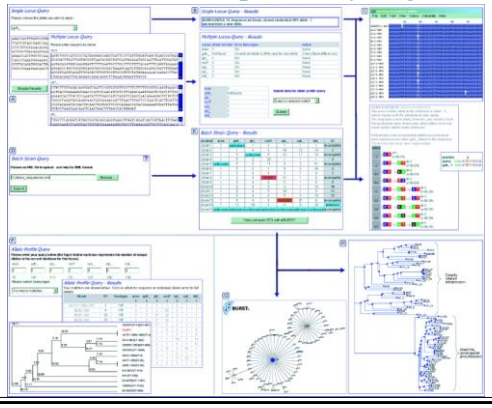


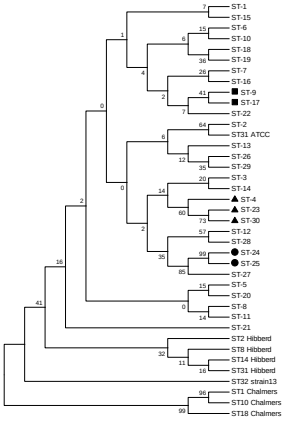
Llanco et al., 2015

Multilocus Sequence Typing

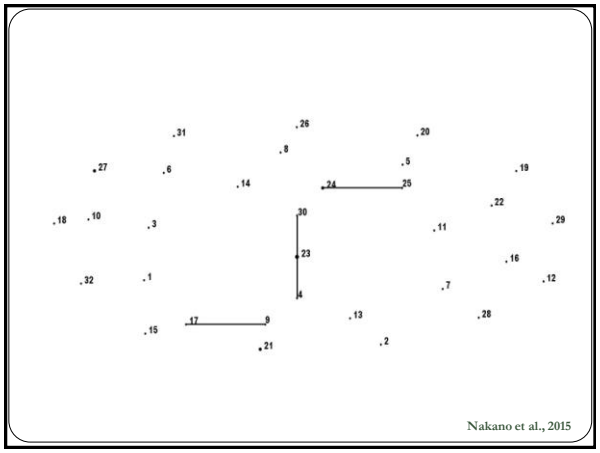
- ✓ Genes constitutivos (housekeeping) - > 6 genes;
- ✓ Sequenciamento de todos os genes em ambos sentidos;
- ✓ Análise e alinhamento - Concatenar;
- ✓ Perfil alélico – Sequence Type;
- ✓ BioNumerics – MEGA6 - eBURST;

Multilocus Sequence Typing

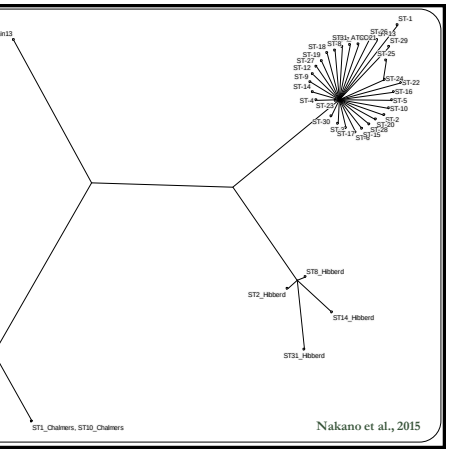




Nakano et al., 2015



Nakano et al., 2015



Nakano et al., 2015