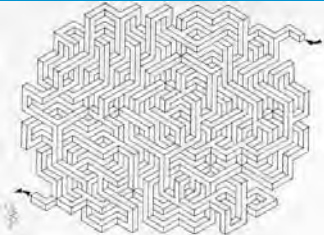


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## DNA and RNA isolation and Sample processing



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### Goals of sample processing

- Concentration
- Homogenisation
- Removal of inhibiting compounds
- Extra requirements depending on subsequent analysis

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### PCR inhibiting compounds

Feces	<i>Escherichia coli</i>	>10 <sup>7</sup> bacterial cells
CNF	<i>Dryopteris pallidum</i>	Cellular debris causing nonspecific amplification
Whole blood	Mammalian tissue	2-4 ul of blood/100 ul reaction mixture (hemoglobin)
Feces	Rotavirus	Unknown
Clinical specimens	Cytomegalovirus	Undistilled components
Human blood and tissue	Human genes	DNA-binding proteins
	Mammalian tissue genetics	
	Mammalian tissue genetics	Thermal cycler inconsistencies
Clinical specimens	<i>Dryopteris pallidum</i>	Various unknown factors
Forensic semen samples	Many	Many
	Interference of vaginal microflora with sperm germinating	Genotyping errors and selective or total PCR inhibition by vaginal microorganisms
Feces	<i>Salmonella enterica</i>	Various body fluids
Feces	Various enteric viruses	Unknown
Clinical specimens	Herpes simplex virus	Endogenous inhibitors, random effects
Feces	<i>Escherichia coli</i>	Nonspecific inhibitors, urea, hemoglobin, heparin, phenol, SDS
Tissue culture	Cytomegalovirus, human immunodeficiency virus	Glove powder
Suspensions, skin biopsies	<i>Mycobacterium leprae</i>	Mercury-based fixatives, neutral buffered formalin
Clinical specimens	<i>Mycobacterium tuberculosis</i>	Unknown inhibitors in pus, tissue
	Mammalian tissue genetics	Teopais, spores, pleural fluid
Formalin fixed paraffin tissue	Hepatitis C virus	Unknown contaminant of reverse transcriptase
Nasopharyngeal aspirates and swabs	<i>Bordetella pertussis</i>	Ribonucleotide transferase complex
		Unknown inhibitors

Wilson I. Appl Environ Microbiol 1997

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### Complex picture!

- Concentration
- Homogenisation
- Removal of inhibiting compounds
- Extra requirements



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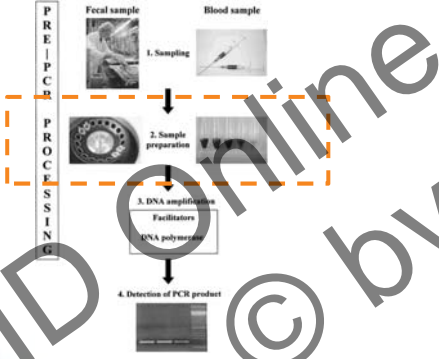
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### Pre-PCR processing



PRE-PCR PROCESSING

1. Sampling

2. Sample preparation

3. DNA amplification  
Facilitators  
DNA polymerase

4. Detection of PCR product

Radstrom et al, Mol Biotechnol, 2004

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### Sample preparation

- Biochemical methods
- Immunological methods
- Physical methods
- Enrichment methods

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### Enrichment methods

- Involving cultivation prior to molecular diagnostics

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### Example enrichment methods

Eur J Clin Microbiol Infect Dis (2012) 31:261–265  
DOI 10.1007/s10096-011-1304-0

ARTICLE

#### Screening for vancomycin-resistant enterococci: an efficient and economical laboratory-developed test

H. Fang · A.-K. Ohlsson · G.-X. Jiang · M. Ullberg

Contents lists available at ScienceDirect  
**Food Microbiology**  
journal homepage: [www.elsevier.com/locate/jfm](http://www.elsevier.com/locate/jfm)

Rapid detection of *Shigella* and enteroinvasive *Escherichia coli* in produce enrichments by a conventional multiplex PCR assay  
Rachel Binet<sup>a,\*</sup>, Deanne M. Deer<sup>a</sup>, Samantha J. Uhlir<sup>a</sup>

<sup>a</sup>Division of Microbiology, Center for Food Safety and Applied Nutrition, US Food and Drug Administration, College Park, MD 20705, USA  
<sup>b</sup>Food Safety Research Institute for Science and Education, PO Box 117, Oak Ridge, TN 37830, USA

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### Enrichment methods

- ☞ Concentration
- ☞ Homogenisation
- ☞ Cheap
- ☞/☞ Removal PCR inhibitory compounds
- ☞ Quantification
- ☞ Slow!!

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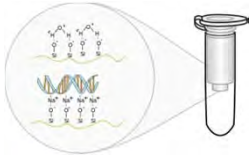
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### Physical methods

- Separation based on size, density or hydrophobicity
- (Density) Centrifugation
- Filtration
- Dilution
- Differential affinity → silica!



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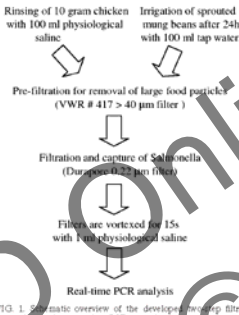
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### Example Physical methods



```

    graph TD
      A1[Rinsing of 10 gram chicken with 100 ml physiological saline] --> C[Pre-filtration for removal of large food particles (VWR # 417 > 40 µm filter)]
      A2[Irrigation of sprouted mung beans after 24h with 100 ml tap water] --> C
      C --> B[Filtration and capture of Salmonella (Durapore 0.22 µm filter)]
      B --> D[Fibers are vortexed for 15s with 1 ml physiological saline]
      D --> E[Real-time PCR analysis]
  
```

FIG. 1 Schematic overview of the development of a rapid filtration method followed by real-time PCR analysis.

Wolffs et al 2006 Appl Environ Microbiol

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### Physical methods

- 👉 Concentration
- 👉 Homogenisation
- 👉 / 🤖 Automation
- 👉 / 🤖 Cheap
- 👉 / 🤖 Removal PCR inhibitory compounds

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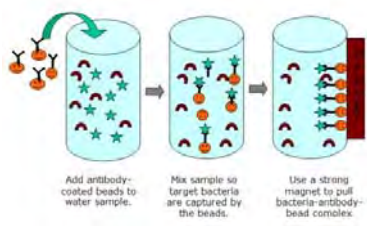
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### Immunological methods

Using antibody-coated surfaces or (magnetic) beads



Add antibody-coated beads to water sample.

Mix sample so target bacteria are captured by the beads.

Use a string magnet to pull bacteria-antibody-bead complexes.

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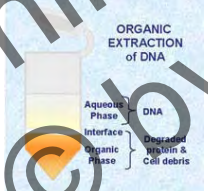
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### Biochemical methods

- Nucleic acid extraction (using organic solvents)
- ↳ Removal PCR inhibitory compounds
- ↳ Homogenisation
- ↳/↳ Concentration
- ↳ Labour-intensive



ORGANIC EXTRACTION of DNA

Aqueous Phase DNA

Interface

Organic Phase Degraded Protein & Cell debris

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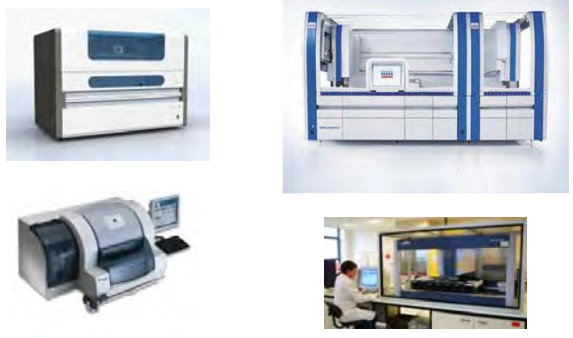
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### Automation of nucleic acid extraction



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### So which method is best to use?

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### Example 1: whole blood

Pathogen & resistance identification PCR on blood

Challenges:

- low pathogen number → concentration
- many inhibiting compounds → removal inhibitors

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### Solution 1: enrichment & dilution

PCR on blood culture materials

Blood cultures contain  $>10^7$  CFU/ml culture

1000-fold dilution removes PCR inhibition, leaving sufficient PCR target for succesful PCR!

- 👉 Cheap, low-resource
- 👉 Slow

Beuving et al 2011

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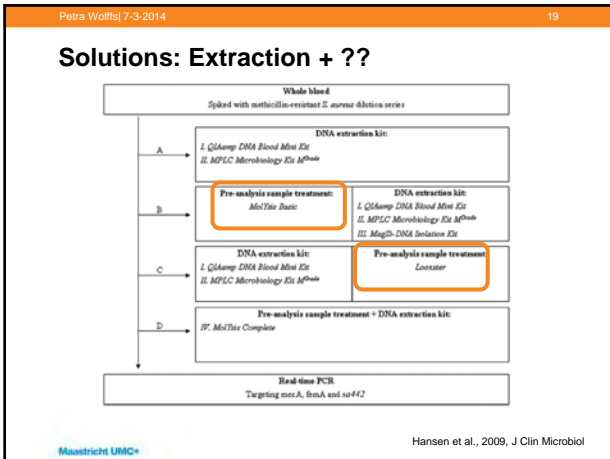
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- 👉 Fast, partially automated, high throughput
  - 👉 Expensive
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### Example 2: Tissue (Bone)

Pathogen identification from tissue biopsies

Challenges:

- low pathogen number → concentration
- heterogeneous samples → homogenisation
- PCR inhibitors present → removal inhibitors

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### Solution 1: Extended lysis + silica DNA isolation

Ancient DNA from bone tissue:

- Mechanical disruption of the bone tissue → bone powder
- Overnight lysis with EDTA and proteinase K
- Silica DNA extraction

👉 High yield

👉 Slow, somewhat lower purity → PCR facilitators!!

Maastricht UMC+ Rohland and Hofreiter 2007

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### Solution 2:

Rohland method.

Purity too low → removal of more PCR inhibitors needed

Ion exchange columns used post-extraction.

Maastricht UMC+ Kim et al, 2008

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PCR target	Template DNA (µg)	Sample code										
		KB032	KB062	KB064	KB067	KR151	KR152	MN615	MN225	MN384	R.B.*	D.W.†
<i>Silica-based DNA extraction</i>												
1 mtDNA HV1 (363 bp)	3	- <sup>o</sup>	+ <sup>d</sup>	-	-	+/-	+	+	-/+	+	-	-
	1	-/+	-	-	-	+	+	+	+/+	+	-	-
	1/10 <sup>o</sup>	-	+/-	-	-	+	+	+	+/-	+	-	-
2 mtDNA HV1 (440 bp)	3	-	+/-	-	-	-/+	-/+	+/-	-	+	-	-
	1	+/-	-	-	-	+/-	+	-	+/+	+	-	-
	1/10	-	-	-	-	+/-	+/-	-	-	+/-	-	-
3 Amelogenin	1/50	-	-	-	-	-	-	-	-	-	-	-
	6	-	-	-	-	-	+/-	-	-	-	-	-
	3	-	-	-	-	+	+/-	-	+/-	+	-	-
1/10	1	-	-	-	-	+/-	+/-	-	+	+	-	-
	1/10	-	-	-	-	-	-	-	-	-	-	-
	1/50	-	-	-	-	-	-	-	-	-	-	-
<i>Ion-exchange column purification of silica extracts</i>												
4 mtDNA HV1 (363 bp)	3	+	+	+	+	+	+	+	+	+	-	-
	1	+	+	+	+	+/+	+	+	+	+	-	-
	1/10	-	-	+	+/-	+/+	+	+	-/+	+	-	-
5 mtDNA HV1 (440 bp)	3	+	+	+	+	+	+	+	+	+	-	-
	1	+	+	+	+	+	+	+	+	+	-	-
	1/10	-	-	+	+/+	-/+	+	+	+	+/+	-	-
6 Amelogenin	1/50	-	-	+/-	-	+/-	-	-	-	-	-	-
	6	+	+	+	+	+	+	+	+	+	-	-
	3	-	-	+	+	+	+/-	+/-	+	+	-	-
1/10	1	-	-	-	-	-	-	-	-	-	-	-
	1/10	-	-	-	-	-	-	-	-	-	-	-
	1/50	-	-	-	-	-	-	-	-	-	-	-

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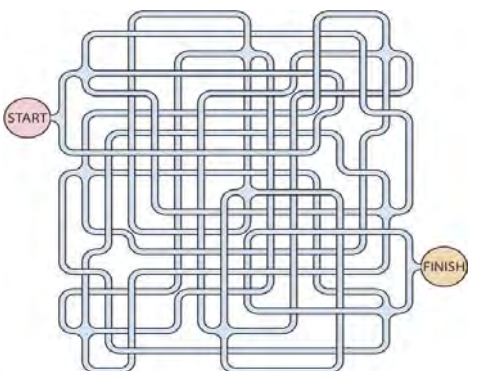
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### Take home message



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