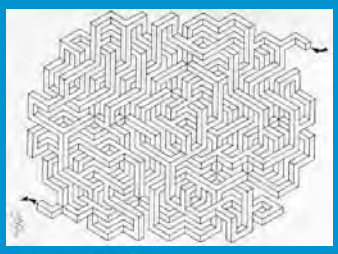
DNA and RNA isolation and Sample processing



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Goals of sample processing

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

PCR inhibiting compounds

Feces	*Escherichia coli*	$>10^3$ bacterial cells
CSF	*Treponema pallidum*	Cellular debris causing nonspecific amplification
Whole blood	Mammalian tissue	≥4 μl of blood/100-μl reaction mixture (hemoglobin)
Feces	Rotavirus	Unknown
Clinical specimens	Cytomegalovirus	Unidentified components
Human blood and tissue	Human genes	DNA-binding proteins
	Mammalian tissue genetics	
	Mammalian tissue genetics	Thermal cycler inconsistencies
Clinical specimens	*Treponema pallidum*	Various unknown factors
	Many	Many
Forensic semen samples	Interference of vaginal microflora with sperm genotyping	Genotyping errors and selective or total PCR inhibition by vaginal microorganisms
Feces	*Salmonella enterica*	Various body fluids
Feces	Various enteric viruses	Unknown
Clinical specimens	Herpes simplex virus	Endogenous inhibitors, random effects
Feces	*Escherichia coli*	Nonspecific inhibitors, urea, hemoglobin, heparin, phenol, SDS
Tissue culture	Cytomegalovirus, human immunodeficiency virus	Glove powder
Suspensions, skin biopsies	*Mycobacterium leprae*	Mercury-based fixatives, neutral buffered formalin
Clinical specimens	*Mycobacterium tuberculosis*	Unknown inhibitors in pus, tissue biopsies, sputum, pleural fluid
	Mammalian tissue genetics	Unknown contaminant of reverse transcriptase
Formalin-fixed paraffin tissue	Hepatitis C virus	Ribonucleotide vanadyl complexes
Nasopharyngeal aspirates and swabs	*Bordetella pertussis*	Unknown inhibitors

Wilson I. Appl Environ Microbiol 1997

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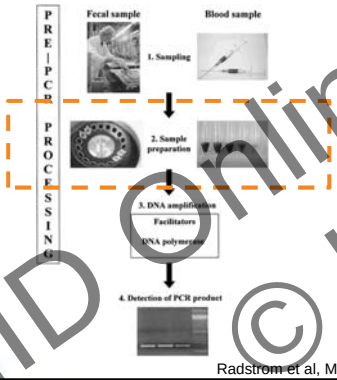
Complex picture!

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

Pre-PCR processing



Radstrom et al, Mol Biotechnol, 2004

Sample preparation

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

Enrichment methods

- Involving cultivation prior to molecular diagnostics

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

Food Microbiology

Rapid detection of *Shigella* and enteroinvasive *Escherichia coli* in produce enrichments by a conventional multiplex PCR assay

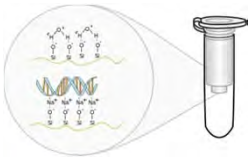
Rachel Binet, Deanne M. Deer, Samantha J. Uhlich

Enrichment methods

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

Physical methods

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



Example Physical methods

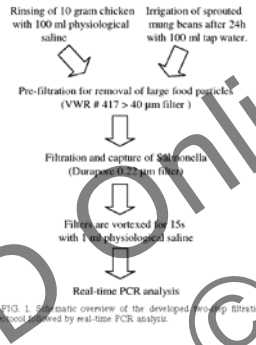


FIG. 1. Schematic overview of the developed two-step filtration protocol followed by real-time PCR analysis.

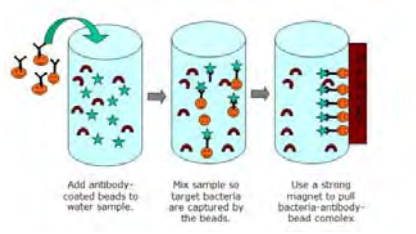
Wolffs et al 2006 Appl Environ Microbiol

Physical methods

- 👍 Concentration
- 👍 Homogenisation
- 👍 / 👎 Automation
- 👍 / 👎 Cheap
- 👍/ 👎 Removal PCR inhibitory compounds

Immunological methods

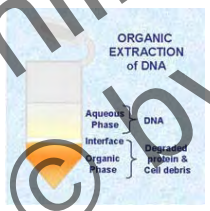
Using antibody-coated surfaces or (magnetic) beads



Biochemical methods

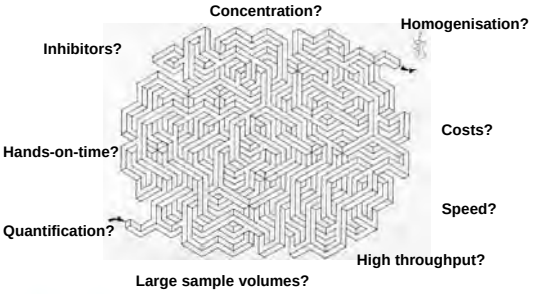
- Nucleic acid extraction (using organic solvents)

- 👍 Removal PCR inhibitory compounds
- 👍 Homogenisation
- 👍/ 👎 Concentration
- 👎 Labour-intensive



Automation of nucleic acid extraction

So which method is best to use?



Example 1: whole blood

Pathogen & resistance identification PCR on blood

Challenges:

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

Solution 1: enrichment & dilution

PCR on blood culture materials

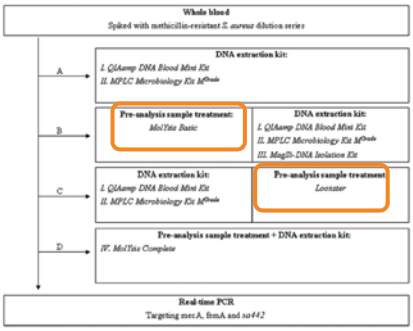
Blood cultures contain >10^7 CFU/ml culture

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

- Cheap, low-resource
- Slow

Beuving et al 2011

Solutions: Extraction + ??



Hansen et al., 2009, J Clin Microbiol

- Fast, partially automated, high throughput
- Expensive

Example 2: Tissue (Bone)

Pathogen identification from tissue biopsies

Challenges:

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

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!!

Rohland and Hofreiter 2007

Solution 2:

Rohland method.

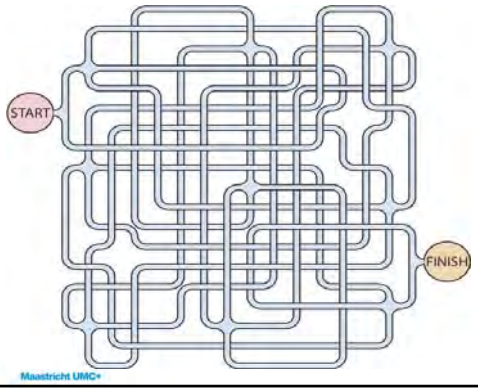
Purity too low → removal of more PCR inhibitors needed

Ion exchange columns used post-extraction.

Kim et al, 2008

	PCR target	Template DNA (µl)	Sample code KR032	KR062	KR064	KR067	KR151	KR152	MN015	MN225	MN384	R.B.[a]	D.W.[b]
Silica-based DNA extraction													
1	mtDNA HV1 (363 bp)	3	–[c]	+[d]	–	–	+[e]/–	+	+	–/+	+	–	–
		1	–/+	–	–	–	+	+	+	+/+	+	–	–
		1/10[f]	–	+/–	–	–	+	+/–	+/–	+	+	–	–
		1/50[f]	–	+/–	–	–	–	–	–	–	+	–	–
2	mtDNA HV1 (440 bp)	3	–	+/–	–	–	–/+	–/+	+/–	–	+	–	–
		1	+/–	–	–	–	+/–	+	–	+/+	+	–	–
		1/10	–	–	–	–	+/–	+/–	–	–	+/–	–	–
		1/50	–	–	–	–	–	–	–	–	–	–	–
3	Amelogenin	6	–	–	–	–	–	+/–	–	–	+	–	–
		3	–	–	–	–	+	+/–	–	+/–	+	–	–
		1	–	–	–	–	+/–	+/–	–	+	+	–	–
		1/10	–	–	–	–	–	–	–	–	–	–	–
		1/50	–	–	–	–	–	–	–	–	–	–	–
Ion-exchange column purification of silica extracts													
4	mtDNA HV1 (363 bp)	3	+	+	+	+	+	+	+	+	+	–	–
		1	+	+	+	+	+/+	+	+	+	+	–	–
		1/10	–	–	+	+/–	+/+	+	–	+	+	–	–
		1/50	–	–	+	–	–	–/+	–	–/+	+	–	–
5	mtDNA HV1 (440 bp)	3	+	+	+	+	+	+	+	+	+	–	–
		1	+	+	+	+	+	+	+	+	+	–	–
		1/10	–	–	+	+	+/+	–/+	–	–	+/+	–	–
		1/50	–	–	+/–	–	+/–	–	–	–	–	–	–
6	Amelogenin	6	+	+	+	+	+	+	+	+	+	–	–
		3	–	–	+	+	+	+/–	+/–	+	+	–	–
		1	–	–	–	+	–	–	+/–	+	+	–	–
		1/10	–	–	–	–	–	–	–	–	–	–	–
		1/50	–	–	–	–	–	–	–	–	–	–	–

Take home message



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