Pre-PCR processing

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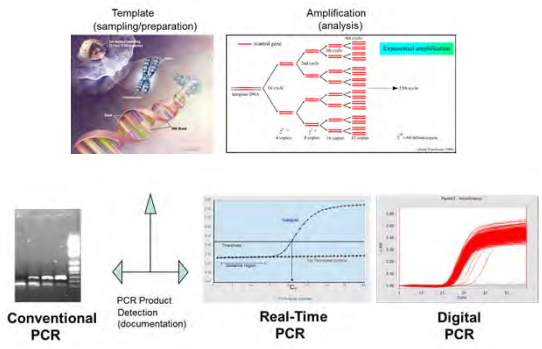
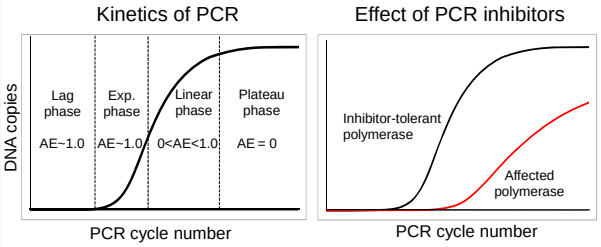
From PCR optimisation to pre-PCR processing

- Reduce PCR inhibition
- Minimise sample preparation
- Enhance PCR
- Concentrate target DNA
- Stabilise AE, qPCR
- Diminish sample variations
- Increase rapidity
- Support automation
- Improve precision

Polymerase Chain Reaction (PCR)



PCR kinetics



AE: Amplification Efficiency

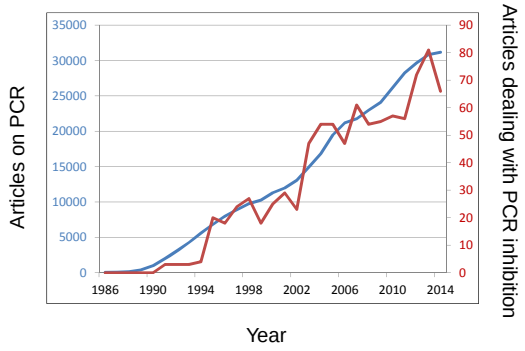
Effect of PCR inhibitors

(i) inhibitors can dramatically affect the detection limit, accuracy and precision

(ii) change the amplification efficiency/kinetics and thus generate ambiguous data in qPCR

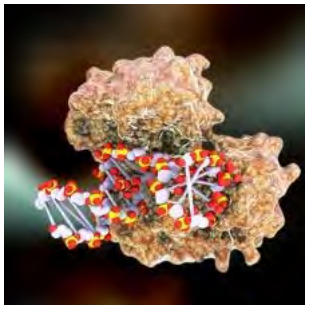
(iii) cause failed amplification

PCR inhibition



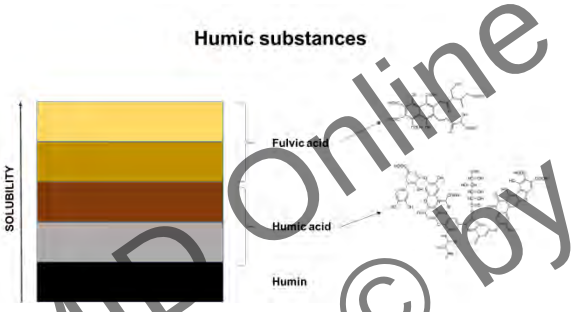
PCR Polymerase - Inhibitors

PCR Inhibitor	Mechanism
Proteinases	Degr. of Polym.
IgG	Binding to DNA
Polysaccharides	Binding to Polym.
Lactoferrin	Release of iron ions
Calcium ions	Competing with Mg^{2+}
Phenol	Denatur. of Polym.
EDTA	Chelation of Mg2+
Heparin	Binding to DNA

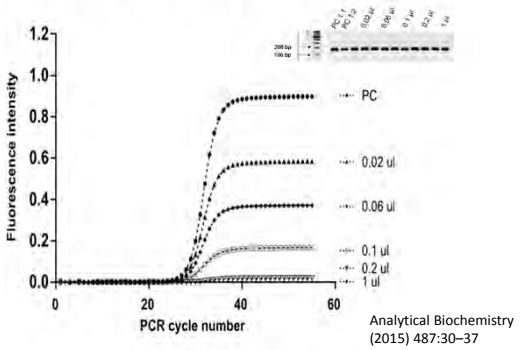


Rådström P, Knutsson R, Wolffs P, Lövenklev M, Löfström C (2004) Pre-PCR processing: strategies to generate PCR-compatible samples. Mol Biotechnol. 26:133-146

Notorious PCR inhibitors

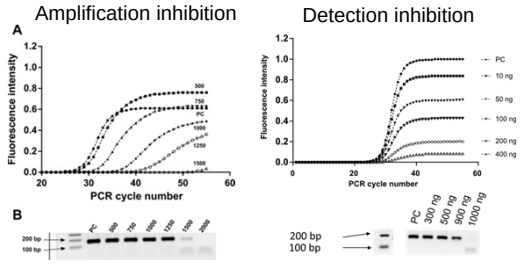


Sediment in qPCR



Analytical Biochemistry (2015) 487:30–37

PCR inhibition caused by fulvic acid and humic acid



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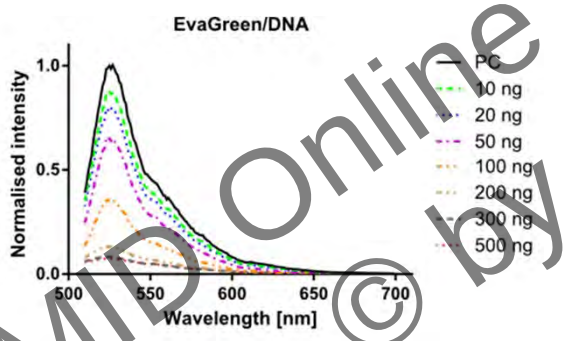
(2015) 487:30–37

Fluorescence quenching- Humic acids



PCR inhibitors may act by:

(i) inactivating the thermostable DNA polymerase
(ii) disturbing the ion composition of the reaction
(iii) capturing nucleic acids

Specific qPCR inhibitors:

(iv) interfering with fluorogenic probes or DNA-intercalating dyes
(v) some compounds may generate background fluorescence or quench the excitation light from the fluorogenic molecules

Different categories of sample preparation methods

Category of sample preparation method	Type of sample preparation method	Example of sample preparation method	Sample	Reference
Biochemical	Adsorption	Lectin-based separation	Beef meat	(Grant et al., 1993)
		Protein adsorption	Blood	(Akane et al., 1994)
	DNA extraction	DNA purification method	Haemolytic serum	(Klein and Juneja, 1997)
		Lytic methods	Blood anti-coagulant	(Nordvåg et al., 1995)
Immunological	Adsorption	Immunomagnetic capture	Blood	(Seesod et al., 1993)
Physical		Aqueous two-phase systems	Soft cheese	(Lantz et al., 1996)
		Buoyant density centrifugation	Minced meat	(Lindqvist, 1997a)
		Centrifugation	Urine	(Gerritsen et al., 1991)
		Dilution	Blood	(Abu Al-Soud et al., 1998)
		Filtration	Milk	(Starbuck et al., 1992)
Enrichment		Cultivation	Meat	(Sharma and Carlson, 2000)

Diagnostic PCR

Pre-PCR Processing

1. **Sampling**
2. **Sample preparation**
3. **DNA amplification**
   - **DNA polymerases**
   - **PCR facilitators**
4. **Detection of PCR products**

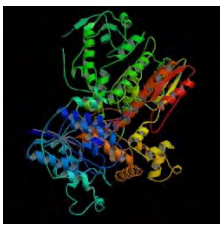
Rådström P et al. (2004) Pre-PCR processing: strategies to generate PCR-compatible samples. Mol Biotechnol 26:133-146

Rådström P et al. (2004) Pre-PCR processing: strategies. In T. Weissensteiner, HG Griffin and A Griffin (ed.), PCR Technology: Current Innovations, Second Edition, CRC Press LLC, p. 37-47

Rådström P et al. (2003) Strategies for overcoming PCR inhibition. In C.W. Dieffenbach and G.S. Dveksler (ed.), PCR Primer: A Laboratory Manual, Second Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, p. 149-161

Choice of DNA polymerase

DNA polymerases from *Thermus aquaticus* are completely inactive in the presence of **0.004%** (v/v) human blood, while the Hot*Tub*, *Pwo*, *rTth* and *Tfl* DNA polymerases are unaffected by the presence of **20%** (v/v) blood



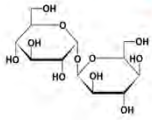
Taq DNA polymerase

Abu Al-Soud W, Rådström P (1998) Appl Environ Microbiol 64:3748-3753
Abu Al-Soud W, Jönsson LJ, Rådström P (2000) J Clin Microbiol 38:345-350
Abu Al-Soud W, Rådström P (2001) J Clin Microbiol 39:485-493
Knutsson R, Löfström C, Grage H, Hoorfar J, Rådström P (2002) J Clin Microbiol 40:52-60
Wolffs P, Grage H, Hagberg O, Rådström P (2004) J Clin Microbiol 42:408-411

PCR facilitators

Proteins
Bovine serum albumin (BSA)
T4 gene 32 protein (gp32)

Biologically compatible solutes
Betaine
L-carnitine
Sorbitol
Trehalose



Non-ionic detergents
NP40
Tween 20

Polymers
PEG400

Organic solvents
DMSO

Amplification facilitators

- improve the specificity of PCR and allow the amplification of GC-rich regions
- increase the fidelity of DNA synthesis
- enhance amplification efficiency in the presence of PCR-inhibitory samples

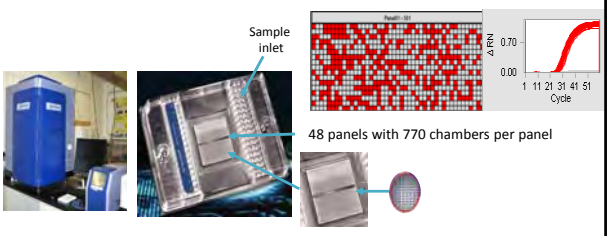
Bovine serum albumen (BSA), for example, has proved to be a particularly potent facilitator by relieving the inhibitory effects by its ability to bind inhibitors

The importance of DNA polymerase and PCR facilitators in diagnostic PCR

Feed sample	PCR amplification mixture[1]			
	Taq[2]	*rTth*, BSA[3]	*Tth*[4]	*Tth*, glycerol[5]
1. Fish meal	-	-	+	+
2. Rapeseed meal	-	-	-	-
3. Soybean	-	-	+	+
4. Soybean (acidified)	-	-	-	+
5. Oats	+	+	+	+
6. Soybean meal	-	-	+	+
7. Wheat	+	+	+	+
8. Meat meal	+	+	+	+
9. Piglett pellets	-	+	+	+
10. Rapeseed	-	-	-	-
11. Palm kernel	-	-	+	+
12. Corn pellets	-	-	-	+
13. Corn glutelin	-	-	+	+
14. Drank	-	-	(U.S)	+
15. Whey	-	-	-	-

[1] - : No amplification, + : Amplification of *invA* , U.S.: unspecific amplification, ND: Not determined.
[2] *Taq* DNA polymerase (Roche Diagnostics Gmbh)
[3] *rTth* DNA polymerase (Applied Biosystems), Facilitator:BSA (Bovine Serum Albumin)
[4] *Tth* DNA polymerase (Roche Diagnostics Gmbh)
[5] *Tth* DNA polymerase (Roche Diagnostics Gmbh), Facilitator: glycerol

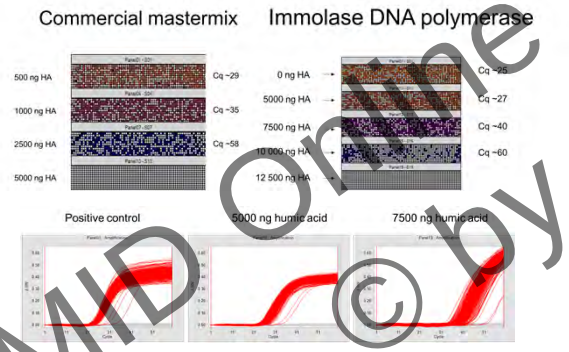
Microfluidics digital PCR



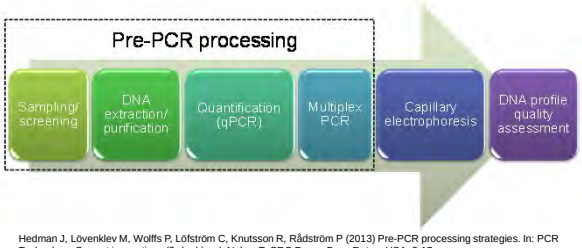
BioMark, Fluidigm

Real-time detection of 37k reactions of 0.85 nL

Digital PCR (Fluidigm)



Forensic DNA analysis



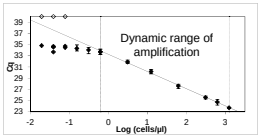
Hedman J, Lövenklev M, Wolffs P, Löfström C, Knutsson R, Rådström P (2013) Pre-PCR processing strategies. In: PCR Technology, Current innovations (3rd ed.), ed. Nolan, T. CRC Press, Boca Raton, USA. 3-17

Hedman J, Rådström P (2013) Overcoming inhibition in real-time diagnostic PCR, Methods Mol Biol 943:17-48

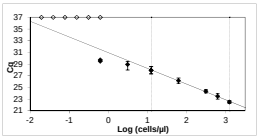
Hedman J, Knutsson R, Ansell R, Rådström P, Rasmusson B (2013) Pre-PCR processing in bioterrorism preparedness: improved diagnostic capabilities for laboratory response networks. Biosecurity and Bioterrorism: Biodefense Strategy, Practice, and Science 11:87-101

Evaluation of alternative DNA polymerases

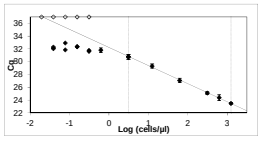
Bio-X-Act Short



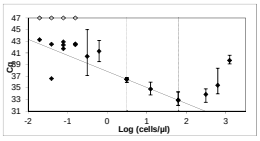
Tth



Taq



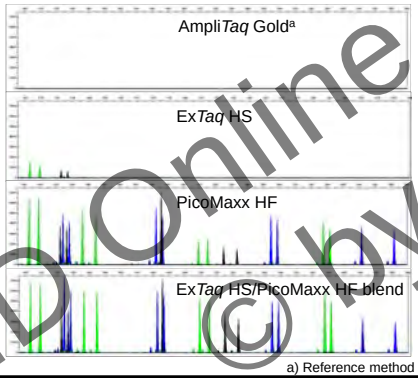
AmpliTaq Gold[a]



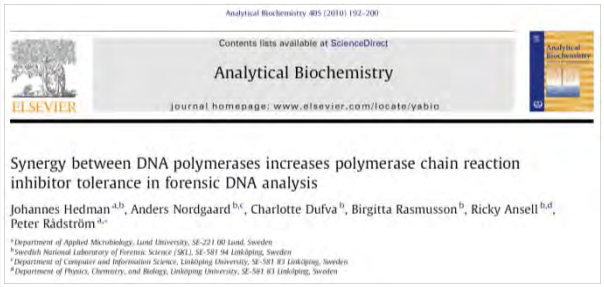
a) Reference method

Alternative DNA polymerases in forensic analysis

DNA 0.19 ng/µL



a) Reference method



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

Synergy between DNA polymerases increases polymerase chain reaction inhibitor tolerance in forensic DNA analysis

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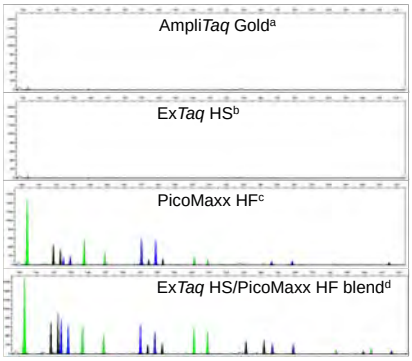
DNA Polymerase Synergy

DNA 0.13 ng/µL



a) Reference method

Forensic DNA Profile Index (FI)

Intensity: Total sum of peak heights (TPH)

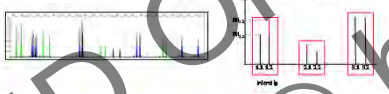
$$TPH = \sum_{i=1}^{M} PH_i; \quad PH_i = PH_{i1} + PH_{i2}$$

Local balance: Mean local balance (MLB)

$$MLB = \frac{1}{M}\sum_{i=1}^{M} LB_i; \quad LB_i = \frac{PH_{i,min}}{PH_{i,max}}$$

Global balance: Shannon entropy (SH)

$$SH = -\sum_{i=1}^{M} p_i \cdot \ln(p_i); \quad p_i = \frac{PH_i}{TPH}$$



$$FI = c_1 \cdot a_1 \cdot tph + c_2 \cdot a_2 \cdot mlb + c_3 \cdot a_3 \cdot sh + K$$

Blood extracts: Evaluation of sample treatment and DNA polymerases

DNA polymerase	Standard extraction[c]	Standard + dilution 1:2	Standard + filtration[d]
AmpliTaq Gold[a]	0.05	0.08	1.64
2x AmpliTaq Gold	0.40	1.75	1.69
Customised blend[b]	10.85	5.59	2.54

Results presented as mean FI values
a) Reference method
b) ExTaq HS/PicoMaxx HF blend
c) Standard extraction is based on Chelex
d) Filtration using Microsep columns and TE buffer

Assessment of routine analysis of crime scene saliva samples

DNA polymerase	Complete profiles (%)	Partial profiles (%)	Blank profiles (%)
Ampli*Taq* Gold[a]	38	47	15
Customised blend[b]	87	8	5

Crime scene samples with DNA concentrations 0.025-0.15 ng/µL

a) Reference method
b) Hedman J, Nordgaard A, Dufva C, Rasmusson B, Ansell R, Rådström P (2010) Synergy between DNA polymerases increases polymerase chain reaction inhibitor tolerance in forensic DNA analysis. Anal Biochem 405:192-200

Enhanced enterotoxin formation

ScienceDaily
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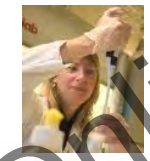


Nina Wallin Carlquist, Doctor of Philosophy in Engineering at the Division of Applied Microbiology, Lund University

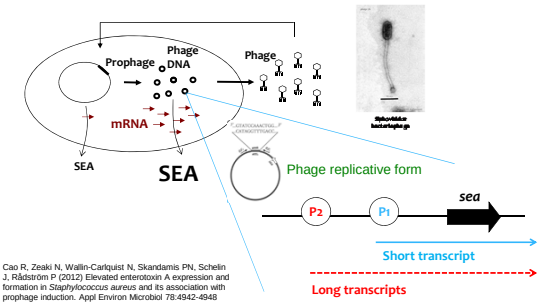
Science News

Share Blog Cite

Small Amount of Common Preservative Increases Toxins from Harmful Bacteria in Food, Study Finds

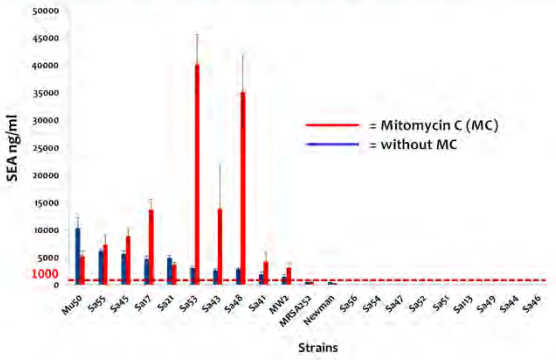
ScienceDaily (June 25, 2010) — In response to consumer demand for more natural food, the food industry has reduced the amount of preservatives in food over recent years. A common preservative is acetic acid, which is used to stop bacterial growth in dressings, sauces, cheese and pickles.

The SEA-prophage is involved in both the **dissemination** of the SEA gene and the **regulation** of the SEA gene

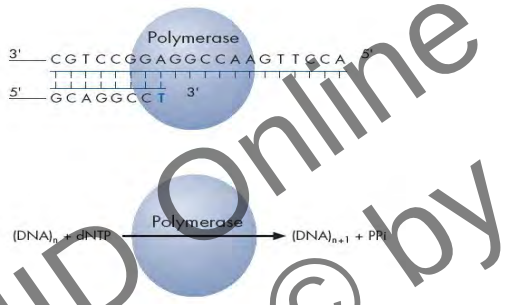


Cao R, Zeaki N, Wallin-Carlquist N, Skandamis PN, Schelin J, Rådström P (2012) Elevated enterotoxin A expression and formation in *Staphylococcus aureus* and its association with prophage induction. Appl Environ Microbiol 78:4942-4948

Prophage induced SEA formation



Amplicon sequencing (NGS)



Ultra-deep 16S rRNA amplicon NGS analysis

Forensic DNA analysis

Lunarc (supercomputer) to use programs on the large data set

High-Throughput Pyrosequencing
Microbial Biofilms in Drinking Water Pipes

Reproducibility for the analytical work-flow

Raw data – approx. 3000 OTU per sample

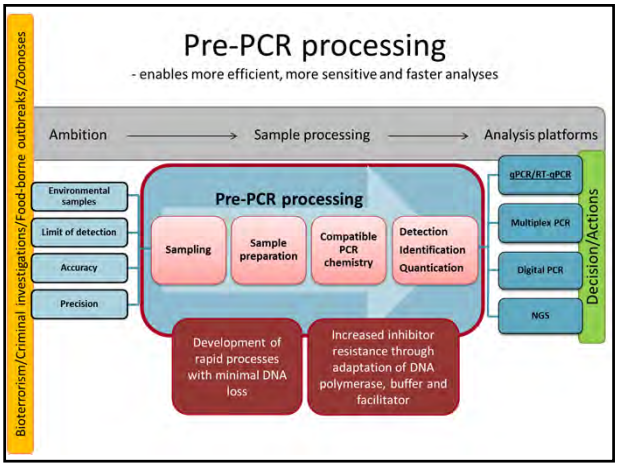
OTU	Prov 5	Prov 6	
MID5_27F_G9PMCDE02HFQX5	2587	1809	Pelagibacter
MID5_27F_G9PMCDE02HWEN	2	4	
MID5_27F_G9PMCDE02IC2M0	5961	3086	Rhizobiales
MID5_27F_G9PMCDE01AK4RV	29	6	
MID5_27F_G9PMCDE01B3KRT	29	5	
MID5_27F_G9PMCDE01D769B	1420	837	not classified
MID5_27F_G9PMCDE01EPOH3	30	23	
MID5_27F_G9PMCDE02GPFKY	10970	14173	Spirigomonadaceae
MID5_27F_G9PMCDE01EX7AD	144	92	
MID5_27F_G9PMCDE02IKY85	17	10	
MID6_27F_G9PMCDE02JRZI2	347	354	
MID6_27F_G9PMCDE02QTBDW	0	4	
MID5_27F_G9PMCDE02GPA4C	17	15	
MID5_27F_G9PMCDE01B3VWQ	13	4	
MID5_27F_G9PMCDE01BE9FV	148	69	
MID6_27F_G9PMCDE01D2ZYB	2	8	
MID5_27F_G9PMCDE02GG1QZ	10	4	
MID5_27F_G9PMCDE01B1FCA	3	0	
MID5_27F_G9PMCDE02JRS51	4	6	
MID5_27F_G9PMCDE02FRCP2	9	5	
MID5_27F_G9PMCDE02G74YT	5	1	
MID6_27F_G9PMCDE02HQRRL	6	10	
MID5_27F_G9PMCDE01EYEX9	5	0	
MID5_27F_G9PMCDE01KXBXO	44	16	
MID6_27F_G9PMCDE01ATDMH	36	31	
MID5_27F_G9PMCDE01BEVAX	7	3	
MID6_27F_G9PMCDE02FUQP9	5	7	
MID6_27F_G9PMCDE01BLJN4	12	10	
MID5_27F_G9PMCDE01EG4EP	7	0	

Microbes Environ (2015) 30:99-107

Phylogenetic tree

Pre-PCR processing

- enables more efficient, more sensitive and faster analyses



Pre-PCR Processing in different PCR-based applications

Application	Objective	DNA polymerase	PCR facilitator	Reference
Diagnostic PCR	To overcome inhibition from meat, blood and feces	r *Tth* (Applied Biosystems)	BSA, gp32, Tween 20, Betaine, PEG 400	(Abu Al-Soud and Rådström 1998), (Abu Al-Soud and Rådström 2000)
	To overcome inhibition from vitreous fluids	*Tth* (Promega Corporation), *Tfl* (Promega Corporation)	TritonX-100	(Wiedbrauk et al. 1995)
	To overcome inhibition from phenol	*Tth* (Amersham)	-	(Katcher and Schwartz 1994)
Reverse Transcription -PCR	To increase sensitivity	r *Tth* (Applied Biosystems)	-	(Schwab et al. 2001)
Real-time PCR	To overcome inhibition from sludge	AmpliTaqGold™ (Applied Biosystems)	Polyvinylpyrrolidone (PVP), gp32	(Monpoeho et al. 2000)
	To overcome inhibition from buffered peptone water (BPW)	r *Tth* (Applied Biosystems)	Glycerol	(Knutsson et al. 2002c)
	To enable detection	15 different DNA polymerases	-	(Kreuzer et al. 2000)
	To increase sensitivity	Platinum *Taq* (Gibco BRL)	-	(Hein et al. 2001)