



Evaluation of probe chemistries and platforms to improve the detection limit of real-time PCR using *Salmonella* as a model

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Food research, Innovation and safety

qPCR 2007

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Introduction

Potential of quantitative real-time PCR for rapid food testing

Need of comparison between different probe technologies and their potential to detect few target DNA in the presence of large background flora

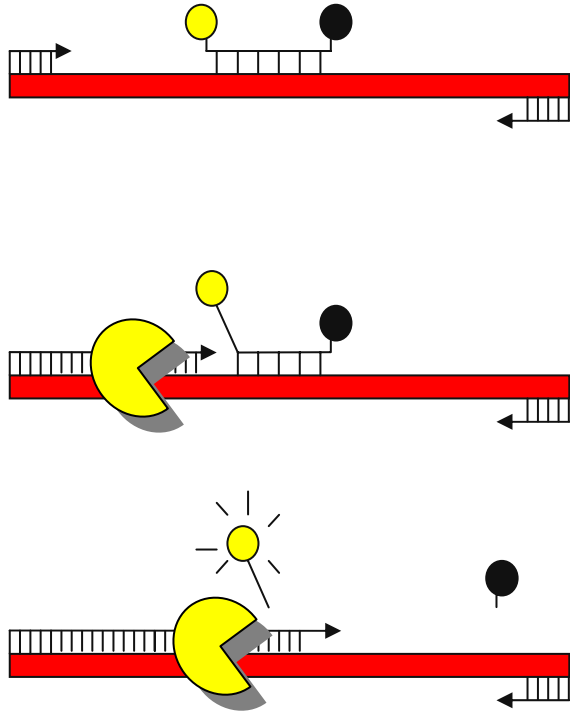
Detection chemistries:

- TaqMan, Scorpions, LNA, MGB, PNA, Molecular Beacons etc.

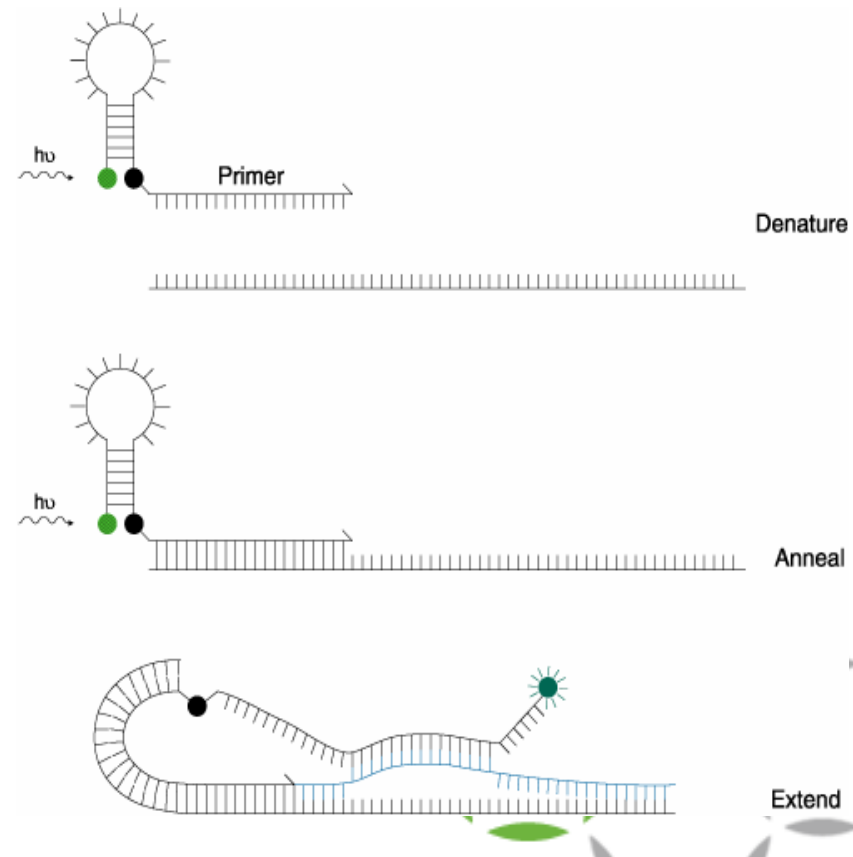


Detection chemistries tested

- TaqMan / LNA

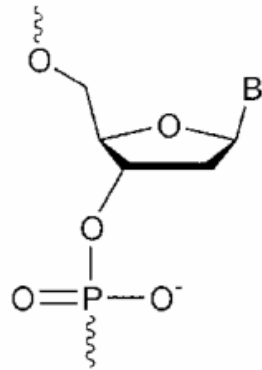


- Scorpion

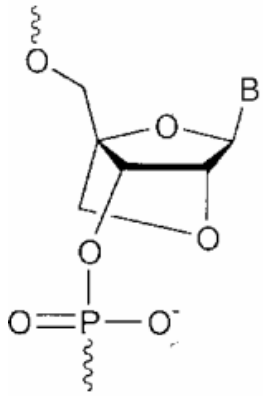




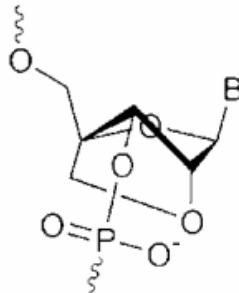
LNA structure



DNA



LNA



LNA probes have a higher melting temperature than conventional TaqMan probes

Locked nucleotides are a nucleic acid analogues containing a bicyclic furanose unit locked in an RNA mimicking sugar conformation





Aims

- Compare different dye / quencher combinations in a TaqMan assay
- Compare to other chemistries
 - Scorpion, LNA
- Compare platforms
- Increase the detection limit of a validated *Salmonella* assay



Experimental setup

Experiments:

Experiment 1
Amplification
Efficiency of
TaqMan probes

Experiment 2
Probe
comparison

Experiment 3
Platform
comparison

Experiment 4
Detection Probability
of best performing
setup

Description:

Purified *Salmonella* gDNA
Comparison between 6
different reporter combinations

Probe comparison
Amplification efficiency of TaqMan
LNA and Scorpion System

Platform comparison
RotorGene 3000
Mx3005p
ABI 7700

**Detection probability using best
performing setup**
Fish-meal, pig feces and chicken
matrices

Detection chemistries:

TaqMan labels:
Oregon Green-Dabcyl
FAM-BHQ1
Cy3-BHQ2
Cy5-BHQ3
FAM-DDQ1
Cy5-DDQ2

TaqMan FAM-BHQ1
Cy5-BHQ3
LNA FAM-BHQ1
Scorpion FAM-BHQ1

TaqMan FAM-BHQ1
LNA FAM-BHQ1

Material and Methods

- Genomic DNA isolated from *Salmonella typhimurium* 51K61,
- Primers and probe sequences and real-time PCR assay adopted from Malorny et.al. 2004 (*ttrRSBSA* locus, 94-bp amplicon)

- Platforms

- Mx3005p (quartz tungsten-halogen source lamp)
- RotorGene 3000 (high-power light emitting diodes)
- ABI 7700 (array of 96 optical fibers)



- Probes

- TaqMan → Six different dye / quencher combinations
- Locked Nucleic Acid (LNA) → FAM-BHQ1
- Scorpion → FAM-BHQ1

- Spiked sample matrices for detection sensitivity:

- Fish-meal
- Pig feces
- Chicken neck skin



Manufactures of probes

Type of probe	Reporter dye ^a	Quencher ^b	T_m ^c	Producer ^d
<i>Taq</i> Man	Oregon Green (488)	Dabcyl	72	MWG
<i>Taq</i> Man	FAM	BHQ1	72	MWG
<i>Taq</i> Man	Cy3	BHQ2	72	MWG
<i>Taq</i> Man	Cy5	BHQ3	72	Eurogentec
<i>Taq</i> Man	FAM	DDQ1	72	Eurogentec
<i>Taq</i> Man	Cy5	DDQ2	72	Eurogentec
Locked nucleic acids (LNA)	FAM	BHQ1	79	Proligo
Scorpion	FAM	BHQ1+ HEG	53	Proligo



Results

Experiment 1, Dye/Quencher comparison:

Probe concentration optimization, run on 10^3 *Salmonella* genomic copies

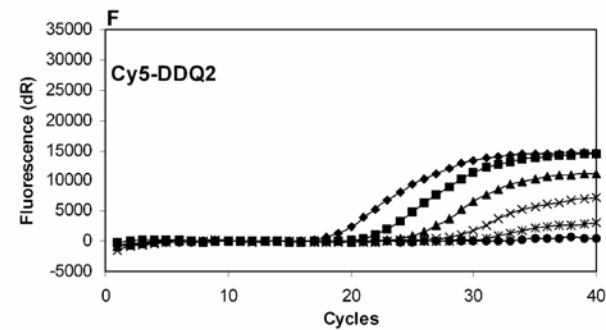
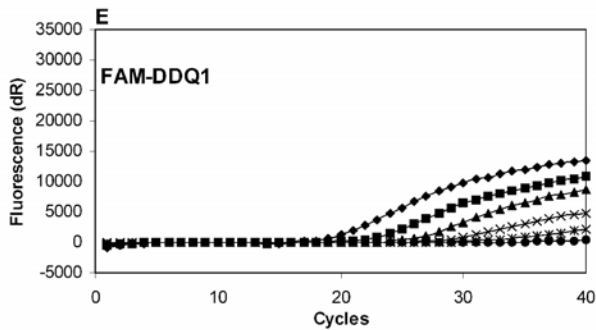
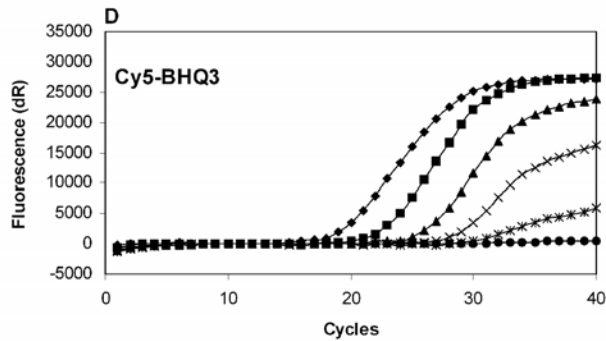
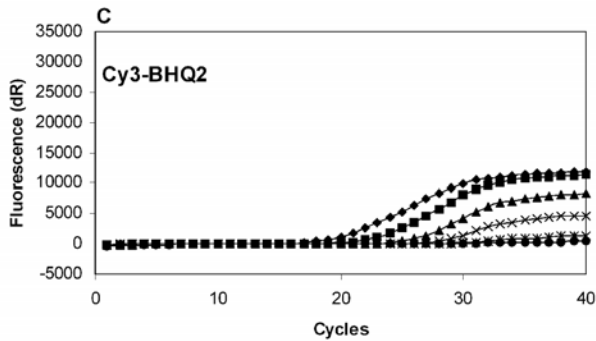
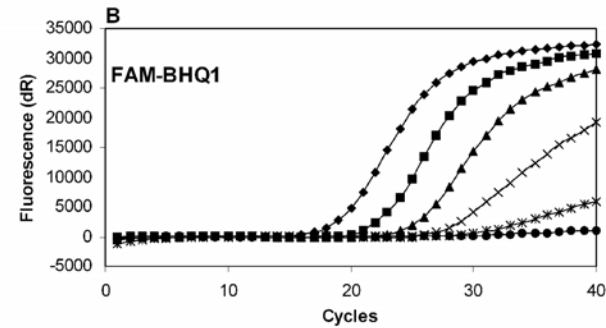
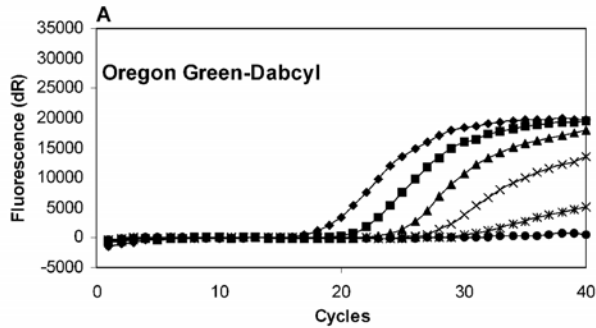
Concentration of probe (nM)	Cy3-BHQ2 ^a (Ct)	Cy5-HQ3 ^a (Ct)
50	28.85	29.05
100	28.25	28.26
150	28.07	27.78
→ 240	27.05	27.19
350	27.34	27.38

Gain setting was also empirically tested and optimized for each dye/quencher combination



Results

Experiment 1, Dye/Quencher comparison:



Results

Experiment 1, Dye/Quencher comparison:

Copies/reaction	Oregon-Dabcyl	FAM-BHQ1	Cy3-BHQ2	Cy5-BHQ3	FAM-DDQ1	Cy5-DDQ2
10^6	16.07 ± 0.54	15.70 ± 0.22	16.82 ± 0.46	15.13 ± 0.38	17.45 ± 0.20	16.50 ± 0.36
10^5	19.90 ± 0.22	18.88 ± 0.22	20.28 ± 0.29	18.06 ± 0.47	20.39 ± 0.65	19.59 ± 0.50
10^4	22.64 ± 0.34	21.91 ± 0.35	23.63 ± 0.29	21.31 ± 0.05	24.16 ± 0.26	22.95 ± 1.68
10^3	25.57 ± 0.73	25.42 ± 0.65	26.92 ± 0.41	24.47 ± 0.18	27.56 ± 0.28	25.65 ± 1.56
10^2	29.10 ± 0.83	27.88 ± 0.50	30.08 ± 0.46	28.98 ± 0.56	30.71 ± 0.59	29.44 ± 1.11
10^1	33.01 ± 2.29	31.35 ± 1.03	32.87 ± 1.60	31.68 ± 0.33	36.50 ± 0.19	33.83 ± 0.83
Efficiency (%)	101.2	109.7	104.2	101.1	88.3	97.0

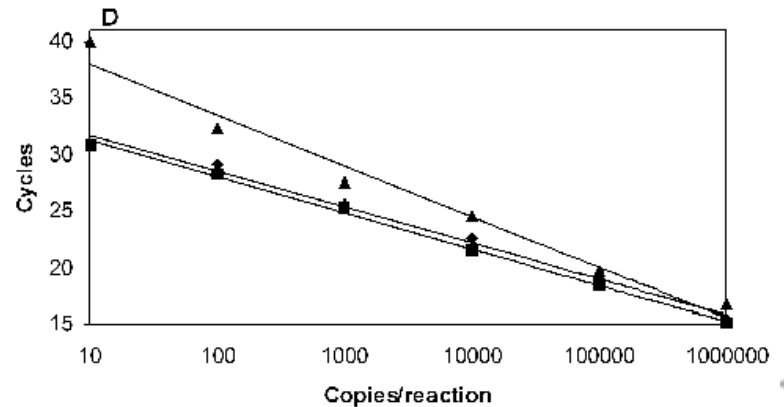
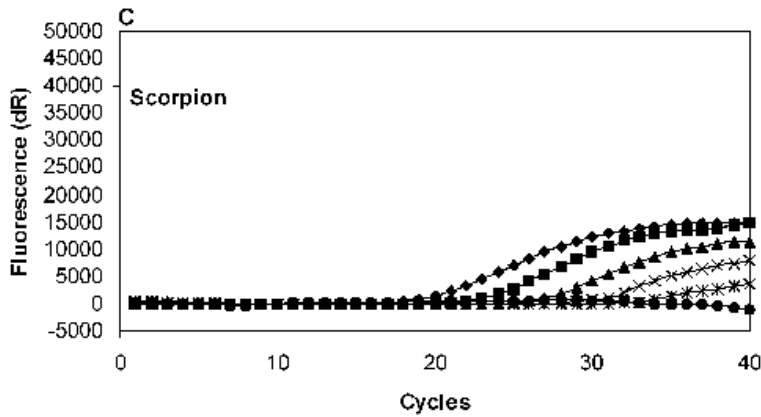
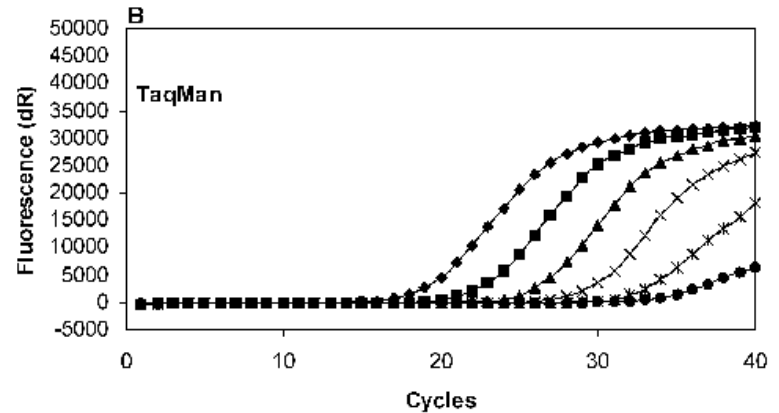
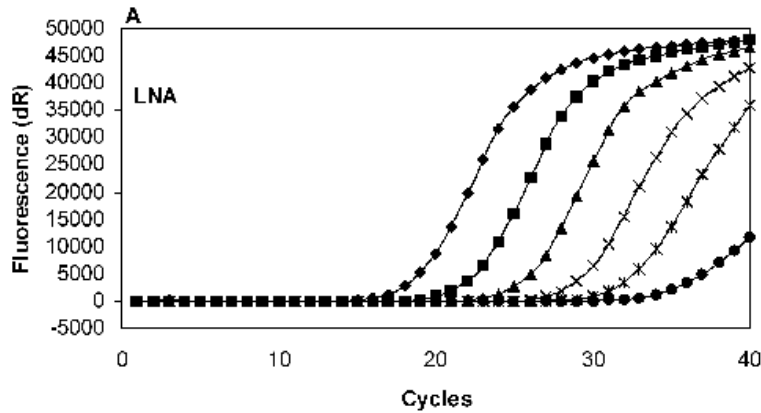


Fam-BHQ1 and **Cy5-BHQ3** generally showed best results



Results

Experiment 2, Probe comparison:



Results

Experiment 2, Probe comparison:

Copies/reaction	<u>TaqMan (FAM-BHQ1)</u>		<u>Locked Nucleic Acids</u>		<u>Scorpion</u>	
	Ct ^a	dR ^b	Ct ^a	dR ^b	Ct ^a	dR ^b
10 ⁶	15.92 ± 0.23	32464	15.37 ± 0.25	47877	17.38 ± 0.60	14943
10 ⁵	19.26 ± 0.19	32161	18.82 ± 0.16	48066	20.68 ± 0.68	14747
10 ⁴	23.17 ± 0.43	30490	22.03 ± 0.14	46670	24.96 ± 0.14	11620
10 ³	26.11 ± 0.09	27321	25.59 ± 0.10	42913	28.13 ± 0.01	7901
10 ²	29.91 ± 0.33	18178	28.63 ± 0.18	36075	32.63 ± 0.49	3650
10 ¹	32.53 ± 1.94	6515	31.7 ± 0.73	11845	No Ct	-
Efficiency (%)	99.9		103.4		83.9	

The LNA probe showed 0.4-1.0 lower Ct values than TaqMan and 1.5-3.0 lower Ct values than the Scorpion



Results

Experiment 3, Platform comparison:

Copies/PCR reaction	<u>Mx3005p</u>		<u>RotorGene</u>		<u>ABI 7700</u>
	TaqMan (Ct)	LNA (Ct)	TaqMan (Ct)	LNA (Ct)	LNA (Ct)
10^6	15.51 ± 0.17	14.46 ± 0.06	14.67 ± 0.73	13.37 ± 0.05	15.60 ± 0.29
10^5	18.80 ± 0.12	17.94 ± 0.16	18.18 ± 0.13	17.04 ± 0.12	18.49 ± 0.06
10^4	22.01 ± 0.16	21.25 ± 0.17	21.22 ± 0.20	20.27 ± 0.14	22.68 ± 1.12
10^3	25.36 ± 0.06	24.38 ± 0.18	24.99 ± 0.50	23.53 ± 0.17	25.45 ± 1.08
10^2	28.03 ± 0.07	27.68 ± 0.15	27.56 ± 0.55	26.88 ± 0.55	28.57 ± 0.49
10^1	31.37 ± 0.70	30.97 ± 0.97	30.92 ± 0.85	30.59 ± 1.65	33.56 ± 1.47
Efficiency	109.4	101.7	103	97	97.8
RSq	0.998	0.996	0.996	0.994	0.98
Slope	-3.116	-3.282	-3.25	-3.395	-3.51



Results

Experiment 4,

Detection in spiked samples with best performing setup

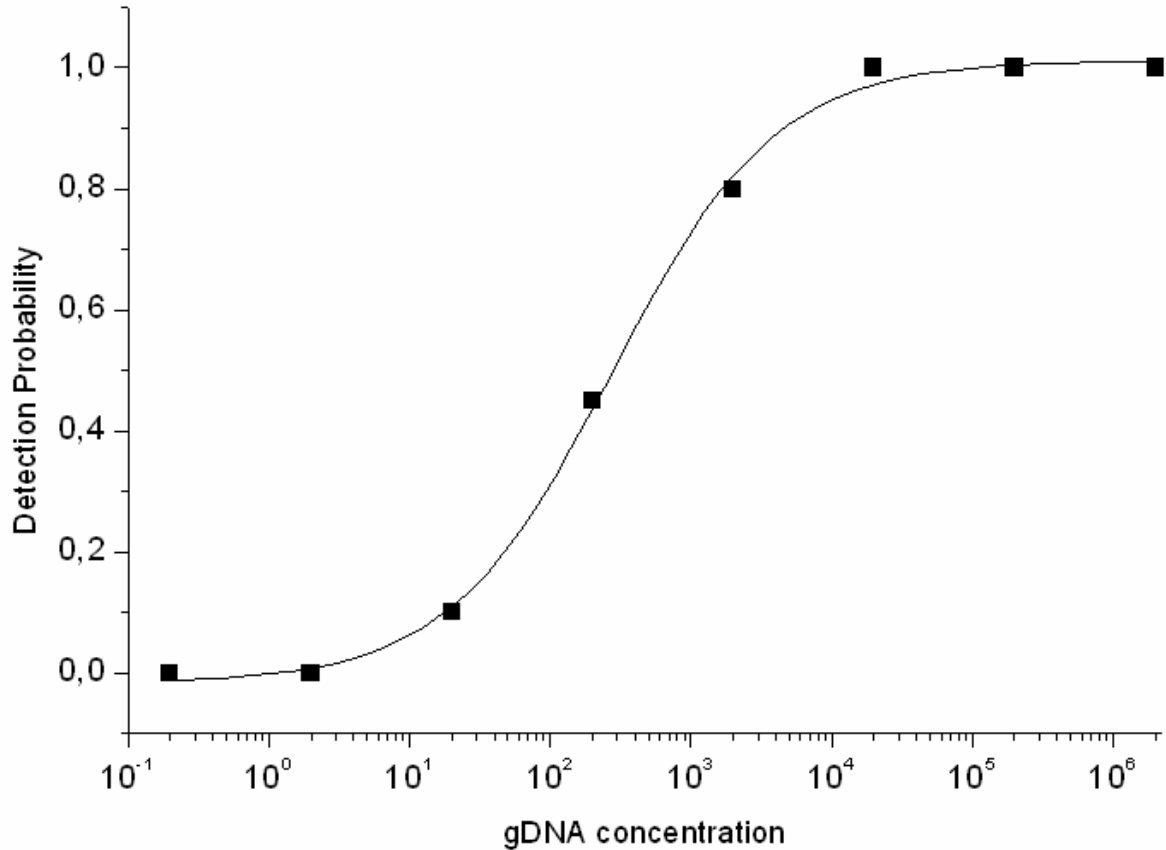
Spiked gDNA (copies/ml)	DNA copies/ reaction ^a	<u>Fish-meal</u>	<u>Pig faces</u>	<u>Chicken neck-skin</u>
		Ct	Ct	Ct
2×10^7	10^6	14.15 ± 0.16	15.04 ± 0.36	14.26 ± 0.16
2×10^6	10^5	18.60 ± 0.22	18.47 ± 0.28	16.81 ± 0.35
2×10^5	10^4	21.62 ± 0.48	21.87 ± 0.81	20.87 ± 0.02
2×10^4	10^3	25.40 ± 0.19	24.12 ± 0.52	24.98 ± 1.36
2×10^3	10^2	35.48 ± 3.98	29.15 ± 1.80	27.21^b
2×10^2	10^1	-	31.81^b	-
2×10^1	10^0	-	-	-



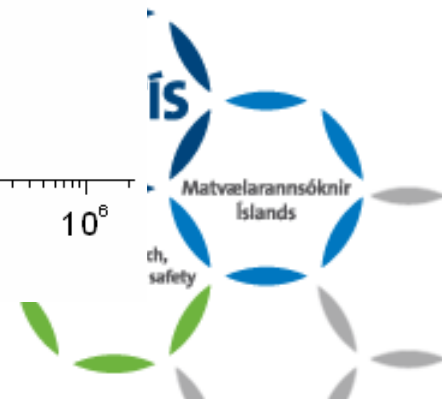
Results

Experiment 4, Detection probability of best performing setup in a pig fecal sample:

5 repl
4 Pcrs
n=20



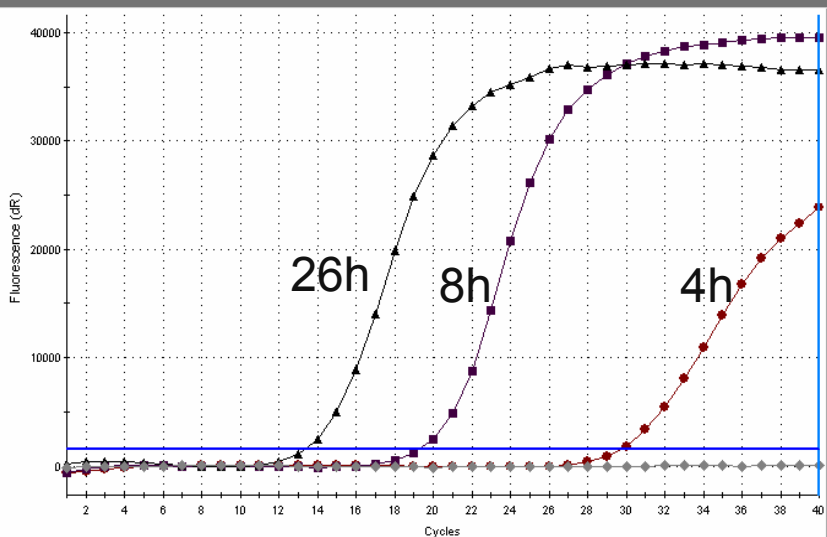
RotorGene
3000



Additional Fish-meal experiments

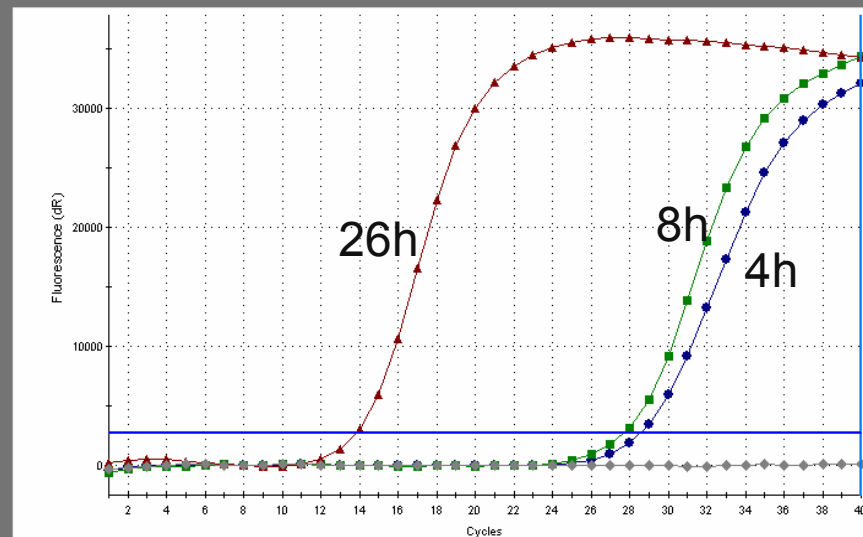
Preenriched up to 26h, 1ml sample, Chelex DNA isolation, run on Mx3000p

Amplification Plots



~1000 Salmonella/g fishmeal

Amplification Plots



~10 Salmonella/g fishmeal

Ongoing collection of fishmeal samples from the industry for the detection of naturally contaminated sample





Conclusions

- Fam-BHQ1 and Cy5-BHQ3 showed the the best performance in a TaqMan assay
- LNA probe showed the best performance compared to other chemistries
 - Detection of purified *Salmonella* genomic DNA
 - Detection of *Salmonella* genomic DNA in the presence of matrix components from fishmeal, pig faces and chicken neck skin.
- Work in progress to achieve NordVal validation of 12-hr detection protocol of *Salmonella* with real-time PCR using the LNA probe.



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Norfa

BÍSN graduates Placement, Leonardo

