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Towards novel validated PCR methods and innovative reference materials for the detection of VTEC Escherichia coli

5th Annual workshop of the National Reference Laboratories for E. coli

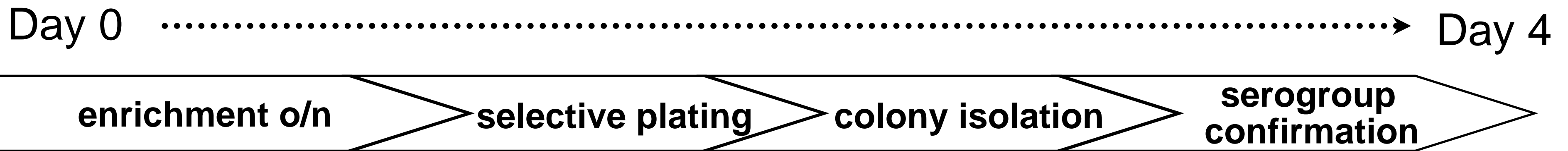
Rome, 08 October 2010

How to validate an analytical method?

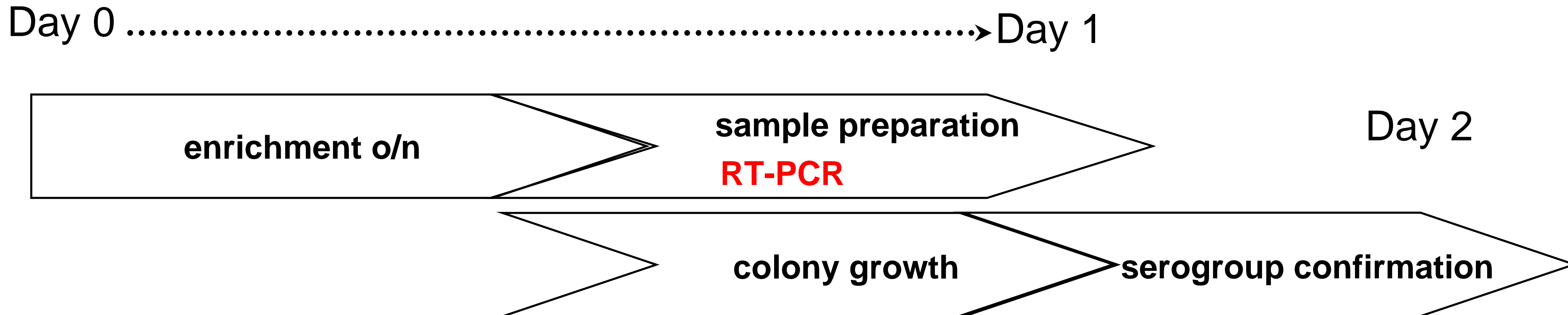
By performing an in-house validation (pre-validation)

By conducting a collaborative study (full-validation)

ISO 16654:2001 for the detection of *E. coli* O157 in food applying immuno-magnetic separation consisting of several time-consuming steps



“Technical Specification” by Working Group 6 of the Technical Committee 275 of the European Normalisation Committee (CEN TC275/WG6); PCR-based



Method Modularity
A model that allows flexibility and enforcement

- We applied the concept called “Method Modularity”
- The PCR “module” is applicable to any DNA template containing a given target.

  Provided that the template DNA is prepared by suitable methods, and fully characterised in terms of quality/quantity prior to use in PCR.

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PCR methods

Target	Forward primer, reverse primer and probe sequences (5'-3')	Amplicon size (bp)	Location within sequence	GenBank accession number
stx1 (Perelle S. et al. Mol Cell Probes 2004 **18**:185–192)	TTTGTYACTGTSACAGCWGAAGCYTTACG CCCCAGTTCARWGTRAGRTCMACRTC **Probe-**CTGGATGATCTCAGTGGGCGTTCTTATGTAA	131	878–906 983–1008 941–971	M16625
stx2 (Perelle S. et al. Mol Cell Probes 2004 **18**:185–192)	TTTGTYACTGTSACAGCWGAAGCYTTACG CCCCAGTTCARWGTRAGRTCMACRTC **Probe-**TCGTCAGGCACTGTCTGAAACTGCTCC	128	785–813 785–813 838–864	X07865
eae (Møller Nielsen E. and Thorup Andersen M. J clin Microbiol 2003	CAT TGA TCA GGA TTT TTC TGG TGA TA CTC ATG CGG AAA TAG CCG TTA **Probe-**ATAGTCTCGCCAGTATTCGCCACCAATACC	102	899-924 1000-979 966-936	Z11541
***rfbE* (O157)** (Perelle S. et al. Mol Cell Probes 2004 **18**:185–192)	TTTCACACTTATTGGATGGTCTCAA CGATGAGTTTATCTGCAAGGTGAT **Probe-**AGGACCGCAGAGGAAAGAGAGGAATTAAGG	88	348–372 412–435 381–410	AF163329
***wbdI* (O111)** (Perelle S. et al. Mol Cell Probes 2004 **18**:185–192)	CGAGGCAACACATTATATAGTGCTTT TTTTTGAATAGTTATGAACATCTTGTTTAGC **Probe-**TTGAATCTCCCAGATGATCAACATCGTGAA	146	3464–3489 3579–3609 3519–3548	AF078736
§*wzx* (O26) (Perelle S. et al. Mol Cell Probes 2004 **18**:185–192)	CGCGACGGCAGAGAAAATT AGCAGGCTTTTATATTCTCCAACTTT **Probe-**CCCCGTTAAATCAATACTATTTCACGAGGTTGA	135	5648–5666 5757–5782 5692–5724	AF529080
***ihp1* (O145)** (Perelle S. et al. Mol Cell Probes 2004 **18**:185–192)	CGATAATATTTACCCCACCAGTACAG GCCGCCGCAATGCTT **Probe-**CCGCCATTCAGAATGCACACAATATCG	132	1383–1408 1500–1514 1472–1498	AF531429
***wzx* (O103)** (Perelle S. et al. J Appl Microbiol 2005 **98**:1162–1168)	CAAGGTGATTACGAAAATGCATGT GAAAAAAGCACCCCCGTACTTAT **Probe-**CATAGCCTGTTGTTTTAT	99	4299–4323 4397–4375 4356–4373	AY532664

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Plasmid collection

pCRL-ECstx1a
’**TTTGTTACTGTGACAGCTGAAGCTTTACG**TTTTCGGCAAATACAGAGG
GGATTTCGTACAACA*CTGGATGATCTCAGTGGGCGTTCTTATGTAA*TGA
CTGCTGAAGATGTT**GATCTTACATTGAACTGGGG** 3’

pCRL-ECstx2a
5’**TTTGTCACTGTCACAGCAGAAGCCTTACG**CTTCAGGCAGATACAGA
GAGAATT*TCGTCAGGCACTGTCTGAAACTGCTCC*TGTGTATACGATGA
CGCCGGGA**GACGTGGACCTCACTCTGAACTGGGG** 3’

pCRL-ECstx2b
5’**TTTGTCACTGTCACAGCAGAAGCCTTACG**GTTCAGGCAAATACAGAG
AGAATT*TCGTC*T*GGCACTGTCTGAAACTGCTCC*TGTTTATACGATGACA
CCGGAA**GAAGTGGACCTCACACTGAACTGGGG** 3’

pCRL-ECstx2c
5’**TTTGTCACTGTCACAGCAGAAGCCTTACG**CTTCAGGCAGATACAGA
GAGAATT*TCGTCAGGCACTGTCTGAAACTGCTCC*TGTGTATACGATGA
CGCCGGGA**GACGTGGACCTCACTCTGAACTGGGG** 3’

pCRL-ECstx2d
5’**TTTGTCACTGTCACAGCAGAAGCCTTACG**CTTCAGGCAGATACAGA
GAGAATT*TCGTCAGGCACTGTCTGAAACTGCTCC*TGTGTATACGATGA
CGCCGGGA**GACGTGGACCTCACTCTGAACTGGGG** 3’

pCRL-ECeae
5’**CATTGATCAGGATTTTTCTGGTGATA**ATACCCGTTTA***GGTATTGGTG***
GCGAATACTGGCGAGACTATTTCAAAAGTAGCGT**TAACGGCTATTTCC**
GCATGAG 3’

pCRL-ECrfbE (O157)
5’**TTTCACACTTATTGGATGGTCTCAA**TTCTAACT*AGGACCGCAGAGGA*
*AAGAGAGGAATTAAGGA***ATCACCTTGCAGATAAACTCATCG** 3’

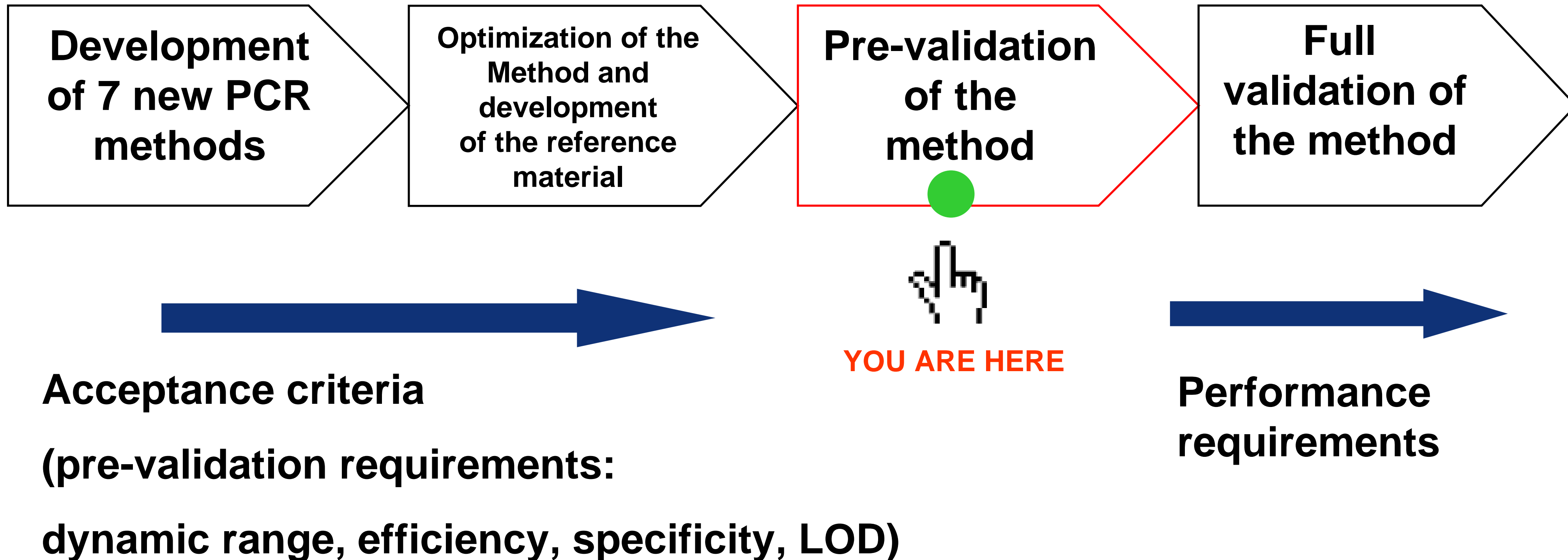
pCRL-ECwbdl (O111)
5’**CGAGGCAACACATTATATAGTGCTTT**GTTACACACTGAAAGTTCTTA
AAAGTGAA*TTGAATCTCCCAGATGATCAACATCGTGAA*TACCTTTGGCT
AACTAAACACCAAATAAAT**GCTAAACAAGATGTTCATAACTATTCAAAA**
A 3’

pCRL-EC§wzx (O26)
5’**CGCGACGGCAGAGAAAATT**ATTAAATGTATTCAGTCTATAGCAA*CCC*
*CGTTAAATCAATACTATTTCACGAGGTTGA*TAAAGCAACATGAATTGAA
ATTAGAACCATAC**AAAGTTGGAGAATATAAAAGCCTGCT** 3’

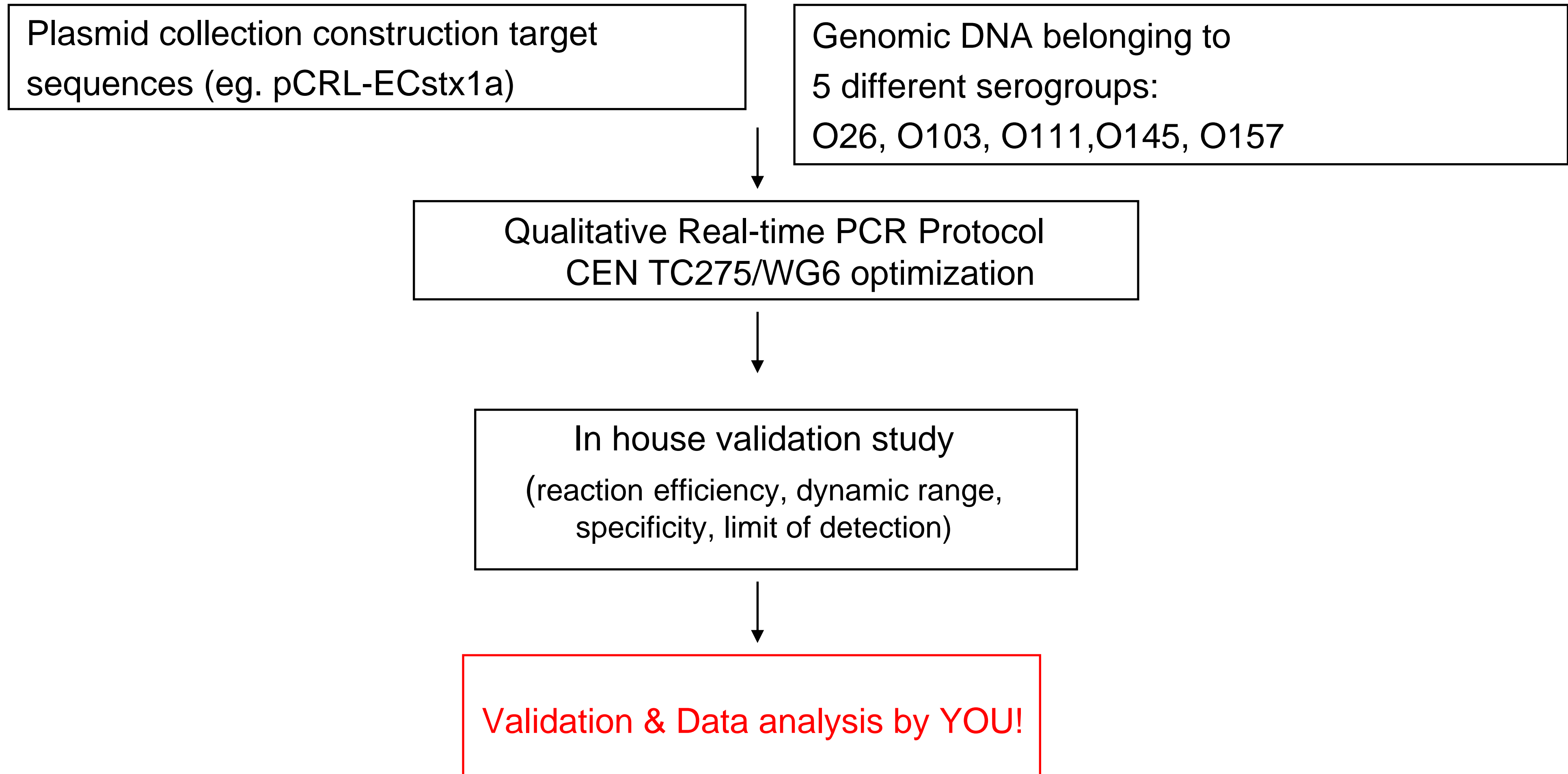
pCRL-ECihp1 (O145)
5’**CGATAATATTTACCCCACCAGTACAG**CCGTACAGACTGACAGCACT
GTCACCGATAAAAACCAGGCAGATCGCGACGCGGCGCAAGATA*CCGC*
*CATTCAGAATGCACACAATATCG*C**AAGCATTGCGGCGGC** 3’

pCRL-ECwzx (O103)
5’**CAAGGTGATTACGAAAATGCATGT**TTTTATTGGCGTGCATCAATTAA
ATTATCCTT*CATAGCCTGTTGTTTTAT*T**ATAAGTACGGGGGTGCTTTTTT**
C 3’

Validation process



Validation scheme



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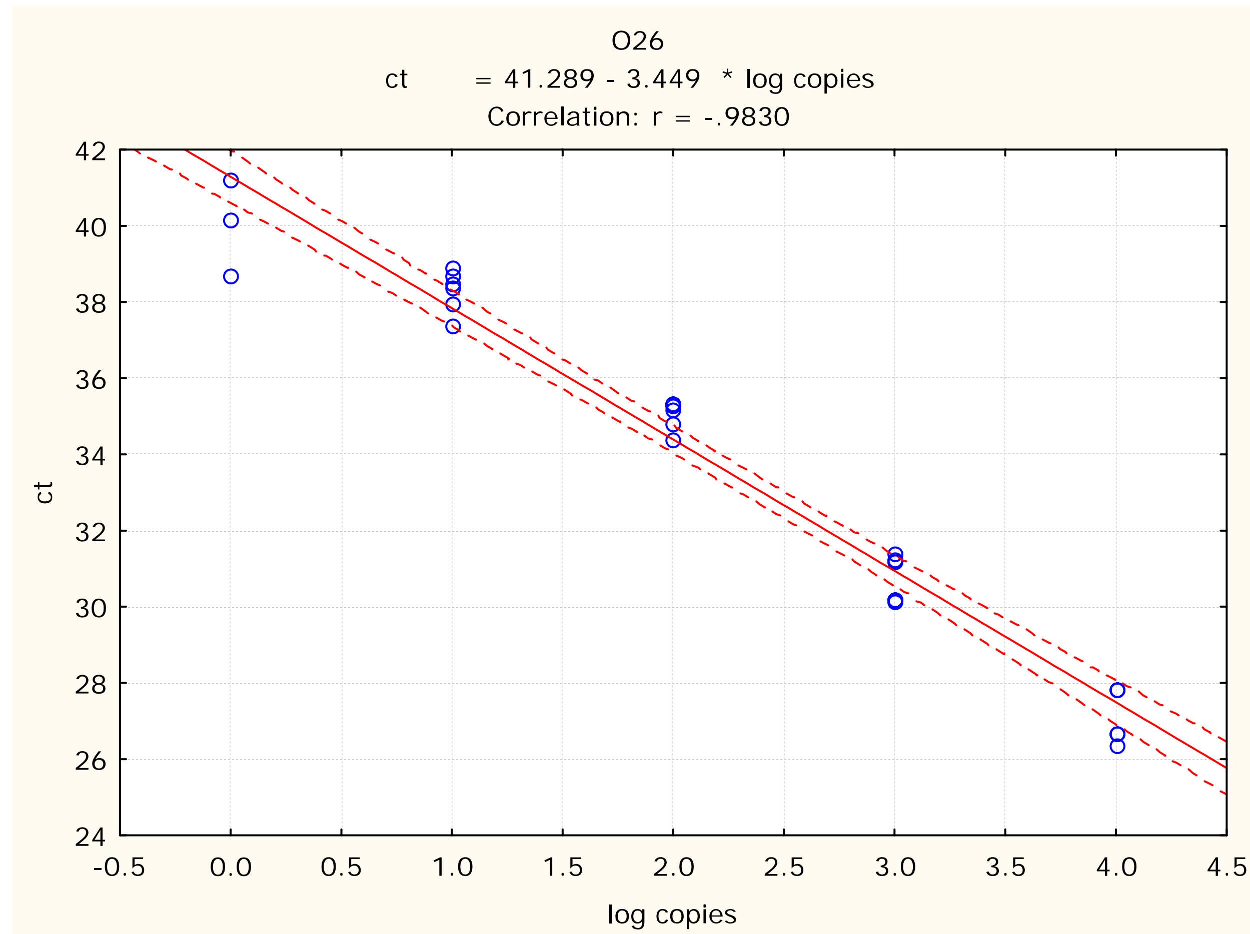
Method	Dynamic Range (gDNA copies) (3 replicates, 3 repetitions for each level)	Efficiency	Annealing temperature
*stx*1	1-10.000	92%	60ºC
*stx*2	1-10.000	89%	60ºC
eae	1-10.000	90%	60ºC
O26	1-10.000	99%	60ºC
O103	1-10.000	80%	No signal above 55ºC due to probe low a.t.
O111	1-10.000	92%	60ºC
O145	1-10.000	84%	60ºC
O157	1-10.000	91%	60ºC

Efficiency formula: ((10^(-1/slope))-1x100)

3,1<Slope<3,6

90%<Efficiency<110%



LOD determination

(60 replicates, 2 repetitions for each level)

Method	Copy number per reaction	Average Ct±SD	Positive (%)
stx1	15	34±0.9	60/60 (100%)
	10	36.5±1.22	57/60 (95%)
	5	37.5±1.29	52/60 (87%)
	0.1	42.3±1.47	3/60 (5%)
stx2	15	34.1±0.78	60/60 (100%)
	10	36.4±1.34	59/60 (98%)
	5	38.3±1.61	56/60 (93%)
	0.1	41.4±1.07	7/60 (12%)
eae	15	34.8±1.49	60/60 (100%)
	10	35.9±1.04	60/60 (100%)
	5	38.0±0.85	59/60 (98%)
	0.1	41.1±1.94	5/60 (8%)

Method	Copy number per reaction	Average Ct±SD	Positive (%)
serogroup O26	20	36.2±0.61	60/60 (100%)
	15	38.7±0.57	59/60 (98%)
	10	37.7±1.22	58/60 (97%)
	5	40.2±1.28	58/60 (97%)
	0.1	41.5±0.08	3/60 (5%)
serogroup O103	15	36.8±0.53	58/60 (97%)
	10	38.5±0.94	60/60 (100%)
	5	38.9±0.81	59/60 (98%)
	0.1	Undetermined	0/60 (0%)
serogroup O111	20	35.11±0.61	60/60 (100%)
	15	38.4±0.98	60/60 (100%)
	10	37.9±0.97	56/60 (93%)
	5	39.7±1.07	58/60 (97%)
	0.1	41.6	1/60 (1.7%)
serogroup O145	20	35.7±0.49	60/60 (100%)
	15	37.5±1.10	60/60 (100%)
	10	36.1±0.95	59/60(98%)
	5	39.0±1.01	57/60 (95%)
	0.1	39.9±0.56	4/60 (6.7%)
serogroup O157	20	36.5±1.14	57/60 (95%)
	15	38.6±1.01	57/60 (95%)
	10	36.4±1.09	59/60 (98%)
	5	40.1±1.58	49/60 (82%)
	0.1	Undetermined	0/60 (0%)

Summary

Method	**Reaction Efficiency**	**Dynamic Range (gDNA copies)**	**LOD (gDNA copies)**
***stx*1**	92%	1-10000	10
***stx*2**	89%	1-10000	10
eae	90%	1-10000	5
O26	99%	1-10000	5
O103	80%	1-10000	5
O111	92%	1-10000	5
O145	84%	1-10000	5
O157	91%	1-10000	10

List of strains used for specificity testing

- *Shigella boydii*
- *Shigella dysenteriae*
- *Klebsiella pneumoniae*
- *Citrobacter freundii*
- *Yersinia enterocolitica*
- *Proteus mirabilis*
- *Campylobacter jejuni*
- *Staphylococcus aureus*
- *Hafnia alvei*
- *Enterobacter sakazakii*
- *Listeria monocytogenes*
- *Salmonella Typhimurium*
- *Salmonella Seftenberg*
- *Salmonella Hadar*
- *Salmonella Enteritidis*
- *Salmonella Cerro*

Results

- Synthetic plasmid collection has been delivered and used as positive control (11 plasmids: pCRL-ECstx1a…)
- All reactions were optimized to perform at 60ºC except O103 serogroup (whose probe annealed at 55ºC)
- All concentration levels used to build up each Dynamic Range were found to be significantly different among them (ANOVA, Scheffé Post-hoc test)
- LOD was fixed at 10 copies (59/60 positive replicates)

Results

- Reaction Efficiency between 80% and 99%
- Dynamic Range between 1 and 10.000 copies
- Plasmids have been used as positive controls
- LOD has been calculated
- Specificity has been tested

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