Detection of Pathogens in Foods and Environmental Samples Using Isothermal Nucleic Acid Amplification

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ABSTRACT

Rapid assays for detection of *Salmonella* spp. and *Listeria* spp. have been developed using the nicking enzyme amplification reaction technology (NEAR™)[1]. The new isothermal nucleic acid assays (ANSR™) produce results in 30–40 minutes following single-stage sample enrichment ranging from 10–24 hours depending on the sample type. The assays utilize molecular beacon probes to generate fluorescent signal which is measured in real time using a simple incubator/fluorescence reader.

An AOAC Performance Tested Method℠ validation study has been conducted for the *Salmonella* assay. Inclusivity was 99.1% in testing of 113 *Salmonella* strains representing both *S. enterica* and *S. bongori* and all genetic subgroups and major serogroups. Exclusivity was 100% in testing of 38 non-salmonellae, mostly closely related Enterobacteriaceae. Comparative testing of 10 varieties of food and environmental samples showed no significant differences in performance between the ANSR and USDA-FSIS[2] or FDA/BAM[3] reference culture procedures as determined by chi-square analysis, with the single exception of a trial with sponge samples from stainless steel surface in which there were significantly more positives by the ANSR method.

The *Listeria* assay targets ribosomal RNA specific to *Listeria* spp. and exhibits a limit of detection of less than 100 cfu/mL in pure culture testing. Method validation is focused on environmental samples, processed meats and seafoods, and dairy products.

ANSR ASSAY

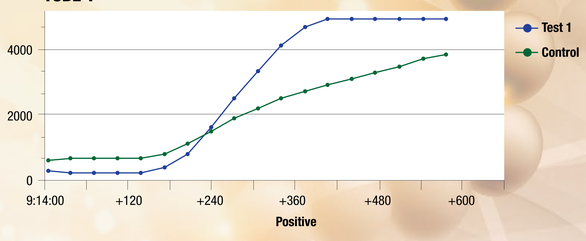
The ANSR assay targets unique sequences in either DNA or RNA. For RNA targets, a reverse transcription step is incorporated to produce complementary DNA.

An oligonucleotide template hybridizes to the target DNA sequence and initiates polymerization. The template contains a binding site for a nicking enzyme. The strand is nicked, recognized as damaged by DNA polymerase, and displaced with a newly synthesized strand. This process repeats, producing multiple copies of DNA complementary to the target sequence. This product is bound by a second template, and the same reactions lead to formation of a second product, homologous to the target sequence. The reactions continue and enter exponential phase.

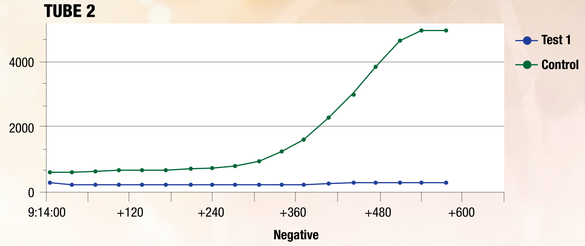
Amplification products are detected using a fluorescent molecular beacon probe. Fluorescence is generated in real time over the course of 10 minutes (*Salmonella* assay) or 20 minutes (*Listeria* assay).

The entire reaction takes place at a constant temperature of 56°C. Reagents are provided lyophilized in strip-tube format. An internal positive control is included to indicate proper reagent function.

TUBE 1



TUBE 2



METHODS

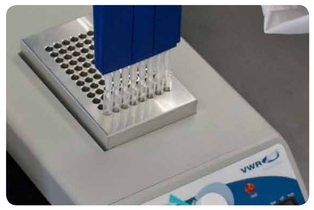
Inclusivity testing: Test strains were grown according to enrichment conditions specified for the ANSR methods and diluted to a level 10 to 100-fold above the limit of detection of the assay before testing (LOD = 10^4 cfu/mL for *Salmonella*, 10^2 cfu/mL for *Listeria*).

Exclusivity testing: Test strains were grown for 16–24 hours under permissive conditions and tested without dilution.

***Salmonella* food and environmental sample testing**: Chicken carcass rinse, raw ground turkey, and raw ground beef samples were enriched in ANSR for *Salmonella* Enrichment Broth 2 for 10–12 hours at 42°C; hot dogs were enriched in ANSR for *Salmonella* Enrichment Broth 1 for 16-24 hours at 36°C; oat cereal and environmental sponge or swab samples were enriched in ANSR for *Salmonella* Enrichment Broth 3 for 16–24 hours at 36°C.

Reference methods: The USDA-FSIS[2] or FDA/BAM[3] culture procedures were used as the reference methods as appropriate for the sample type.

Statistical analysis: Results were analyzed using the Mantel-Haenszel chi-square test for unpaired data[4].



Salmonella Assay

1. Add 50 µL enriched sample to lysis tube.
2. Add 450 µL lysis buffer. Incubate 20 minutes at 80°C.
3. Transfer 50 µL lysate to ANSR reaction tubes in reader. Cap tubes and start reader.
4. Results are reported in 10 minutes.

Listeria Assay

1. Add 50 µL enriched sample to lysis tube.
2. Add 450 µL reconstituted lysis reagent. Incubate 10 minutes at 37°C.
3. Transfer lysis tubes to 80°C and incubate 10 minutes.
4. Transfer 50 µL lysate to ANSR reaction tubes in reader. Cap tubes and start reader.
5. Results are reported in 20 minutes.

SALMONELLA ASSAY INCLUSIVITY

Species/subspecies	# strains	# positive[a]
I *S. enterica* subsp. *enterica*	90	89
II *S. enterica* subsp. *salamae*	10	10
III *S. enterica* subsp. *arizonae*	2	2
IV *S. enterica* subsp. *houtenae*	4	4
V *S. bongori*	6	6
VI *S. enterica* subsp. *indica*	1	1
TOTAL	113	112

[a] Tested at ~10^5 cfu/mL

SALMONELLA ASSAY EXCLUSIVITY

Genus	# species	# strains	# positive[a]
Citrobacter	4	4	0
Cronobacter	1	1	0
Edwardsiella	2	2	0
Enterobacter	6	7	0
Escherichia	5	5	0
Hafnia	1	1	0
Klebsiella	3	3	0
Kluyvera	1	1	0
Morganella	1	1	0
Pantoea	1	1	0
Pasteurella	1	1	0
Proteus	4	4	0
Providencia	4	4	0
Pseudomonas	1	1	0
Serratia	2	2	0
TOTAL	37	38	0

[a] Tested at ~10^9 cfu/mL

LISTERIA ASSAY INCLUSIVITY

Species/subspecies	# strains	# positive[a]
L. grayi	3	0[b]
L. innocua	9	9
L. ivanovii	4	4
L. monocytogenes	26	26
L. seeligeri	4	4
L. welshimeri	5	5
TOTAL	51	48

[a] Tested at ~10^9 cfu/mL [b] All 3 positive when grown under non-selective conditions

LISTERIA ASSAY EXCLUSIVITY

Genus	# strains (species)	# positive[a]
Bacillus	4	0
Brevibacillus	1	0
Brochothrix	1	0
Enterococcus	4	0
Geobacillus	1	0
Gordonia	1	0
Kocuria	2	0
Kurthia	2	0
Lactobacillus	4	0
Lactococcus	1	0
Micrococcus	1	0
Rhodococcus	2	0
Staphylococcus	3	0
Streptococcus	5	0
TOTAL	32	0

[a] Tested at ~10^9 cfu/mL

ANSR FOR _SALMONELLA_ RESULTS WITH RAW MEATS

Sample	Serovar	Level (cfu/portion)	N	ANSR 10 h	ANSR 12 h	ANSR confirmed	USDA method	x^2 10 h[a]	x^2 12 h[a]
Raw ground turkey 25 g	*S.* Heidelberg	0.90	20	Not done	15	15	19		3.06
	-	0.0	5	Not done	0	0	0		-
Raw ground turkey 25 g[b]	*S.* Heidelberg	0.67	20	Not done	7	7	10		0.90
	-	0.0	5		0	0	0		-
Chicken carcass rinse 35 mL	Nat. contam.[c]	-	20	Not done	7	6	8		0.43
Raw ground turkey 25 g	*S.* Newport	0.90	20	12	13	13	14	0.43	0.11
	-	0.0	5	0	0	0	0	-	-

[a] Chi-square > 3.84 indicates a significant difference at $P < 0.05$ [b] Trial performed by independent laboratory [c] Naturally contaminated; *Salmonella* spp. of serogroups C_2, D, and E_4 were isolated

ANSR FOR _SALMONELLA_ RESULTS WITH PROCESSED FOODS

Sample	Serovar	Level (cfu/portion)	N	ANSR 16 h	ANSR 24 h	ANSR confirmed	FDA method	USDA method	x^2 16 h[a]	x^2 24 h[a]
Hot dogs 25 g	*S.* Oranienburg	0.90	20	8	9	9		9	2.44	1.58
	-	0.0	5	0	0	0		0	-	-
Hot dogs 325 g	*S.* Oranienburg	0.09	20	11	11	11		13	0.41	0.41
	-	0.0	5	0	0	0		0	-	-
Oat cereal 25 g	*S.* Agona	2.3	20	13	15	15	13		0.00	0.46
	-	0.0	5	0	0	0	0		-	-

[a] Chi-square > 3.84 indicates a significant difference at $P < 0.05$

ANSR FOR _SALMONELLA_ RESULTS WITH ENVIRONMENTAL SAMPLES

Surface	Serovar	Level (cfu/portion)	N	ANSR 16 h	ANSR 24 h	ANSR confirmed	FDA method	x^2 16 h[a]	x^2 24 h[a]
Stainless steel	*S.* Heidelberg + competitor cocktail[b]	140/1,500	20	20	20	20	8	16.7	16.7
	-	-	5	0	0	0	0	-	-
Plastic	*S.* Javiana	21,700	20	11	11	11	16	2.78	2.78
	0	0	5	0	0	0	0	-	-
Sealed concrete	*S.* Infantis	21	20	13	15	15	13	0.00	0.46
	-	0	5	0	0	0	0	-	-
Ceramic tile	*S.* Meleagridis	110	30	19	19	19	24	2.02	2.02
	-	-	5	0	0	0	0	-	-
Ceramic tile[c]	*S.* Meleagridis	53	20	10	10	10	7	0.90	0.90
	-	-	5	0	0	0	0	-	-
Rubber	*S.* Arizonae	210	20	7	8	8	7	0.00	0.10
	-	-	5	0	0	0	0	-	-

[a] Chi-square > 3.84 indicates a significant difference at $P < 0.05$ [b] *Enterobacter aggiomerans, Escherichia coli and Bacillus subtilis* [c] Trial performed by independent laboratory

SUMMARY

- The ANSR *Salmonella* and *Listeria* assays demonstrate broad inclusivity and high specificity.
- Performance of the ANSR for *Salmonella* assay was determined to be equivalent to that of the FDA/BAM and USDA-FSIS reference culture methods for the food and environmental sample types evaluated.
- The ANSR assays require only simple instrumentation and a minimum number of steps.
- Assay results are available after only 30–40 minutes for *Salmonella* and *Listeria*, respectively.
- Current work is focused on additional commodity validation for *Salmonella* and *Listeria* and development of ANSR assays for other pathogens.

REFERENCES

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