G.B. English

Product Number:
9870

NEOGEN®
ANSR®
for Salmonella

BY AFNOR CERTIFICATION
NF VALIDATION

NEO 35/02-05/13*
Alternative Analytical
Methods For Agribusiness
nf-validation.afnor.org/en

The ANSR *Salmonella* method has been certified NF VALIDATION as alternative to the reference method NF EN ISO 6579, according to the ISO 16140-2 protocol, for the detection of *Salmonella* spp. in all products for human consumption and animal feed products. For more information about the end of validity of the NF VALIDATION certification, please refer to the certificate NEO 35/02-05/13 available on the website: nf-validation.afnor.org/en or on request from NEOGEN.

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ASNR for *Salmonella*

Product Number: 9870

Intended Use

The ANSR® for *Salmonella* method provides for rapid and accurate detection of *Salmonella* spp. in a wide variety of foods and environmental samples. Serovars belonging to both *Salmonella enterica* and *Salmonella bongori* can be detected with the ANSR method.

In the context of NF VALIDATION certification, the NEOGEN® ANSR for *Salmonella* test has been validated for the detection of *Salmonella* in all products for human consumption and animal feed products.

Assay Principles

ANSR for *Salmonella* is an isothermal, amplified nucleic acid assay. The ANSR method is based on nicking enzyme amplification reaction (NEAR) technology. Target nucleic acid is amplified through a mechanism of polymerization from the ends of nicks created in double-stranded DNA by the action of a specific endonuclease. Amplified target sequences are detected in real time using fluorescent molecular beacon probes.

A two-stage lysis is performed, first at 37 ± 2°C for 10 minutes, then at 80 ± 2°C for 20 minutes. Next, a portion of the lysed sample is transferred to a strip tube containing lyophilized ANSR reagents. The tubes are sealed and incubated at 56 ± 1°C on the ANSR reader. Results are generated by the reader and displayed in the ANSR software within 10 minutes. Positive results may be confirmed from the enrichment cultures following standard procedures. Each tube of ANSR reagents contains an internal positive control, ensuring the reagents are functioning properly.

Intended User

The ANSR for *Salmonella* test is designed for use by personnel with appropriate training in microbiology. Training in the use of the ANSR test system is available through NEOGEN.

Materials Provided

1. 12 strips of 8 cluster tubes, 1.2 mL
2. 12 strips of 8 reaction tubes, 200 µL, containing lyophilized ANSR for *Salmonella* reagents in 2 sealed foil pouches with desiccant pack
3. 12 strips of 8 permanent caps for reaction tubes
4. 1 bottle lysis reagent suspension buffer, 60 mL
5. 3 vials containing lyophilized lysis reagents
6. 1 kit insert

Equipment Required

1. ANSR reader (NEOGEN item 9828)
2. Computer and software for connection to ANSR reader (NEOGEN item 9832)
3. 1 dual heater block with aluminum block inserts for 1.2 mL cluster tubes, 80 ± 2°C and 37 ± 2°C (NEOGEN items 9836-DUAL48, 9829-48) or 2 single heater blocks with aluminum block inserts for 1.2 mL cluster tubes, 80 ± 2°C and 37 ± 2°C (NEOGEN item 9836D or equivalent)
4. Pipettor, 20–200 µL (NEOGEN item 9276 or equivalent)
5. Pipettor, 100–1000 µL (NEOGEN item 9463 or equivalent)
6. Pipette tip rack, 100–1000 µL, sterile (NEOGEN item 9487 or equivalent)
7. Pipettor, 10–100 µL, 8-channel (NEOGEN item 9388 or equivalent)
8. Pipette tips, 100 µL, sterile, filtered (NEOGEN item 9389 or equivalent)
9. Stomacher or equivalent (optional)
10. Vortex, adjustable speed (NEOGEN item 9494 or equivalent)
11. 3 thermometers, traceable (NEOGEN item 9518 or equivalent)
12. Timer, 3-channel (NEOGEN item 9426 or equivalent)
13. Optional-for-use heater block with 0.2 mL reaction tube aluminum block insert, 56 ± 1°C (NEOGEN item 9836D or equivalent)
14. Webcam (NEOGEN item WEBCAM)
15. ANSR Ethernet cable (NEOGEN item 9835)
16. 10 mL pipette pump (NEOGEN item 9277 or equivalent)
17. Pipettes, sterile serological (NEOGEN item 9415 or equivalent)
18. 40-slot, 20 mm test tube rack, autoclavable (NEOGEN item 9553 or equivalent)

Other Material Required

1. Stomacher-type bags for sample enrichment. Filtered bags are recommended (NEOGEN item 6827 or equivalent)
2. Graduated cylinder, 250 mL (NEOGEN item 9368 or equivalent)
3. 1 L purified water

Media Enrichment Broth Required

1. Buffered Peptone Water (BPW) (NEOGEN item NCM0015 or equivalent)
2. Phosphate Buffered Saline (PBS) (NEOGEN item 8425 or equivalent)
3. Supplement A (NEOGEN item ES/SM3-B)
4. Supplement B (NEOGEN item ES/SM2-B)

Storage

Store ANSR reagents at 2–8°C. After removing reaction tubes from the foil pouch, promptly reseal the pouch. Leave the desiccant pack in the pouch at all times.

Precautions

1. Use good microbiology laboratory practices, such as ISO 7218.
2. Dispose of used pipette tips in a covered container containing a fresh solution of 10% bleach. The 10% bleach solution should be made fresh each day. Undiluted stock solutions of bleach should be used within 30 days of opening.
3. Discard bleach solution and tips as regular waste at the end of each day.
4. Do not use reagents beyond the expiration date.
5. Use of enrichment media and incubation times or temperatures other than those specified may lead to erroneous results.
6. Remove the reaction tubes from the foil pouch just before use and keep covered until heating process begins. Reseal the pouch containing the remaining reaction tubes to avoid prolonged exposure to light. More than 15 minutes of total exposure time may lead to erroneous results.
7. Do not, under any circumstance, remove caps from reaction tubes after the assay has been started. This is essential in order to prevent accidental contamination of the environment with amplification products.
8. Exercise care in all pipetting steps to avoid cross-contamination of samples.
9. Complete all assay steps in sequence, avoiding delays between steps.

10. Tap reaction tubes on bench top to make sure lyophilized reagents are at the bottom of the tube prior to adding lysed sample.
11. The laboratory equipment (pipettes, tubes, etc.) must not circulate from one work station to another.
12. Use powder-free gloves. Change gloves often, especially if you suspect they are contaminated.
13. Clean work spaces periodically with at least 10% bleach and other decontaminating agent.
14. It is strongly advised to work under a hood or a PCR workstation during lyses and amplification steps.

Preparation of Enrichment Broth

1. Rehydrate 20 g of BPW (NEOGEN item NCM0015 or equivalent) with 1 L sterile water.
2. Autoclave for 121°C for 15 minutes.
3. Add 3 mg of supplement A and 3 mg of supplement B to the autoclaved media.
   Example: If the vial supplied contains 20 mg, reconstitute with 10 mL sterile deionized water.
   Next, add 1.5 mL of Supplement A and 1.5 mL Supplement B to 1 L of BPW. Therefore, for a single sample, 0.338 mL of reconstituted Supplement A and 0.338 mL of reconstituted Supplement B should be added to 225 mL of BPW.

Sample Enrichment

Please refer to commodity group table at the back of this kit insert to select the appropriate protocol.
Note: If the level of microflora is unknown, please choose the most selective enrichment. In the context of NF VALIDATION, samples more than 25 g have not been tested. For preparations of initial suspensions, follow instructions of ISO 6579-1. Protocol 1 is not recommended for marinated, cured, and smoked fish and meat types.

Protocol 1: Unprocessed raw food (including frozen) with high background microflora as well as animal feedstuffs and environmental samples (e.g., raw meat)

1. Weigh 25 g sample in a Stomacher-type bag.
2. Add 225 mL BPW supplemented with selective reagents to the bag. Homogenize (Stomacher, etc.) the sample as appropriate for the sample type.
3. Incubate the culture at 41.5 ± 1°C for 22 ± 2 hours.

Protocol 2: Processed food with low background microflora (e.g., milk powder)

1. Weigh 25 g sample in a Stomacher-type bag.
2. Add 225 mL BPW to the bag. Homogenize (Stomacher, etc.) the sample as appropriate for the sample type.
3. Incubate the culture at 34–38°C for 22 ± 2 hours.

Lysis REAGENT solution preparation

1. Reconstitute 1 vial of lyophilized lysis reagents with 18 mL of lysis buffer by adding the buffer to the reagent vial. Swirl gently to mix.
   Note: 1 vial of lysis reagents is enough for approximately 32 samples. Prepared lysis reagent solution can be stored at 2–8°C for up to 30 days.

ANSR Test Procedure

Prior to starting the assay:

1. Remove the foil pouch containing the reaction tubes from the refrigerator and allow the kit to warm at room temperature for 15 minutes. To avoid excess light exposure, leave reaction tubes in foil pouch until they are needed.
   Note: Keep the lysis buffer solution in the refrigerator until ready to use.
2. Preheat one lysis heater block to 80 + 2°C. Preheat the second lysis heater block to 37 + 2°C. If using the optional single heater, preheat to 56 ± 1°C. Use the thermometer for the temperature reading.
3. Connect the ANSR reader to the computer via USB or Ethernet and turn the computer on.
4. Turn on the ANSR reader. The reader will preheat to 56 + 1°C.
5. Start the ANSR software and click the connect button. Input sample IDs, lot number, and user information.
   Note: For instructions on using the reader and software, see the user guide that came with the ANSR reader. ANSR software versions up to 1.8.3 were used in the context of NF VALIDATION. Please check with your technical representative for the latest version.

Assay Procedure

1. Add 50 µL enrichment culture (or dilution) to a 1.2 mL cluster tube(s) using 100 µL filtered tips.
   Note: Cluster tubes may be pulled apart to provide the number of tubes needed.
2. Add 450 µL lysis reagent solution to each cluster tube(s) containing culture. Be sure to switch pipette tips between samples.
   Note: Return the lysis reagent solution to the refrigerator after use (within 1 hour).
3. Incubate the cluster tube(s) at 37 ± 2°C for 10 minutes.
4. Immediately transfer the cluster tube(s) to the 80 ± 2°C heater block and incubate for 20 minutes.
   Note: The 80 ± 2°C incubation time may be extended to a total of 60 minutes for the purpose of managing staggered assay start times.
5. For 3 to 5 minutes before the end of the lysis step, preheat the ANSR reagents to 56 ± 1°C by placing the reaction tube(s) in the ANSR reader. Optional: A separate heat block can be used. It should be heated to 56 ± 1°C.
   Note: The strip of reaction tubes may be cut to provide the number of tubes needed. Keep all unused tubes in the sealed foil pack. Ensure the pellet in the reaction tube(s) is at the bottom by tapping the tubes gently on the bench top.
6. After the completion of the 20–60 minute lysis incubation, remove and discard the caps from the reaction tube(s) in the ANSR reader.
   Important: Proceed with steps 7–9 without delay. The transfer of the sample from the lysis tubes to the reaction tubes should be completed within 1 minute.
7. Using an 8-channel pipette and 100 µL filtered tips, carefully transfer 50 µL from the top third of the lysed sample(s) in the cluster tube(s) to the reaction tube(s). Debris may accumulate at the bottom of the lysis tube(s) that will interfere with assay performance. Avoid transfer of debris by aspirating from the top third of the lysis tube(s). Do not prime the pipette tips and do not mix before aspirating. Place the provided permanent cap(s) on the reaction tube(s).
   Caution: Ensure that the pellet is not touched with the pipette when transferring the lysed sample as this can lead to erroneous results
   Note: Lysed sample may be transferred from the same cluster tube a maximum of 3 times.
8. Remove the strip(s) of tubes from the reader (or 56 ± 1°C heat block if one was used) and vortex briefly (about 2 seconds). Quickly visually check each reaction tube to assure that no bubbles are on the bottom or in the middle of the sample. A quick tap of the tubes should release any bubbles from the bottom or middle to the top. Then place back into the reader without delay. Close the reader's lid.
   Note: The reader will not provide accurate results if the lid is open. Keep the lid closed at all times while the assay is running. Contamination may occur if the permanent caps are not placed on the reaction tubes and/or if the permanent caps are removed.
9. Click start in the ANSR software to begin the 10 minute assay.
10. Results will be displayed as positive, negative or invalid by the software at the end of the assay. If the result displays an invalid, the test must be repeated. A single repeat test can be run starting at step 6. Alternatively, start over from step 1 if lysis from step 6 has been at 80 ± 2°C for more than 60 minutes.

Interpretation of Results

Each tube of ANSR reagents contains an internal positive control. A positive control curve will develop in the case of a valid assay. In the case of an invalid result, the positive control curve should be examined and the assay repeated. The sample matrix may be tested for inhibitory effects by performing an assay on a 1:10 dilution of the enrichment culture. The ANSR software will indicate the test results as positive or negative for the presence of *Salmonella* spp. in the enriched sample. In addition, the real-time fluorescence curve generated from the assay can be viewed.

Confirmation

In the context of the NF VALIDATION certified method, all positive ANSR results need to be confirmed in one of two ways:

1. Using standard tests described in the standardized CEN or ISO methods (including the purification step). For the confirmation test, it is necessary to start from the buffered peptone water enrichment broth after the full 20 ± 2 hours enrichment at 41.5°C.
2. Subculturing 0.1 mL of the BPW enrichment into 10 mL of RVS Broth. Incubate at 41.5°C for 24 hours before subculturing onto a selective agar plate (followed by the OXOID latex test, performed directly on isolated colonies).
3. Using any other method certified NF VALIDATION and based on a principle different from that used in ANSR test. The validated protocol of this second method must be followed entirely; the confirmation is carried out from the buffered peptone water enrichment broth, if this step is common to both methods.
   Note: In the event of discordant results (presumptive positive with the alternative method, non-confirmed by one of the means described above) the laboratory must follow the necessary steps to ensure the validity of the result obtained.

For Use as a Confirmation Test from Colony Picks (AOAC Official Method 2013.14)

ANSR for *Salmonella* can be used to determine if a presumptive colony on selective/differential or non-selective agar media is *Salmonella* spp. Colonies picked from the following media types have been tested and found to produce accurate results: Hektoen enteric agar, xylose lysine deoxycholate agar, bismuth sulfite agar, brilliant green sulfa agar, xylose lysine tergitol agar (XLT4), double-modified lysine iron agar and tryptic soy agar.

Procedure

Pick a colony with an inoculating loop or needle and resuspend the colony in 0.5 mL diluent. Vortex, or otherwise mix, to ensure the colony is completely resuspended. Proceed with the ANSR Test Procedure, followed by the Colony Picks Assay Procedure using 50 µL of the resuspension as sample. A positive test result indicates *Salmonella* spp. A negative test result indicates not *Salmonella* spp.

Colony Picks Assay Procedure

1. Add 50 µL colony resuspension to a 1.2 mL cluster tube(s) using 100 µL filtered tips.
   Note: Cluster tubes may be pulled apart to provide the number of tubes needed.
2. Add 450 µL lysis reagent buffer to each of the cluster tube(s) containing sample.
   Note: The colony assay procedure only has one lysis step at 80 ± 2°C. There is no 37 ± 2°C step needed.
3. Immediately transfer the cluster tube(s) to the 80 ± 2°C heater block and incubate for 20 minutes.
   Note: The 80 ± 2°C incubation time may be extended to a total of 60 minutes for the purpose of managing staggered assay start times.
4. For 3–5 minutes before the end of the lysis step, preheat the ANSR reagents to 56 ± 1°C by placing the reaction tubes in the ANSR reader. Optional: A separate heat block can be used. It should be heated to 56 ± 1°C. **Note**: The strip of reaction tubes may be cut to provide the number of tubes needed. Keep all unused tubes in the sealed foil pack. Ensure the pellet in the reaction tube(s) is at the bottom by tapping the tubes gently on the bench top.
5. After the completion of the 20–60 minute lysis incubation, remove and discard the caps from the reaction tube(s) in the ANSR reader.
   Important: Proceed with steps 7–9 without delay. The transfer of the sample from the lysis tubes to the reaction tubes should be completed within 1 minute.
6. Using an 8-channel pipette and 100 µL filtered tips, carefully transfer 50 µL from the top third of the lysed sample(s) in the cluster tube(s) to the reaction tube(s). Debris may accumulate at the bottom of the lysis tube(s) that will interfere with assay performance. Avoid transfer of debris by aspirating from the top third of the lysis tube(s). Do not prime the pipette tips and do not mix before aspirating. Place the provided permanent cap(s) on the reaction tube(s).
   Note: Lysed sample may be transferred from the same cluster tube a maximum of 3 times.
7. Remove the strips(s) of tubes from the reader (or 56 ± 1°C heat block if one was used) and vortex briefly (about 2 seconds). Quickly visually check each reaction tube to assure that no bubbles are on the bottom or in the middle of the sample. A quick tap of the tubes should release any bubbles from the bottom or middle to the top. Then place into the reader without delay. Close the reader's lid.
   Note: The reader will not provide accurate results if the lid is open. Keep the lid closed at all times while the assay is running. Contamination may occur if the permanent caps are not placed on the reaction tubes and/or if the permanent caps are removed.
8. Click start in the ANSR software to begin the 10 minute assay.
9. Results will be displayed as positive, negative or invalid once the assay is finished. If the result displays an invalid, the test must be repeated. A single repeat test can be run starting at step 5. Alternatively, start over from step 1 if lysis has been at 80 ± 2°C for more than 60 minutes.

Disposal

Enrichment cultures and used lysis tubes should be disposed of as biohazard waste. The preferred method of treatment for biohazard waste is autoclaving. Items that cannot be autoclaved, such as used reaction tubes and caps, should be immersed in a fresh 10% bleach solution that is made daily. Consult the safety advisor for your facility for detailed instructions.

Do not remove permanent caps, for any reason, from the ANSR reaction tubes once the assay has started, even when disposing of them. Reaction tubes can be disposed of as non-biohazardous waste. It is recommended that they be placed in sealable plastic bags and immediately disposed of to protect against accidental opening.

Customer Service
NEOGEN Customer and Technical Services can be contacted through NEOGEN.com and product training is available by request.

SDS Information Available
Safety data sheets are available for all test kits at NEOGEN.com or by calling 800.234.5333 or 517.372.9200.

Terms and Conditions
NEOGEN's full terms and conditions are available online.

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Commodity Group Table

Protocol	Product examples (meat)	Product examples (dairy)	Product examples (Seafood and vegetables)	Product Examples (Eggs)	Product Examples (Feeds)
1	Turkey Chicken Salami	Raw milk Pasteurised milk Pasteurised cheese	Fish Frozen fish Frozen vegetables Fresh Seafood Fresh Vegetables	–	Raw products Low moisture products Heat Processed Products
2	Ready-to-eat products	Milk powder	Ready-to-eat seafood Sandwiches	Egg Powder Liquid Egg Products Egg based Products	–