

## Routine Salmonella Serotype Identification

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### I. Description

Premi®Test Salmonella is a DNA-based detection method for the detection of *Salmonella* spp. and the identification of the Salmonella serotype when *Salmonella* spp. is present.

The *Salmonella* serotypes currently identified are shown in appendix 1.

### II. Kit components (for 72 samples)

Components (article nr.)	Description	Storage conditions
<b>Box RT</b>		
9-0020 Colony samplers	1 vial sterile colony samplers (±)	Room
9-0015 Lysis Buffer	1 bottle 10 ml	Room
9-0007 Detection Buffer	1 bottle 80 ml	Room
9-0008 Blocking Buffer	1 bottle 20 ml	Room
9-0014 Staining Solution	1 bottle 5 ml	Room temperature
10-0003 Array Tubes	6 bags of each 4 AT's (total 24)	Room
<b>Box -/-20°C</b>		
9-0022 Blue tray	24x3 tubes, 2.5 µl reagent/tube	-/- 20°C
9-0021 Solution A	1 tube (amber cap ●) 600 µl	-/- 20°C
9-0003 Solution B1	1 tube (purple cap ●) 1600 µl	-/- 20°C
9-0004 Solution B2	1 tube (blue cap ●) 120 µl	-/- 20°C
9-0005 Solution C1	1 tube (yellow cap ●) 1600 µl	-/- 20°C
9-0006 Solution C2	1 tube (red cap ●) 120 µl	-/- 20°C
9-0027 Conjugate	1 tube (green cap ●) 100 µl	-/- 20°C

Positive and negative controls are built into the system. It is, however, strongly recommended to use a positive and negative control for each series of reactions.

### III. Shelf life and Storage

Once received, the components of the kit must be stored at -/-20°C and room temperature. Please check the individual components for optimal storage conditions directly upon receiving of the kit. Reagents stored at the appropriate storage conditions can be used until the expiration date indicated on the boxes.

### IV. Materials required but not supplied with the kit:

Two different starter sets are available for Premi®Test Salmonella.

#### Basic starter set

- Micro Array Reader ATR-03
- Check-Points software

#### Total starter set

- Basic starter set
- PCR instrument
- Thermo Mixer: Eppendorf Comfort with thermoblock for 1.5/2ml tubes

#### Equipment not supplied

- Vortex mixer
- Personal computer
- Two bench top microfuges for PCR tubes (spectrafuge mini)

#### Supplies

- Disposable laboratory (powder free) gloves
- Pipettes & disposable (preferably filter-) tips for volumes of 1 to 1000 µl
- 1.5 ml tubes (Eppendorf)
- 10 ml tubes
- Selective enrichment agar, MSRV: (Modified Semisolid Rappaport- Vassiliadis) supplier: OXOID
- Non-selective isolation agar (nutrient agar)

#### V. Precautions and recommendations for best results

- The test must be performed by adequately trained personnel.
- Food samples and enrichment cultures must be treated (and discarded) as potentially infectious material.
- Spinning down for a few seconds is done in the various steps to ensure all material is properly collected on the bottom of the tubes.
- The quality of the results depends on strict compliance with the following good laboratory practice, especially concerning PCR:
  - Separate the activities in steps A, B and C, from the activities in the detection step, by using separate laboratory equipment and materials (pipettes, tubes, centrifuges etc.). Preferably use a dedicated room to separate the detection step from the activities in steps A, B and C. Alternatively work in a specialized hood for steps A, B and C.
  - Do not use reagents after their expiration date.
  - Before use, thaw frozen reagents **gradually** at room temperature and vortex after thawing to ensure homogeneity. After vortexing briefly spin down the solution to avoid contamination when opening the lid.
  - Periodically, verify the accuracy and precision of pipettes, as well as correct functioning of the instruments.
  - Change gloves frequently, especially if contamination is suspected or possible.
  - Clean work spaces daily with a 5% hypochlorite solution (bleach).

## VI. Protocol Premi®Test Salmonella

It is strongly recommended that the full protocol is read before using the test.

The protocol consists of the following steps:

1. Sampling
2. Lysis
3. DNA recognition step A
4. DNA recognition step B
5. DNA recognition step C
6. Detection step

### 1. Sampling

#### Non-selective isolation agar (nutrient agar)

- Dispense 100 µl Lysis Buffer into a 1.5 ml tube. Use a separate tube for each sample.
- Pierce a single colony, keeping the colony sampler in vertical position and pierce through the agar till the bottom. (It is advised to wear gloves.)
- Place the colony sampler in the 100 µl Lysis Buffer and twist the sampler between thumb and index finger while remaining in the buffer. Remove the sampler and discard. Close the tube, vortex and continue with step 2

#### MSRV

- The sample must be treated and inoculated according to a modification of ISO 6579 (2002), where a semi solid medium (MSRV) is used as the single selective enrichment. The semi-solid medium should be incubated at 41.5°C for minimum 24 (+/- 3) hours.  
MSRV: CM0910 and supplement SR0161E (OXOID)
- Dispense 100 µl Lysis Buffer into a 1.5 ml tube. Use a separate tube for each sample.
- Take a 5 µl aliquot from the MSRV plate suspected to contain *Salmonella* using a pipette and disposable tips. Pipet gently to avoid enclosure of air bubbles.  
Take the sample from the migration boundary. If migration is not evident, sample the centre of the inoculation point.
- Suspend the 5 µl aliquot in the 100 µl Lysis Buffer. Close the tube, vortex and continue with step 2.

### 2. Lysis

- Transfer the 1.5 ml tubes with the resuspended cells to a heating block and incubate at 99°C for 15 min. For this purpose the Thermo Mixer may be used: set the Thermo Mixer to 99°C and 400 rpm. This 15 minutes incubation at 99°C disrupts the cells and releases the DNA into the Lysis Buffer.
- Vortex and cool down to room temperature by placing the tubes on the table. (When the samples aren't used directly, store the tubes at -20°C).
- (Thaw and) vortex before continuing with step 3.

### 3. DNA recognition step A

- First add 5 µl solution A (cap colour amber ●) to every reaction tube of the strip (supplied with the kit). Next add 10 µl of DNA extract (step 2) of each sample. Please write down the sample reference for each tube of the strip.

- Close the tubes, spin down briefly using the minifuge to collect both sample and solution A at the bottom of the tubes. Mix well by tapping against each strip, make sure that the blue colour of the solution is homogeneous. Again spin down briefly using the minifuge.

- Note:**
- The reaction tubes supplied with the kit are prefilled with a small amount of blue-coloured reagent. Proper mixing of this reagent, solution A and the sample is crucial for optimal performance.
  - Solution A is frozen and should be thawed at room temperature and mixed properly before use.
  - When the DNA extracts have been stored at  $-20^{\circ}\text{C}$ , please thaw the sample properly and mix well.
  - When closing the tubes of the strip(s), don't use an excessive amount of pressure as the cap may distort, and the sample may then evaporate during DNA recognition steps A, B and C.
  - To avoid sample contamination, use a new pipette tip for every sample.
- Place the strip(s) in the PCR instrument and run the CP step A program (total sample volume 18  $\mu\text{l}$ ). The program will run for approximately 2.5 hours.

#### 4. DNA recognition step B

- Prepare B-mix in a 1.5 ml tube for recognition step B, while step A is proceeding. Take solution B1 (cap colour purple  $\color{purple}\bullet$ ) and solution B2 (cap colour blue  $\color{blue}\bullet$ ) from the freezer.

Solution B1 is frozen (B2 is not), and should be thawed properly at room temperature, mixed well, and spin down briefly before use. Use the pipetting scheme at the back of this protocol to prepare the required amount of B-mix. First add the required amount of B1 solution to the tube. Then dispense solution B2 in solution B1 by pipetting up and down 3 times. Mix very well by vortexing and spin down briefly.

- Store the B-mix in the refrigerator until step A is finished.
- Briefly spin down the reaction tubes after step A is finished.
- Add 15  $\mu\text{l}$  of the freshly prepared B-mix to each sample in the strip(s). Close the tubes, mix by tapping each strip, and spin down briefly.
- Place the strip(s) in the PCR instrument and run the CP step B program (total sample volume 33  $\mu\text{l}$ ). The program will run for approximately 1 hour.

#### 5. DNA recognition step C

- Prepare C-mix in a 1.5 ml tube for recognition step C approximately 10 minutes before the end of step B. Take solution C1 (cap colour yellow  $\color{yellow}\bullet$ ) and solution C2 (cap colour red  $\color{red}\bullet$ ) from the freezer. Solution C1 is frozen (C2 is not), and should be thawed properly at room temperature, mixed well, and spin down briefly before use. Use the pipetting scheme at the back of this protocol to prepare the required amount of C-mix. First add the required amount of C1 solution to the tube. Then dispense solution C2 in solution C1 by pipetting up and down 3 times. Mix very well by vortexing and spin down briefly.
- Store the C-mix in the refrigerator until step B is finished.
- Briefly spin down the reaction tubes after step B is finished.
- Add 15  $\mu\text{l}$  of the freshly prepared C-mix to each sample in the strip(s). Close the tubes, mix by tapping each strip, and spin down briefly.
- Place the strip(s) in the PCR instrument and run the CP step C program (total sample volume 48  $\mu\text{l}$ ). The program will run for approximately 1.5 hour.
- Transfer the reaction tubes to the area where the detection step is carried out. Briefly spin down the reaction mixture.
- Store the reaction mixtures at  $4^{\circ}\text{C}$  when the detection step is carried out within 24 hours. Alternatively, store the samples at  $-20^{\circ}\text{C}$  when the detection step is carried out within two weeks.

#### 6. Detection step



Figure 1: the ArrayTube (AT) and the ATR-03 Micro Array reader.

- Note:**
- The CLONDIAG ArrayTube™ DNA microarray platform is sold under licence from CLONDIAG chip technologies GmbH.

- 1- Start preparing the required number of ArrayTubes (AT's) for detection approximately 10 minutes before the end of step C program. Heat the Thermo Mixer to  $50^{\circ}\text{C}$ .

- Note:**
- one AT is required for every 3-tube strip.

- 2- Remove the AT's from their package(s) and place them in the Thermo Mixer at  $50^{\circ}\text{C}$ .

- Note:**
- Be careful when removing or adding liquid with the pipette from or to the AT, do not touch the micro array at the bottom of the tube at any time. Pipet all material in or out the AT at the side of the bottom of the tube without touching the array.

- 3- Add 300  $\mu\text{l}$  of Detection Buffer to every AT and shake the tubes for 2 minutes (400 rpm) in the Thermo Mixer. It is not necessary to close the tubes.

- 4- Remove the Detection Buffer from the AT's and repeat step 3.

- 5- Replace the Detection Buffer by 300  $\mu\text{l}$  of fresh Detection Buffer.

Take the samples from step C. Samples stored longer than 2 hours after step C was finished should be heated in the PCR instrument at  $95^{\circ}\text{C}$  for 2 minutes (CP Melt program of the PCR instrument). Briefly spin down the reaction mixture.

Transfer 10  $\mu\text{l}$  reaction mixture from each tube of one strip to the corresponding AT (in total 30  $\mu\text{l}$  per AT). The total volume of the AT will be 330  $\mu\text{l}$ .

The lid of the AT should be labelled for reference.

- Note:**
- Samples may contain a white-coloured precipitate. This is due to denaturation of one of the reaction components, a protein stabilizer. The presence of this precipitate has no effect on the result of the detection step and can be ignored when adding the sample.

- When adding samples to the AT do not remove the AT from the Thermo Mixer, to prevent the buffer from cooling down. Add the sample directly into the Detection Buffer of the AT by pipetting up and down.

- With the completion of step 5, three samples have been added to one AT.

- 6- Close the lids of the AT's and shake the tubes for 30 minutes at  $50^{\circ}\text{C}$  (400 rpm).

- Note:**
- Close the lid of the AT properly, to prevent the AT from drying out.

- 7- After 30 minutes, replace the Detection Buffer with 300  $\mu\text{l}$  of Blocking Buffer: do this with one AT at a time! Remove the AT from the Thermo Mixer, discard the Detection Buffer using a pipette and immediately replace with 300  $\mu\text{l}$  Blocking Buffer using a fresh pipette tip. Place the AT back in the thermoblock at  $50^{\circ}\text{C}$  and proceed to the next AT until all the AT solutions have been replaced with Blocking Buffer. It is important to replace Detection Buffer by Blocking Buffer in the ATs one by one, taking out one AT while the remaining AT's stay in the Thermo Mixer at  $50^{\circ}\text{C}$ . Shake for 5 minutes at  $50^{\circ}\text{C}$  (400 rpm).

**Optional:** Evaporated water condensed on the lid of the AT may be removed with a pipette, e.g. before removing the Detection Buffer containing the sample.

**Note:** – Transfer the liquids you remove from the AT's to a disposable tube, and dispose it the same day with the other laboratory waste.

- 8- Replace the Blocking Buffer with 300 µl of fresh Blocking Buffer. Adjust the temperature of the Thermo Mixer to 30°C and incubate for 10 minutes. During this incubation time the Thermo mixer can cool down from 50°C to 30°C.
- 9- In the mean time prepare a dilution of the conjugate solution (cap colour green ●) with Detection Buffer using the pipetting scheme at the back of this protocol. For this purpose a 1.5 ml tube or a 10 ml tube may be used depending on the amount required. Dispense the conjugate solution in the Detection buffer by pipetting up and down 3 times. Mix well by vortexing for 30 seconds.

**Note:** – Conjugate dilutions have to be made fresh, and should be used on the day of preparation.

- 10- Remove the Blocking Buffer completely and add 150 µl of the conjugate dilution. Incubate for 15 minutes at 30°C (400 rpm).
- 11- Remove the conjugate dilution from the AT's and add 600 µl of Detection Buffer. Shake the tubes for 2 minutes at 30°C (400 rpm).
- 12- Replace the Detection Buffer by 600 µl of fresh Detection Buffer, and shake the tubes again for 2 minutes at 30°C (400 rpm).
- 13- Remove the Detection Buffer from the AT's and add 150 µl of Staining Solution to each AT. Incubate for 15 minutes at room temperature.  
Make sure to store the bottle with staining solution in the dark after use.
- 14- In the mean time:
  - Start the computer
  - Start software on computer by double-clicking the Check-Points icon located on the desktop of the computer.

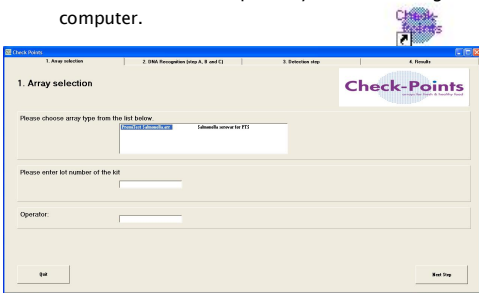


Figure 2: screen '1'. Array Selection"

- Double Click on "PremiTest Salmonella" in sheet 1 "Array selection" of the Check-Points software as shown in figure 2.  
Fill in the lot number of the kit in the appropriate field (optional) and the name of the Operator (optional), followed by pressing the "Next Step" button.
- In the sheet "DNA Recognition (A, B and C)" fill in the sample codes as shown in figure 3 and 4

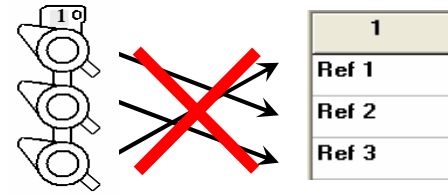
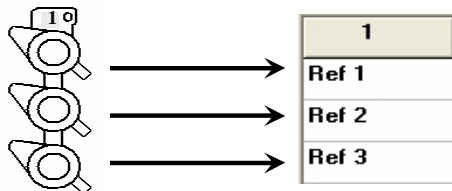


Figure 3: Example of filling in sample references in the software and assigning samples to the corresponding tubes

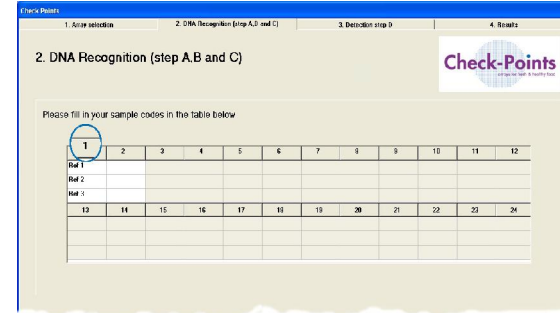


Figure 4: screen '2'. DNA Recognition (A, B and C)"

- Note:** – Samples without the reference filled in will not be analysed.
- When the sample references are filled in, additional remarks can be added per sample (optional) by double-clicking on one of the sample reference fields. A pop-up will appear (see fig 5) allowing remarks to be added to individual samples or to all samples, by marking the box of the sample in question.

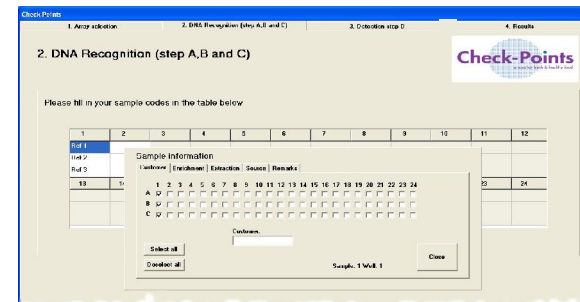


Figure 5: Pop-up for additional sample details.

- 15- Go to the sheet Detection step in the Check-Points' software (see figure 6), by pressing the "Next Step" button



Figure 6: Detection-step in Check-Points' software

16- The software will indicate the sample references which have to be put into the reader first and will ask for an 8 digit AT LOT number, located on the top of the label of the AT.

17- Fill in the AT lot number on the screen, place the AT into the reader and then press the "Confirm" button

**Note:** – Pressing "Confirm" leads to immediate scanning of the AT.

Press "Confirm" only after the full incubation period of 15 minutes is completed.

– When pressing "Confirm", if the AT is not in the reader, simply put the AT in the reader and press "Confirm" again.

– Scanning the AT after the incubation time (over 15 minutes) may lead to incorrect results!

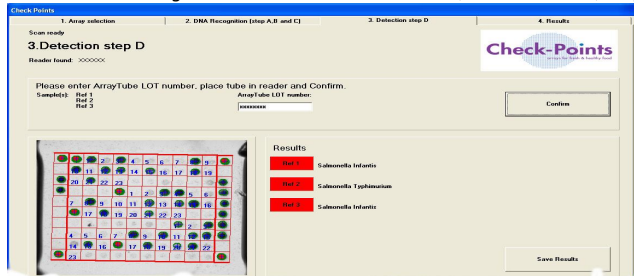


Figure 7: Presentation of the final result of an ArrayTube in the CP software

18- After pressing "Confirm", the results are shown (see figure 7). By pressing 'Save Results', the results will be saved in the database. The software will indicate which sample should be analysed next. Repeat from step 16 until all AT's are analysed.

19- When all AT's are analysed, a new window with the summary of the results will appear, which can also be printed (Press 'Print' button). Press the Quit button to end this run of analyses.

### Limitations of the test

The Premi® Test Salmonella uses highly specific DNA markers to identify the Salmonella serotype. The presence or absence of a range of these markers determines the serotype. The correlation between this DNA marker profile (expressed as genovar score) and the serotype has been tested and validated extensively for the serotypes listed in appendix 1 [1, as well as unpublished results]. Additional work with a wide range of serotypes has indicated that the vast majority of serotypes yield a unique genovar score. However, as more than 2600 Salmonella serotypes have been described in literature, it cannot be excluded that certain, exotic serotypes generate an identical genovar score. Therefore, the Premi® Test Salmonella result may need to be confirmed by traditional methodology in specific cases (e.g. for regulatory samples).

The Premi® Test Salmonella has been developed to serotype Salmonella enterica subsp. enterica bacteria. The test also recognizes the other Salmonella species and subspecies, but does not identify them all. Consequently, the test indicates the presence of Salmonella in these cases as a genovar score without a specific serotype, except for Salmonella bongori. Other (biochemical) tests are required to identify these strains.

The Premi® Test Salmonella is able to fully serotype non-motile or monophasic Salmonella. So, for example a monophasic S. Typhimurium strain will be typed as S. Typhimurium. A specific exception is S. 1,4,[5],12:i:-. When the specific DNA marker for the FliA gene is absent the score will be the S. 1,4,[5],12:i:-. In other cases S. 1,4,[5],12:i:- strains are identified as S. Typhimurium.

For known overlapping scores the probability score of the dominant serotype is based on the prevalence of the respective serotypes according to the database of the Global Salmonella Survey of the World Health Organization (WHO). (<http://www.who.int/salmsurv/en/>).

More details about the overlap in genovar scores as well as frequent updates of the Serotype list (appendix 1) can be found on the Premi® Test Salmonella website. [http://www.dsm.com/en\\_US/html/premitest/premitest\\_salmonella.htm](http://www.dsm.com/en_US/html/premitest/premitest_salmonella.htm).

1: Wattiau, P. et al. Evaluation of the Premi® Test Salmonella, a commercial low-density DNA microarray system intended for routine identification and typing of Salmonella enterica. International Journal of Food Microbiology (2008). Vol 123, p 293-298.

### Appendix 1:

#### Salmonella serotypes identified with Premi® Test Salmonella\*:

1	Abony	31	Heidelberg <sup>1</sup>	61	Reading
2	Adelaide	32	Ibadan	62	Rissen
3	Agona <sup>4</sup>	33	Indiana	63	Saintpaul <sup>4</sup>
4	Albany	34	Infantis <sup>1,2,4</sup>	64	San Diego
5	Altona	35	Isangi	65	Schwarzengrund
6	Anatum	36	Javiana	66	Senftenberg
7	Bareilly	37	Kedougou	67	Stanley
8	Berta	38	Kentucky	68	Teitelkebir
9	Blockley	39	Kottbus	69	Tennessee
10	Bovismorbificans	40	Lexington	70	Thompson
11	Braenderup <sup>4</sup>	41	Liverpool	71	Typhi
12	Brandenburg	42	Livingstone <sup>3</sup>	72	Typhimurium <sup>1,2</sup>
13	Bredeney	43	London	73	Urbana
14	Carrau	44	Manhattan	74	Virchow <sup>2</sup>
15	Cerro	45	Mbandaka <sup>3</sup>	75	Weltevreden
16	Chandans	46	Meleagridis	76	Worthington
17	Choleraesuis	47	Minnesota	77	Yoruba
18	Coeln	48	Montevideo <sup>1,4</sup>	78	S. 1,4,[5],12:i:- <sup>1</sup>
19	Cubana	49	Muenchen		
20	Derby	50	Muenster		
21	Dublin	51	Newport <sup>1,4</sup>		
22	Duisburg	52	Ohio		
23	Enteritidis <sup>1,2,4</sup>	53	Oranienburg <sup>4</sup>		
24	Gallinarum	54	Oslo		
25	Give	55	Quakam		
26	Gloucester	56	Panama		
27	Goldcoast	57	Paratyphi B		
			Paratyphi B v.		
28	Grumpensis	58	Java <sup>4</sup>		
29	Hadar <sup>2</sup>	59	Poona		
30	Havana	60	Pullorum		

\* Serotypes not included in this test may yield a Genovar score. (e.g. Salmonella, genovar 428).

This list, which will be extended with more serotypes on a regular basis, also includes the monophasic serotypes.

1 FSIS notice 04-026 (February 2006)

2 Regulations (EC) N.1003/2005 and (EC) N.1168/2006

3 Salmonella serotypes frequently found by EFSSA in broiler flocks

4 Most frequently reported Salmonella serotypes from human sources reported tot CDC in 2004

Pipetting scheme for B or C mix:  
conjugate dilution:

Pipetting scheme for  
conjugate dilution:

samples	µl B1 or C1	µl B2 or C2
1 - 3	45	3
4 - 6	90	6
7 - 9	135	9
10 - 12	180	12
13 - 15	225	15
16 - 18	270	18
19 - 21	315	21
22 - 24	360	24
25 - 27	405	27
28 - 30	450	30
31 - 33	495	33
34 - 36	540	36
37 - 39	585	39
40 - 42	630	42
43 - 45	675	45
46 - 48	720	48
49 - 51	765	51
52 - 54	810	54
55 - 57	855	57
58 - 60	900	60
61 - 63	945	63
64 - 66	990	66
67 - 69	1035	69
70 - 72	1080	72

AT's	µl conjugate solution	µl Det. Buf.
1	5	495
2	5	495
3	5	495
4	10	990
5	10	990
6	10	990
7	15	1485
8	15	1485
9	15	1485
10	20	1980
11	20	1980
12	20	1980
13	25	2475
14	25	2475
15	25	2475
16	30	2970
17	30	2970
18	30	2970
19	35	3465
20	35	3465
21	35	3465
22	40	3960
23	40	3960
24	40	3960

Despite the utmost care in the development and preparation of the protocol, DSM nor Check-Points® can take any responsibility for any errors, omissions and/or future changes herein.