Venor®GeM Classic

Mycoplasma Detection Kit for conventional PCR including Taq Polymerase

INSTRUCTIONS FOR USE

FOR USE IN RESEARCH AND QUALITY CONTROL

Symbols

Lot No.

Order No.

Expiry date

Storage temperature

Number of reactions

Manufacturer

INDICATION

The *Venor®GeM Classic Kit* is designed for the detection of Mollicutes, such as *Mycoplasma*, *Acholeplasma*, and *Spiroplasma,* in cell cultures and other biological matrices.

EXPLANATION OF THE TEST

The *Venor®GeM Classic Assay* is based on PCR amplification, as the established method of choice for rapid, robust and sensitive detection of mycoplasma contamination. The assay is targeting a highly conserved region within the mycoplasma genome to detect all mollicute species included in EP 2.6.7 and many more as listed in section “Assay Characteristics”.

The assay is suitable for the direct detection in cell culture supernatants usually applicable in research, or for performing the “cell culture enrichment” method, or after DNA extraction. The kit fully complies with the requirements of EP 2.6.7.

Almost all mycoplasma species will be detected in a single experiment, whereas eukaryotic and bacterial DNA is not amplified. The procedure takes less than 3 hours, and, in contrast to other methods like luminescence-linked enzymology, fluorescent staining or culture methods, there is no need for vital cells. Notably, the detection by PCR is considered to be superior in terms of sensitivity and precision.

This new version of the *Venor®GeM Classic* kit is now more convenient and contains already DNA Taq polymerase lyophilized in the *Classic PCR Mix* .

TEST PRINCIPLE

Mycoplasma are specifically detected by amplifying the 16S rRNA coding region in the mycoplasma genome. Depending on the mycoplasma species the amplicon is ~270 bp in size. The kit contains lyophilized components such as *Classic PCR Mix*, *Internal Control DNA*, and *Positive Control DNA* as well as *10x Reaction Buffer* and *PCR grade Water*.

The user instructions include protocols for both screening of cell culture supernatant as well as EP compliant testing with defined DNA extraction and sample volume specifications.

The *Classic PCR Mix* contains dUTP instead of dTTP to facilitate precursor amplicon degradation by use of uracil-DNA glycosylase (UNG). Thus, the probability of false-positive result is minimized. Please note that UNG is not included in the *Venor®GeM Classic Kit*. The *Internal Control DNA* as well the *Positive Control DNA* are means to assess the assays’ performance. The *Internal Control DNA* gives rise to a 191 bp amplicon.

KIT COMPONENTS

Each kit contains reagents for 25, 50, 100, or 250 reactions. The expiry date of the unopened package is marked on the package label. The kit components must be stored at +2 to +8 °C until use. The rehydrated components must be stored at ≤ -18 °C.

Component	Quantity				Cap Color
	25 Reactions Order No. 11-1025G	50 Reactions Order No. 11-1050G	100 Reactions Order No. 11-1100G	250 Reactions Order No. 11-1250G	
Classic PCR Mix	1 vial, lyophilized	2 vials, lyophilized	4 vials, lyophilized	10 vials, lyophilized	red
10x Reaction Buffer	1 vial, 0.5 ml	1 vial, 0.5 ml	1 vial, 0.5 ml	2 vials, 0.5 ml	blue
Positive Control DNA	1 vial, lyophilized	1 vial, lyophilized	1 vial, lyophilized	1 vial, lyophilized	green
Internal Control DNA	1 vial, lyophilized	1 vial, lyophilized	1 vial, lyophilized	1 vial, lyophilized	yellow
PCR grade Water	1 vial, 2.0 ml	1 vial, 2.0 ml	2 vials, 2.0 ml	4 vials, 2.0 ml	white

The lot-specific quality control certificate (*Certificate of Analysis*) can be downloaded from our website (www.minerva-biolabs.com).

USER-SUPPLIED CONSUMABLES AND EQUIPMENT

The *Venor®GeM Classic Kit* contains PCR reagents for the specific detection of mycoplasma. Additional PCR consumables and equipment is supplied by the user:

- PCR cycler and suitable PCR reaction tubes
- 1.5 ml reaction tubes, DNA-free
- Microcentrifuge for 1.5 ml and PCR reaction tubes
- Pipettes with corresponding filter tips (10, 100, and 1000 µl)
- Agarose gel electrophoresis system including dye, marker and loading buffer
- Requirement for EP 2.6.7 compliant testing:
  DNA extraction kit, e.g. *Venor®GeM Sample Preparation Kit*
  10 mM Tris-HCl, pH 8.4
- Optional for process validation and EP 2.6.7 compliant testing:
  Internal Control DNA extra (Cat. No. 11-1905)
  10CFU™ Sensitivity Standards available for all EP listed mycoplasma species
- Optional for carry-over prevention: Uracil DNA glycosylase (UNG)

SAMPLES FOR CELL CULTURE SCREENING

Samples should be obtained from cell cultures with 80 to 90 % confluence. Cell culture supernatant is very well suited for the mycoplasma test without the need of additional sample preparation. However, PCR inhibiting substances accumulate in the medium of cell cultures, which limits the sample volume per PCR or makes it necessary to extract the DNA prior to the PCR test (see “DNA extraction” below for further information). Note that penicillin or streptomycin in the culture media are not known to inhibit mycoplasma nor affect the tests’ sensitivity.

The average mycoplasma titer in cell culture is $\sim 10^6$ particles per ml with a maximum of 10^8 particles per ml. Within this range, a sufficient amount of mycoplasma DNA is present in the supernatant for successfully applying the PCR test. Prepare the PCR template in order to lyse mycoplasma and deactivate DNAses as follows:

1.	Transfer 100 µl of the supernatant from the cell culture to a sterile reaction tube. Close the lid tightly.
2.	Incubate the sample supernatant at 95 °C for 5 minutes.
3.	Centrifuge the sample at max. speed for 15 s to pellet any cellular debris.
4.	Use up to 2 µl directly for PCR, or store the sample for up to 6 days at 4 °C or at ≤–18 °C for long time storage.

Cell pellets cannot be used directly for the test due to cell debris that will interfere with the PCR reaction. Higher sample volumes or other biological materials such as foetal calf serum (> 5 %), vaccines, cryo stocks, and paraffin-embedded samples require DNA extraction as well.

SAMPLES FOR EP 2.6.7 COMPLIANT TESTING

EP 2.6.7 compliant testing requires DNA extraction. The material or volume may vary. Follow the sample concentration protocol on next page if the sample volume is higher than 200 µl. Specimen should be stabilized after sampling if the DNA extraction cannot be done immediately. Please follow the stabilization protocol in theses cases:

Sample stabilization (optional)

Cell culture samples are likely to contain high concentrations of DNases which will degrade mycoplasma DNA even at lower temperatures. Therefore we recommend the following steps to stabilize the sample. This step is not necessary if DNA extraction is performed immediately after sample collection.

1.	Transfer 500 µl of the supernatant from the cell culture into a 1.5 ml reaction tube. Close the lid tightly.
2.	Incubate the sample at 95 °C for 10 minutes.
3.	Centrifuge the sample at max. speed briefly (15 s) to pellet cellular debris.
4.	Use the sample for DNA extraction. Store the sample for up to 6 days at 4 °C or at ≤ –18 °C for long time storage.

SPECIMEN FOR EP 2.6.7 COMPLIANT TESTING (CONTINUED)

DNA Extraction (required)

A substantial body of evidence shows that DNA extraction is required to achieve the highest level of sensitivity. Numerous DNA extraction methods are established for a vast variety of sample materials. However, the DNA extraction method must be compatible for mycoplasma genomes. For EP compliant testing, the DNA method must be tested in combination with the PCR kit.

We recommend our *Venor®GeM Sample Preparation Kit* (Cat. No. 56-1010/-1050/-1200). This DNA extraction kit was tested extensively. The protocol for DNA extraction is described in detail in the instructions for use of the DNA extraction kit.

Sample concentration (optional)

For sample volumes > 200 to 1000 µl, a concentration step is recommended to achieve optimal sensitivity. Please note that the sample concentration works only with intact cells. Therefore, any step to disrupt cells such as heat inactivation prior to sample concentration must be avoided. Samples up to 200 µl volume can be processed directly without a concentration step.

1.	Transfer up to 1 ml supernatant of the sample into a 1.5 ml reaction tube.
2.	Centrifuge the sample at ≥ 10,000 x g for 15 min (or ≥ 13,000 x g for 6 min) to pellet mycoplasma particles.
3.	Discard the supernatant and re-suspend the pellet in 200 µl Tris buffer (10 mM Tris-HCl, pH 8.4).
4.	Vortex the sample briefly and proceed immediately with sample stabilization or DNA extraction.

RECOMMENDATIONS

According to EP 2.6.7 a sensitivity of 10 CFU/ml must be demonstrated. The sample material can be spiked with 10 CFU of mycoplasma by using special reference materials (*10CFU™ Sensitivity Standard*s, Cat. No. 102-0002) and processed in parallel.

The *Internal Control DNA* of *Venor®GeM Classic Kit* is used to validate the DNA extraction step as well. Please note, that the actual sample volume that will be spiked is not relevant for the required volume of *Internal Control DNA*. The volume of *Internal Control DNA* depends on the final elution volume (containing the DNA extract) of the DNA extraction step. In general, add 5 µl per 10 µl DNA extract directly to the sample, vortex briefly and process the DNA extraction as described. (Example: add 30 µl *Internal Control DNA* to the original sample if the elution volume will be 60 µl Elution Buffer.) Do not add further *Internal Control DNA* to the PCR master mix if the internal control was already added to the sample before. *Internal Control DNA* can be purchased separately (*Internal Control DNA Extra*, Cat. No. 11-1905).

REMARKS ON EP 2.6.7 COMPLIANT VALIDATION

Please note that validation data are provided for information purpose only, containing basic information on specificity and sensitivity. EP 2.6.7 clearly states “Where commercial kits are used …, documented validation points already covered by the kit manufacturer can replace validation by the user. Nevertheless, the performance of the kit with respect to its intended use has to be demonstrated by the user (e.g. detection limit, robustness, cross-detection of other classes of bacteria.”. Please contact us for assistance.

PRECAUTIONS

The *Venor®GeM Classic Kit* is intended for in vitro use only. The kit should be used by trained laboratory staff only.

All samples should be considered as potentially infectious and handled with all due care and attention. Always wear suitable lab coat and disposable gloves.

This kit does not contain hazardous substances. Remnants can be discarded according to local regulations.

Cross contamination may lead to false-positive test results. Thus all tests should be performed according to good laboratory practice.

IMPORTANT NOTES

⇒ These instructions must be understood to successfully use the *Venor®GeM Classic Kit*. The components supplied should not be mixed with reagents from different lot and used as an integral unit. The reagents of the kit must not be used beyond their shelf life.

⇒ Follow the exact protocol. Any deviation may affect the test method and can affect the results.

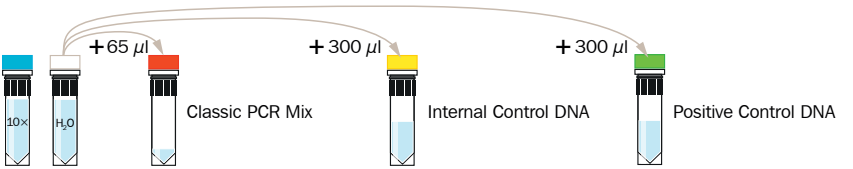
⇒ Avoid cross contamination by preparing the Positive Controls after the Negative Controls and Test Reactions

⇒ Set up at least one negative control sample (non template control) in each PCR. Use fresh cell culture medium or elution buffer for the NTC in case of extracted DNA.

⇒ PCR inhibition is likely to be caused by the sample matrix, or, in case of extracted DNA, caused by the elution buffer. Thus we recommend our *Venor®GeM Sample Preparation Kits*. Any other DNA extraction kit needs to be qualified.

PROCEDURE - OVERVIEW

1. Reagent preparation

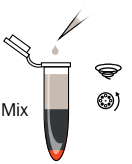


5 min briefly 5 sec

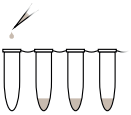
2. Reaction mix preparation

for cell culture screening

mix components*
15.5 µl H_2O
2.5 µl 10×
2.5 µl Classic PCR Mix
2.5 µl IC



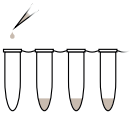
aliquot 23 µl



for EP 2.6.7 testing

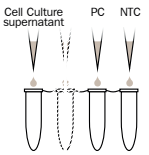
mix components*
7.5 µl H_2O
2.5 µl 10×
2.5 µl Classic PCR Mix
2.5 µl IC

aliquot 15 µl



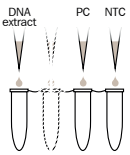
3. Add samples

+ 2 µl



briefly

+ 10 µl



briefly

4. PCR amplification

1 cycle	94 °C for 2 min
39 cycles	94 °C for 30 sec
	55 °C for 30 sec
	72 °C for 30 sec
hold	4 - 10 °C

5. Gel electrophoresis

+ add vortex incubate centrifuge * example composition

This procedure overview is not a substitute for the detailed manual.

PROCEDURE - STEP BY STEP

⇒ Set up negative and positive controls with each test.

1. Reagent preparation

1.	*Classic PCR Mix* *Internal Control DNA* *Positive Control DNA*	red cap yellow cap green cap	Centrifuge all components at max. speed for 5 sec
2.	*Classic PCR Mix*	red cap	Add 65 µl PCR grade Water (white cap) For sample kit only: Add 15 µl PCR grade Water
3.	*Internal Control DNA*	yellow cap	Add 300 µl PCR grade Water (white cap)
4.	*Positive Control DNA*	green cap	Add 300 µl PCR grade Water (white cap)
5.	*Classic PCR Mix* *Internal Control DNA* *Positive Control DNA*	red cap yellow cap green cap	Incubate at room temperature for 5 min
6.	*Classic PCR Mix* *Internal Control DNA* *Positive Control DNA*	red cap yellow cap green cap	Vortex briefly and spin down for 5 sec

After reconstitution, the reagents must be stored at ≤–18 °C. Repeated freeze-thaw-cycles should be avoided. For small sample numbers, we recommend to prepare aliquots of reconstituted *Classic PCR Mix*, the *Positive Control DNA* and the *Internal Control DNA*.

2. Reaction mix preparation

Prepare the required amount of reaction mix at room temperature in a 1.5 ml reaction tube for all control and test reactions.

2a) Reaction mix for cell culture screening (2 µl sample volume)

		for 1 reaction	for 25 reactions
1.	*PCR grade Water*	15.5 µl	387.5 µl
	10x Reaction Buffer	2.5 µl	62.5 µl
	Classic PCR Mix	2.5 µl	62.5 µl
	Internal Control DNA	2.5 µl	62.5 µl
2.	Vortex the reaction mix briefly and spin down for 5 s.		
3.	Pipet 23 µl to each PCR tube, discard remaining material.		

2b) Reaction mix for EP 2.6.7 compliant testing (10 µl sample volume)

		for 1 reaction	for 25 reactions
1.	*PCR grade Water*	7.5 µl	187.5 µl
	10x Reaction Buffer	2.5 µl	62.5 µl
	Classic PCR Mix	2.5 µl	62.5 µl
	Internal Control DNA *	2.5 µl	62.5 µl
	* Add water instead if the *Internal Control DNA* was added to the sample prior to DNA extraction.		
2.	Vortex the reaction mix briefly and spin down for 5 s.		
3.	Pipet 15 µl to each PCR tube, discard remaining material.		

3. Add samples

⇒ Set up positive and negative control samples (non template control) in each PCR.

3a) for cell culture screening (2 µl sample volume)

1.	Negative Controls: add 2 µl *PCR grade Water* (white cap).
2.	Samples: add 2 µl of cell culture supernatant or DNA extract.
3.	Positive Control: add 2 µl *Positive Control DNA* (green cap).
4.	Close and spin all PCR tubes briefly, load the PCR cycler and start the program.

3b) for EP 2.6.7 compliant testing (10 µl sample volume)

1.	Negative Controls: add 10 µl elution buffer from DNA extraction kit (ref. chapter “Sample Material“).
2.	Samples: add 10 µl DNA extract.
3.	Positive Control: add 2 µl *Positive Control DNA* (green cap) and 8 µl of *PCR grade Water* (white cap).
4.	Close and spin all PCR tubes briefly, load the PCR cycler and start the program.

4. Start PCR amplification

1.	Place the PCR tubes in the cycler and close the lid tightly.		
2.	Program the PCR cycler or check stored temperature profiles.		
	1 cycle	94 °C for 2 min	
	39 cycles	94 °C for 30 sec	
		55 °C for 30 sec	
		72 °C for 30 sec	
	Hold between +4 °C to +10 °C		
3.	Start the program.		

5. Agarose gel electrophoresis

⇒ Use your established gel electrophoresis system, agarose gel and DNA stain if compatible with PCR products between 200 and 300 bp. Otherwise follow this example:

1.	Prepare a 1.5 to 2.0 % agarose gel including DNA stain (~5 mm thick, 5 mm comb).		
2.	Mix 5 µl from each PCR reaction with a suitable loading buffer and load the mix. Note: Bromophenol blue will run similarly to ~270 bp PCR fragments and may therefore mask the PCR product. Make sure to use bromophenol blue in a low concentration or other dyes such as Orange G or Xylene Cyanol.		
3.	Perform the gel electrophoresis (e.g. 20 min at 100 V). Visualize the PCR results on a suitable transilluminator.		
4.	Expected amplicon sizes:	Internal control *Mycoplasma spp.*	191 bp 265-278 bp

RESULT INTERPRETATION

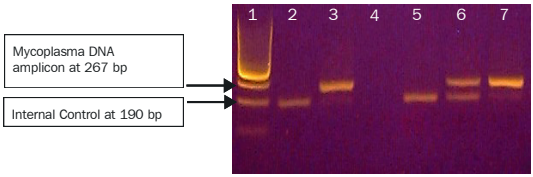
The *Internal Control DNA* gives rise to a distinct 191 bp band in every lane indicating a successfully performed PCR. This band will fade out with increased amounts of primary target amplification (e.g. mycoplasma DNA input of > 10^3 copies per PCR. The initial concentration of positive control DNA is higher than 10^4 copies per PCR. Consequently, the internal control is usually not visible in the positive control reaction.

Other PCR products may be visible in the gel as faint, diffuse bands of different sizes (neither 191 bp nor ~270 bp). This does not indicate positive results. These products are unspecific and caused by non-specific annealing (e.g. high DNA input of > 100 µg/ml). Also, primer self-annealing may give rise to a band of 80-90 bp in size. This again does not affect the sensitivity and precision or results of the test.

If the PCR test shows inhibition due to the sample (lower band intensity compared to negative control) a DNA extraction needs to be performed prior to re-testing the sample (see chapter "Specimen").

Detection of *Mycoplasma* band at 265-278 bp	Internal control band at 191 bp	Interpretation
Positive	Irrelevant	Mycoplasma present in the sample
Negative	Negative	PCR inhibition
Negative	Positive	No mycoplasma detectable in the sample

Fig. 1: A typical agarose gel image



1: 100 bp DNA Ladder
2: negative control reaction
3: positive control reaction
4: inhibited sample
5: negative sample
6: positive sample, weak contamination
7: positive sample, strong contamination

ANALYTICAL CHARACTERISTICS OF THE TEST

An extensive validation study is available on request.

Analytical Sensitivity

The detection limit depends on the species and ranges from ≤ 2.5 to ≤ 10 CFU/ml using the EP 2.6.7 compliant protocol with 10 µl sample volume per PCR reaction. For all EP 2.6.7 listed *Mycoplasma* species the required detection limit of 10 CFU/ml was reached with a pre-analytical DNA extraction using the *Venor® GeM Sample Preparation Kit*.

Species	Detection limit LOD_{95} [CFU/ml]
Acholeplasma laidlawii	≤ 2.5
Mycoplasma hyorhinis	≤ 2.5
Mycoplasma fermentans	≤ 2.5
Mycoplasma orale	≤ 10
Mycoplasma synoviae	≤ 10

Species	Detection limit LOD_{95} [CFU/ml]
Mycoplasma pneumoniae	≤ 10
Mycoplasma arginini	≤ 10
Mycoplasma gallisepticum	≤ 10
Spiroplasma citri	≤ 10

Cross Reactivity

Cross-reactivity with eukaryotic DNA origin could not be found. Unspecific PCR products such as faint, diffuse bands of different sizes are rarely observed (see chapter "Result Interpretation). The kit will not detect any of the phylogenetically related microorganisms, such as *Clostridium*, *Lactobacillus* and *Streptococcus*. Likewise, the waterborne germ *Burgholderia* is not detected. The test is positive for *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Bacillus subtilis* at concentrations above 10^4 genomes/µl.

Sequence Alignment

A substantial number of *Mycoplasma* sequences have been published. The primers of the kit were aligned against the NCBI data and scrutinized for homologies within the target region of the 16S rRNA. The following table shows all mycoplasma species with relevant sequence homologies and, thus, presumptively positive PCR results. The table also shows species that will not be detected:

Positive tested *Mollicutes*	Negative tested		
	EP listed bacteria	Other microorganisms	Mammals
Acholeplasma laidlawii	*Clostridium acetobutylicum*	*Chlamydia trachomatis*	Vero-B4
Mycoplasma arginini	*Lactobacillus acidophilus*	*Legionella pneumophila*	Per.C6
Mycoplasma arthritidis	*Streptococcus pneumoniae*	*Micrococcus luteus*	RK13
Mycoplasma fermentans		*Candida albicans*	CHO-K1
Mycoplasma genitalium		*Enterococcus faecalis*	Murine genomic DNA
Mycoplasma hominis		*Enterobacter aerogenes*	Calf thymus DNA
Mycoplasma hyorhinis		*Escherichia coli*	Foetal bovine serum
Mycoplasma orale		*Proteus mirabilis*	Horse serum
Mycoplasma penetrans		*Bacillus cereus*	Goat serum
Mycoplasma pneumoniae			
Mycoplasma salivarium			
Mycoplasma synoviae			
Ureaplasma urealyticum			

Related Products

Contamination Control Kits for conventional and real-time PCR

11-7024/-7048/-7096/-7240	Venor®GeM Advance Mycoplasma Detection Kit	24/48/96/240 tests
11-8025/-8050/-8100/-8250	Venor®GeM OneStep Mycoplasma Detection Kit	25/50/100/250 tests
11-9025/-9100/-9250	Venor®GeM qEP Mycoplasma Detection Kit	25/100/250 tests
11-91025/91100/91250	Venor®GeM qOneStep Mycoplasma Detection Kit	25/100/250 tests
12-1025/-1050/-1100/-1250	Onar® Bacteria Detection Kit	25/50/100/250 tests

Sample Preparation

56-1010/1050/1200	Venor®GeM Sample Preparation Kit	10/50/200 extractions

Mycoplasma Elimination

10-0200/0500/1000	Mynox® Mycoplasma Elimination Reagent	2/5/10 treatments
10-0201/0501/1001	Mynox® Gold Mycoplasma Elimination Reagent	2/5/10 treatments

Genomic DNA Extracts - Specificity Standards 10 ng ± 2 ng / vial

51-0129	*Mycoplasma arginini*
51-0112	*Mycoplasma orale*
51-0115	*Mycoplasma gallisepticum*
51-0119	*Mycoplasma pneumoniae*
51-0124	*Mycoplasma synoviae*
51-0117	*Mycoplasma fermentans*
51-0130	*Mycoplasma hyorhinis*
51-0116	*Acholeplasma laidlawii*
51-0164	*Spiroplasma citri*

See Minerva homepage for further available species

10CFU™ Sensitivity Standards 3 vials with 10 CFU each, 2 vials negative control

102-1003	*Mycoplasma arginini*	
102-2003	*Mycoplasma orale*	
102-3003	*Mycoplasma gallisepticum*	
102-4003	*Mycoplasma pneumoniae*	
102-5003	*Mycoplasma synoviae*	
102-6003	*Mycoplasma fermentans*	
102-7003	*Mycoplasma hyorhinis*	
102-8003	*Acholeplasma laidlawii*	
102-9003	*Spiroplasma citri*	
102-0002	*Mycoplasma* Set, all EP 2.6.7 listed species	2 vials per species, 10 CFU each

PCR Clean™ (formerly DNA Remover™)

15-2025/15-2200	DNA Decontamination Reagent, spray bottle/refill bottles	250 ml/4x 500 ml
15-2201	Wipes	120 wipes in a dispenser box
15-2202	Wipes, refill packs	5 x 120 wipes in a bag

Mycoplasma Off™

15-1000	Surface Disinfectant Spray, spray bottle	1000 ml
15-5000	Surface Disinfectant Spray, refill bottles	5 x 1000 ml
15-1001	Surface disinfectant Wipes in dispenser box	120 wipes
15-5001	Surface Disinfectant Wipes, refill pack	5 x 120 wipes

ZellShield™

13-0050/-0150	Contamination Prevention Reagent 100x concentrate	50 ml/ 3 x 50 ml

WaterShield™

15-3015/-3020/-3050	Water Disinfection Additive for incubators and water baths 200x concentrate	30x5ml / 3x50ml / 500ml

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