Etest®

Cefotaxime/cefotaxime + clavulanic acid

Ceftazidime/ceftazidime + clavulanic acid

Cefepime/cefepime + clavulanic acid

For *in vitro* confirmation of ESBL

INTENDED USE

Etest® ESBL Cefotaxime/cefotaxime + clavulanic acid (CT/CTL), Ceftazidime/ceftazidime + clavulanic acid (TZ/TZL) and Cefepime/cefepime + clavulanic acid (PM/PML) strips are designed to confirm the presence of clavulanic acid inhibitable ESBL (Extended Spectrum Beta-Lactamase) enzymes in *Escherichia coli*, *Klebsiella pneumoniae* and *K. oxytoca* and other relevant species within Enterobacteriaceae. The suspected presence of ESBL in strains with phenotypic susceptibility patterns where MIC values of aztreonam, cefotaxime, ceftazidime, ceftriaxone or cefpodoxime are ≥ 1 µg/mL, can be confirmed using both Etest CT/CTL and TZ/TZL strips. When testing organisms where inducible chromosomal AmpC ß-lactamases can interfere with the clavulanic acid synergy e. g . *Enterobacter* species, it may be appropriate to use Etest PM/PML in conjunction with Etest CT/CTL and TZ/TZL for confirmation of Etest ESBL. Equally, Etest PM/PML can be used to test strains showing non-determinable ESBL results with CT/CTL and TZ/TZL.

SUMMARY AND EXPLANATION

ESBLs are plasmid-mediated enzymes that have evolved through point mutations of key amino acids in parent TEM and SHV enzymes. Although ESBLs vary in their ß-lactam substrate affinity and enzyme kinetics, they essentially inactivate all penicillins and cephalosporins. They are generally inhibited by ß-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam but inhibitor resistant TEM (IRT) ESBL enzymes have been reported. Currently, ESBLs are not active against cefamycins and carbapenems. Multiple ESBL enzymes (1 to 5) and chromosomally or plasmid encoded AmpC ß-lactamases may be simultaneously produced in the same clinical isolate. Plasmids encoding for ESBL may also carry resistance genes for trimethoprim/sulfamethoxazole and aminoglycosides. Chromosomal fluoroquinolone resistance is commonly found in ESBL producing strains.

ESBLs have been selected for after many years of extensive use of expanded spectrum cephalosporins (ESC) such as ceftazidime. Although *K. pneumoniae* and *E. coli* are the main pathogens producing ESBLs, more recently *K. oxytoca, Enterobacter cloacae, E. aerogenes, Serratia marcesens, Citrobacter diversus, Providencia stuartii, Proteus mirabilis, Salmonella typhimurium, Pseudomonas aeruginosa, Burkholderia cepacia* and *Acinetobacter* spp. have also been reported to produce ESBL. ESBLs are often produced by nosocomial pathogens found in intensive care units, oncology, burn and neonatal wards, nursing homes and in infections associated with indwelling devices. Several ESBL outbreaks have occurred in the USA, South America and Western Europe and prevalence is increasing worldwide.

Besides being associated with high morbidity and mortality, few options remain for treatment of infections involving ESBLs. The efficacy of ß-lactam therapy, including the use of ESC, is compromised while co-resistance to trimethoprim/sulfamethoxazole, aminoglycosides and fluoroquinolones has been reported. Therefore, accurate *in vitro* detection of ESBLs is essential to guide therapy selection and allow for efficient infection control interventions to be rapidly implemented. Current susceptibility test methods are not useful for ESBL detection because MIC values and zone sizes of ESBL producers overlap those of susceptible strains. CLSI recommends ESBL screen criteria for *K. pneumoniae*, *K. oxytoca* and *E. coli* of MIC ≥ 8 µg/mL to cefpodoxime, MIC ≥ 2 µg/mL to ceftazidime, cefotaxime, ceftriaxone or aztreonam, or zone diameters of ≤ 17 mm for cefpodoxime, ≤ 22 mm for ceftazidime, ≤ 27 mm for aztreonam or cefotaxime or ≤ 25 mm for ceftriaxone. CLSI screen recommendations for *P. mirabilis* are MIC ≥ 2 µg/mL for cefpodoxime, ceftazidime and cefotaxime, or zone diameters of ≤ 22 mm for cefpodoxime and ceftazidime or ≤ 27 mm for cefotaxime. The CLSI MIC confirmatory criterion for ESBL is a reduction of ≥ 3 $\log_2$ dilutions for either cefotaxime or ceftazidime in the presence of 4 µg/mL clavulanic acid.

PRINCIPLES OF USE

Etest ESBL CT/CTL, TZ/TZL and PM/PML strips (Figure 1) consist of a thin, inert and non-porous plastic carrier. One side of the strip is calibrated with the MIC reading scales in µg/mL while the reverse surface carries two predefined exponential gradients. CT codes for the cefotaxime (0.25-16 µg/mL) gradient and CTL the cefotaxime (0.016-1 µg/mL) plus 4 µg/mL clavulanic acid. TZ codes for the ceftazidime (0.5-32 µg/mL) gradient and TZL the ceftazidime (0.064-4 µg/mL) plus 4 µg/mL clavulanic acid. PM codes for the cefepime (0.25-16 µg/mL) gradient and PML the cefepime (0.064-4 µg/mL) plus 4 µg/mL clavulanic acid. The test is set up according to standard Etest procedures for Gram negative aerobes, however, an inhibition ellipse may be observed at each end of the strip (Figure 4).

Testing must be done with at least **both Etest CT/CTL and TZ/TZL strips.** The presence of ESBL is confirmed by the appearance of a phantom zone or deformation of the CT, TZ or PM ellipse (**READING AND INTERPRETATION**, Figures 5 and 6) or when either the MIC of CT, TZ or PM is reduced by ≥ 3 $\log_2$ dilutions in the presence of clavulanic acid. Non-determinable results with Etest CT/CTL and TZ/TZL should be confirmed using Etest PM/PML strips.

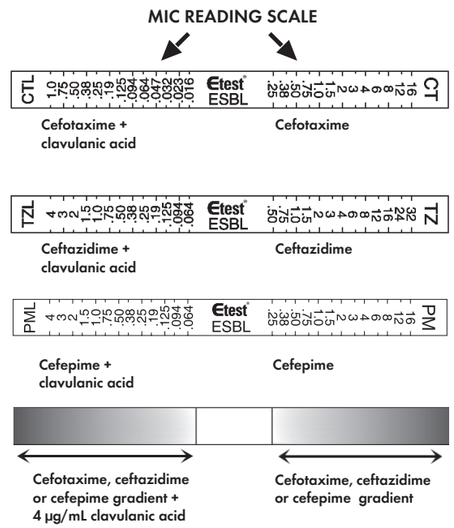

Figure 1. Configuration of Etest ESBL strips

REAGENTS

- 100 or 30 reagent units of either Etest CT/CTL, TZ/TZL or PM/PML
- 1 package insert

STORAGE

All unopened packages and unused Etest ESBL strips must be stored at -20 °C or the temperature denoted on the package until the given expiry date. Unused strips must be stored in an airtight storage container with colour indicating desiccant. The batch number and expiry date should be clearly marked on the package and/or storage container.

Protect Etest ESBL strips from moisture, heat and direct exposure to strong light at all times.

Prevent moisture from penetrating into or forming within the package or storage container. Etest ESBL strips must be kept dry.

HANDLING

Before using Etest ESBL strips from an unopened package, visually inspect to ensure the package is intact. Do not use the strips if the package has been damaged.

When removed from the -20 °C freezer, allow the package or storage container to reach room temperature for about 30 minutes. Moisture condensing on the outer surface must evaporate completely before opening the package.

Open the package according to the instructions. When handling Etest ESBL strips manually, grip only the strip at the area Etest ESBL. **Do not touch the surface of the strip with the antibiotic gradient i.e. the side opposite the scale.** Strips can be placed in an applicator tray until ready to use (Figure 2). The vacuum pen Nema C88™ (AB BIODISK) can be used to efficiently apply the strips to the agar surface.

PRECAUTIONS AND WARNINGS

- Etest ESBL is intended for *in vitro* diagnostic use only.
- Although the procedure is straightforward, proper use of the system requires the judgement of skilled personnel trained in microbiology and antimicrobial susceptibility testing.
- Etest ESBL should be used strictly according to the procedures described herein.
- Aseptic procedures should be used at all times when handling bacterial specimens and established precautions against microbiological hazards strictly adhered to. Agar plates should be sterilised after use, before discarding.
- Occasionally, static electricity can cause two or more strips to stick together. Make sure that you separate the strips and apply only one at a time onto the agar surface.
- Due to the instantaneous release of antibiotic, Etest ESBL strips cannot be moved once in contact with the agar surface.
- Please consult Etest references and technical information (www.abbiodisk.com), and read the package insert thoroughly before using Etest ESBL for the first time.

PROCEDURE

Materials required but not provided:

- Mueller Hinton agar plates (depth of 4 ± 0.5 mm)
- Sterile saline (0.85% NaCl)
- Sterile loops, swabs (not too tightly spun), test tubes, pipettes and scissors
- Forceps, Etest manual applicator or Biotools™ (Retro C80™, Nema C88, AB BIODISK)
- 0.5 McFarland turbidity standard
- Incubator (35 ± 2 °C)
- Quality control organisms
- Storage container with desiccant
- Additional technical information from www.abbiodisk.com/ Etest Technical Manual

Agar medium

Ensure that the agar depth is 4.0 ± 0.5 mm, pH 7.3 ± 0.1 and results fulfil specifications (**QUALITY CONTROL**, Table 2).

Inoculum preparation

Emulsify several well-isolated colonies from an overnight agar plate in saline to achieve a turbidity equivalent to a 0.5 McFarland standard. When the inoculum is correct, a confluent or almost confluent lawn of growth will be obtained after incubation. Perform regular colony counts to **verify that your procedure gives the correct inoculum density in terms of CFU/mL.**

Note:

As the ESBL amount is inoculum dependent, too heavy or too light an inoculum may affect results. Excess enzyme may quench the clavulanic acid component in the test and potentially reduce the MIC ratio of CT/CTL, TZ/TZL or PM/PML and give a false negative result. On the contrary, too little enzyme may give a lower MIC for CT, TZ or PM, and reduce the CT/CTL, TZ/TZL and PM/PML ratio.

Inoculation

Dip a sterile, non-toxic swab into the inoculum suspension. Remove excess fluid by pressing the swab against the inside wall of the test tube. Swab the entire agar surface three times, rotating the plate approximately 60 degrees each time to ensure an even distribution of inoculum. Alternatively, use Retro C80 (rota-plater) to efficiently streak the inoculum over the agar surface. Allow excess moisture to be absorbed for about 15 minutes so that the **surface is completely dry** before applying Etest ESBL strips.

Application

Check that the inoculated agar surface is **completely dry** before applying Etest ESBL strips. Open the package and handle the strips as described under **HANDLING**. Etest ESBL strips can be applied to the inoculated agar surface with forceps, a manual Etest Applicator, or Nema C88 (Figure 3). Always place the strip on the agar with the MIC scale facing upward i.e. towards the opening of the plate, and the antibiotic gradient on the agar surface. If incorrectly placed upside down, no ellipse will form because the antibiotic cannot diffuse across the non-porous plastic strip.

Ensure the whole length of the strip is in complete contact with the agar surface. If necessary, remove air pockets by pressing gently on the strip with forceps, always moving from the lowest concentration upwards. Small bubbles under the strip will not affect results. Once applied, the strip cannot be moved as the antibiotic is instantaneously released into the agar.

Figure 2. ESBL strips in an Etest Applicator tray.

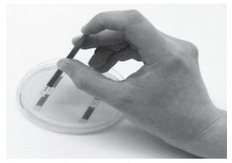

Figure 3. Applying Etest strips with the manual Etest Applicator

Incubation

Incubate the agar plates in an inverted position at 35 ± 2 °C for 16-20 hours in ambient atmosphere.

READING AND INTERPRETATION

Reading

When bacterial growth is visible, read the CT, CTL, TZ, TZL, PM and PML MIC values where the respective inhibition ellipses intersect the strips (Figure 4). Growth along the entire gradient i.e. no inhibition ellipse indicates that the MIC is greater than or equal to (≥) the highest value on the reading scale. An inhibition ellipse below the gradient indicates a MIC less than (<) the lowest value on the scale.

When mutant colonies are present in the inhibition ellipse, read the MIC where these colonies are completely inhibited. For MIC values in the high range, inhibition ellipses may be very small or not clearly discernable. Occasionally, a "rounded" zone (phantom zone) may be seen below the CTL, TZL or PML gradients and an ellipse may/may not be seen around the CT, TZ or PM ends (Figure 5). The CT, TZ or PM inhibition ellipse may also be deformed at the tapering end (Figure 6). The presence of a phantom zone or ellipse deformation is a unique advantage of the Etest ESBL technique. It clearly indicates ESBL detected at unusual ratios of synergy between the ß-lactam substrate CT, TZ, or PM and the clavulanic acid diffusing across from the CTL, TZL or PML sections. Different growth inhibition patterns are illustrated in Figures 4-7.

Different growth-inhibition patterns:

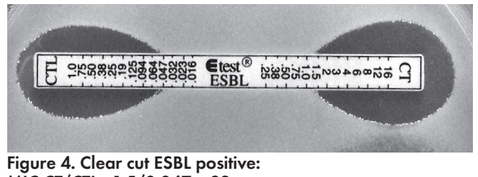

Figure 4. Clear cut ESBL positive: MIC CT/CTL =1.5/0.047 = 32

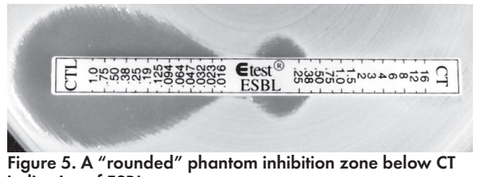

Figure 5. A "rounded" phantom inhibition zone below CT indicative of ESBL.

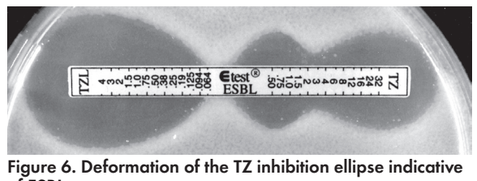

Figure 6. Deformation of the TZ inhibition ellipse indicative of ESBL.

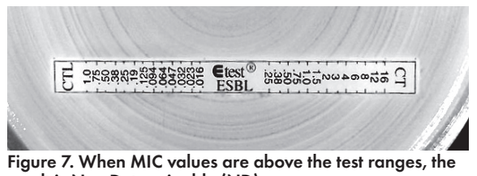

Figure 7. When MIC values are above the test ranges, the result is Non-Determinable (ND).

Interpretation

Table 1: Guidelines for interpretation of Etest ESBL.

ESBL	MIC Ratio	Reporting
Positive	CT ≥ 0.5 and CT/CTL ≥ 8 OR TZ ≥ 1 and TZ/TZL ≥ 8 OR PM ≥ 0.25 and PM/PML ≥ 8 OR "Phantom" zone or deformation of the CT, TZ or PM ellipse	ESBL producer and resistant to all penicillins, cephalosporins and aztreonam (CLSI M100-S series).
Negative	CT < 0.5 or CT/CTL < 8 AND TZ < 1 or TZ/TZL < 8	ESBL non-producer and report actual MICs of relevant drugs as determined by a MIC method.
Non-determinable (ND)	CT > 16 and CTL > 1 AND TZ > 32 and TZL > 4 AND PM > 16 and PML > 4 OR When one strip is ESBL negative and the other ND	ESBL non-determinable and report actual MICs of relevant drugs as determined by a MIC method. If ESBL is suspected, confirm results with genotyping.

Examples of how to interpret MIC ratios:

CT/CTL	8/0.125 = 64	= ESBL +
TZ/TZL	>32/<0.064 = >500	= ESBL +
PM/PML	1/<0.064 = >15	= ESBL +
CT/CTL	4/>1 = <4	= ESBL -
TZ/TZL	1/0.5 = 2	= ESBL -
CT/CTL	0.25/0.19 = 1.3 (CT < 0.25)	= ESBL -
TZ/TZL	1/4[1] = 0.25	= ESBL -
PM/PML	<0.25[2]/0.38 = <0.65	= ESBL -
TZ/TZL	>32/>4 = out of range	= ND [3]
CT/CTL ESBL negative and TZ/TZL ND		= ND [4]

Notes:

1) When MICs of CTL, TZL or PML are higher than CT, TZ or PM respectively, it may reflect the induction of ß-lactamase production by clavulanic acid.
2) When PM < 0.25, the result for the Etest PM/PML strip is considered negative.
3) When the MIC values are above the test ranges, the result is ND = Non-determinable. This may suggest the presence of IRT (inhibitor resistant TEM), AmpC enzymes or that the MIC values are outside the test range. When this occurs with both CT/CTL and TZ/TZL, additional testing with PM/PML is recommended.
3) When one result is ESBL negative and the other ND, the interpretation for the strain should be ND.

QUALITY CONTROL

Quality control strains should be tested by the method outlined under **PROCEDURE** to check the quality of the reagents and the ability of the test to confirm ESBL production. The expected MIC values and interpretation for control strains are provided in Table 2. *E. coli* ATCC® 35218 produces TEM-1 ß-lactamase (non-ESBL) and serves as a negative control for the test. *K. pneumoniae* ATCC 700603, an ESBL positive genotype, provides on-scale MIC values and serves as a positive control, either by MIC ratio criteria or the deformed ellipse phenomenon. Careful attention should be given to maintenance and storage of *K. pneumoniae* ATCC 700603 as spontaneous loss of the plasmid encoding the ESBL has been documented and may give QC results outside the acceptable limits. When CTL, TZL and PML MIC values are above specifications, this may imply either degradation of clavulanic acid or excessively high inoculum. Check the storage and handling of strips and repeat the test using the correct inoculum.

Table 2. Quality control specifications for Etest ESBL CT/CTL, TZ/TZL and PM/PML strips.

Strain	MIC (µg/mL) Cefotaxime (CT)	Cefotaxime + clavulanic acid (CTL)	ESBL Interpretation[1)]
E. coli ATCC 35218	≤0.25[2)]	0.016 - 0.064	Negative
K. pneumoniae ATCC 700603	1 - 4[3)]	0.125 - 1	Positive
P. mirabilis[4)] ATCC BAA-856	0.5 - 1	0.032 - 0.064	Positive
Strain	**Ceftazidime (TZ)**	**Ceftazidime + clavulanic acid (TZL)**	**ESBL Interpretation[1)]**
E. coli ATCC 35218	≤0.5[2)]	≤0.064[2)]	Negative
K. pneumoniae ATCC 700603	8 - ≥32	0.125 - 0.5	Positive
Strain	**Cefepime (PM)**	**Cefepime + clavulanic acid (PML)**	**ESBL Interpretation[1)]**
P. aeruginosa ATCC 27853	0.5 - 2	1 - 4	Negative
K. pneumoniae ATCC 700603	0.25 -1[3)]	0.064 - 0.25	Positive

Notes:
1) See **READING AND INTERPRETATION**.
2) MIC value below the strip range.
3) MIC ratio may be ≤ 8 but deformation of the CT or PM ellipse is indicative of ESBL.
4) Mortensen *et al*. (2005). *JCM*. **43**(5).

PERFORMANCE CHARACTERISTICS

Several *in vitro* studies have compared the performance of Etest ESBL CT/CTL, TZ/TZL and PM/PML strips to ESBL genotype characterisation and/or the CLSI agar dilution method and confirmatory criteria for ESBL. In an FDA criteria study (Bolmström *et al*), Etest detected ESBL enzymes produced by a total of 73 genotypically characterised strains in studies performed at different independent sites. Comparison of Etest performance to the CLSI method based on 479 strains comprising clinical and challenge organisms tested at three independent sites (328 positive ESBL phenotypes and 151 negative controls) is summarised in Table 3.

Table 3. Etest ESBL CT/CTL and TZ/TZL performance compared to CLSI reference method.

Agreement N (%)	CLSI ESBL+	CLSI ESBL-
Etest ESBL +	324 (99)	0
Etest ESBL -	0	144 (95)
Etest ND	4 (1)	7 (5)

LIMITATIONS

1. Inhibitor resistant TEM (IRT) enzymes cannot be detected by Etest ESBL strips.
2. An ESBL negative result with elevated MICs to CT/TL and TZ/TZL may be due to an IRT, AmpC or an ESBL masked by the concurrent presence of these enzymes and/or other resistance mechanisms. Etest PM/PML may be additionally tested in these cases.
3. Strains showing non-determinable (ND) results with CT/CTL and TZ/TZL strips should be further tested using PM/PML strips. If all results are found to be non-determinable, these strains should be further investigated by genotyping.
4. Performance of Etest ESBL is based on the use of at least both TZ/TZL and CT/CTL strips simultaneously. The use of only one Etest ESBL strip to confirm the presence of ESBL is not valid.

REFERENCES AND BIBLIOGRAPHY

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7. CLSI M7-A7, January 2006. *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically*.
8. CLSI M100-S series, latest edition.

WARRANTY AND DISCLAIMER

EXPRESS LIMITED WARRANTY AND DISCLAIMER

AB BIODISK expressly warrants that Etest ESBL CT/CTL, TZ/TZL and PM/PML will detect clavulanic acid inhibitable Extended-Spectrum Beta-Lactamases (ESBLs), if the procedures, precautions and limitations indicated in this package insert are strictly complied with. If the Etest ESBL strip does not do so, AB BIODISK shall refund the cost of the product or replace the defective test strips.

AB BIODISK makes no other warranties, expressed or implied, including the implied warranty of merchantability or fitness for particular purpose.

Any change or modification of the product instructions may affect results. AB BIODISK shall not be liable for any damages resulting from product tampering, variance in transportation, stated storage, handling, testing procedures, precautions and other instructions of the most recently revised version of the package insert.

Etest® ESBL

For *In Vitro* Confirmation of ESBL

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