NEBNext Enzymatic Methyl-seq Kits

NGS METHYL-SEQUENCING (EM-SEQ™)

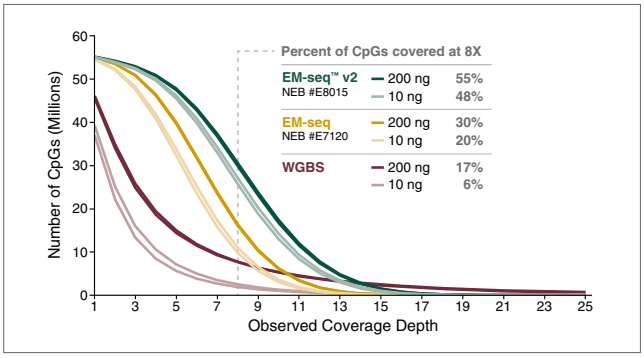
NEW ENGLAND Biolabs®

NEBNext Enzymatic Methyl-seq v2 (EM-seq)

NEBNext Enzymatic Methyl-seq (EM-seq) is a high-performance enzyme-based alternative to bisulfite sequencing for the identification of 5mC and 5hmC on the Illumina platform. Unlike harsh bisulfite treatment, EM-seq minimizes DNA damage, resulting in superior detection of 5mC and 5hmC from fewer sequencing reads.

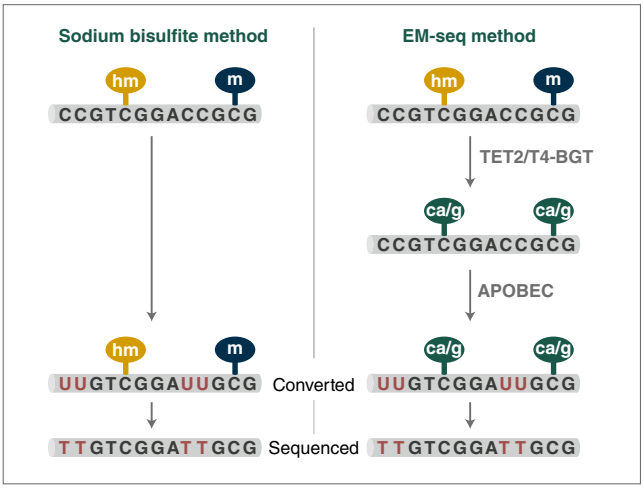
The new NEBNext Enzymatic Methyl-seq v2 Kit has a wider input range (as low as 100 pg), a faster, more streamlined workflow and improved performance compared to the original EM-seq kit (NEB #E7120).

The NEBNext Enzymatic Methyl-seq v2 Kit includes conversion reagents, library prep reagents and the EM-seq Adaptor. Multiple sets of the required index primers (NEBNext LV Unique Dual Index Primers) are available separately, enabling greater flexibility in multiplexing. For enzymatic fragmentation of DNA designed for use with EM-seq, see NEBNext UltraShear (see page 4).



NEBNext EM-seq v2 identifies more CpGs than WGBS and the original EM-seq, at lower sequencing coverage depth

EM-seq v2 (NEB #E8015), EM-seq (NEB #E7120) and WGBS libraries were prepared from 200 ng and 10 ng of NA12878 DNA (sheared to ~350 bp), spiked with unmethylated lambda and CpG-methylated pUC19. Libraries were sequenced on an Illumina NovaSeq 6000. For accurate comparison of the original EM-seq and WGBS data with EM-seq v2 data, we evaluated data from approximately 625 million 100 base reads for each library aligned to a composite human T2T, lambda and pUC19 reference genome using bwa-meth. The T2T genome covers a maximum of 67.8 million CpGs when the top and bottom strands are counted independently. EM-seq v2 and EM-seq covered over 54 million CpG sites for both 200 ng and 10 ng inputs; however, WGBS libraries covered only 46 million and 39 million for 200 ng and 10 ng inputs respectively at 1X coverage. The dashed lines represent coverage of 8X. The table lists the percentage of CpG sites covered by different libraries at 8X coverage level.



EM-seq conversion method

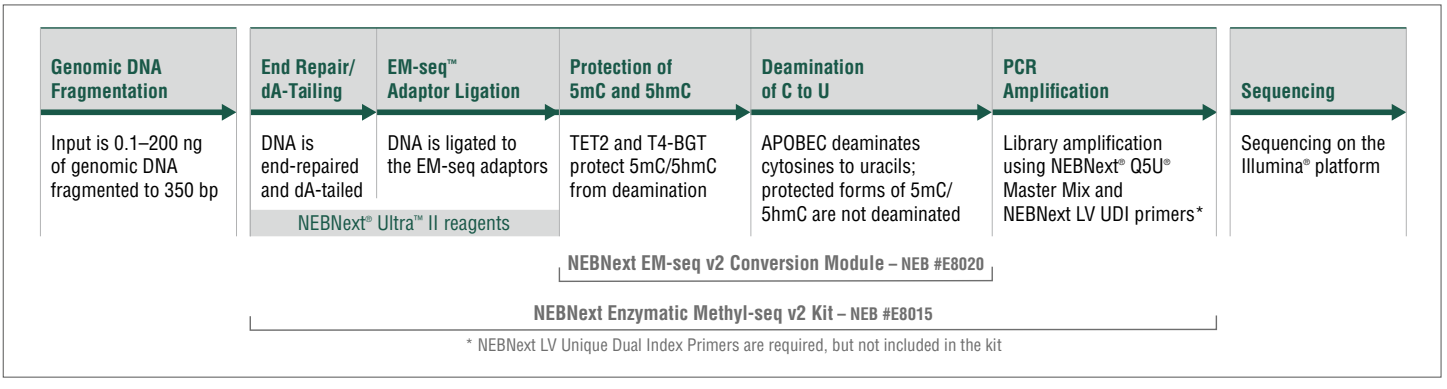
The harsh sodium bisulfite treatment deaminates unmodified cytosines to uracil. In the EM-seq workflow, 5mC and 5hmC are first protected using the enzymes TET2 and T4-BGT. Unmodified cytosines are then deaminated by the APOBEC enzyme to uracil, while the protected 5mC and 5hmC are not converted. During Illumina sequencing, 5mCs and 5hmCs are represented as cytosine, while unmodified cytosines are represented as thymine.

Advantages

- Superior sensitivity of 5mC and 5hmC detection
- 100 pg – 200 ng input range
- Detection of more CpGs with fewer sequencing reads
- Even GC coverage
- High performance library preparation and larger library insert sizes
- Index primers supplied separately

NEBNext Enzymatic Methyl-seq is an enzymatic alternative to bisulfite conversion with superior performance. For more information, including extensive performance data, visit **NEBNext.com.**

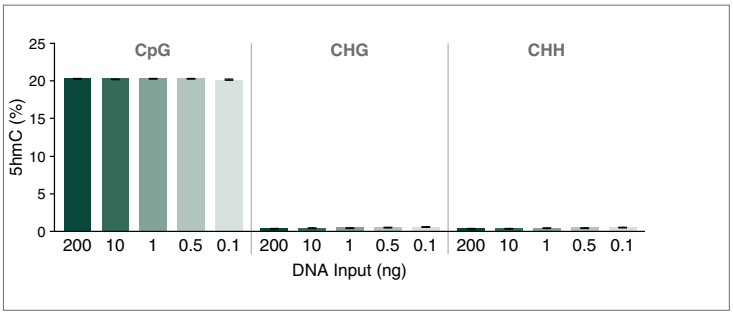
NEBNext Enzymatic Methyl-seq (EM-seq) v2 workflow



NEBNext Enzymatic 5hmC-seq (E5hmC-seq)

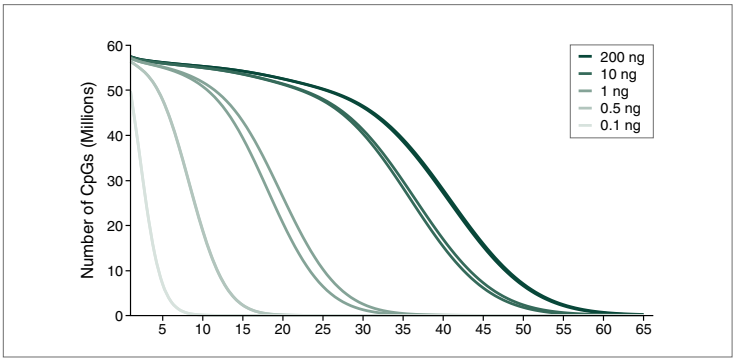
While NEBNext Enzymatic Methyl-seq (EM-seq) detects both 5mC and 5hmC, it does not distinguish between them. Specific detection of 5hmC sites is now enabled by the NEBNext Enzymatic 5hmC-seq Kit (E5hmC-seq). The kit includes NEBNext Ultra II library prep reagents, and 5hmC is detected using a two-step enzymatic conversion workflow, that minimizes damage to DNA and allows discrimination of 5hmC from both cytosine and 5mC after Illumina sequencing. E5hmC-seq data can also be subtracted from EM-seq data, allowing determination of the precise location of individual 5mC and 5hmC sites.

5hmC detected by E5hmC-seq in human brain gDNA is consistent across inputs



200–0.1 ng of human brain genomic DNA was sheared to 350 bp (Covaris ME220) and E5hmC-seq libraries were prepared and sequenced on an Illumina NovaSeq 6000 (2 x 150 bases). Approximately 1.9 billion (200 ng, 10 ng and 1 ng) or 715 million (0.5 ng and 0.1 ng) reads for each library were aligned to a composite human T2T, lambda and T4 reference genome using bwa-meth, and methylation information was extracted from the alignments using MethylDackel. Values shown are the average of two technical replicates and error bars show standard deviation. Detected 5hmC levels are similar between all inputs in the CpG, CHH and CHG contexts.

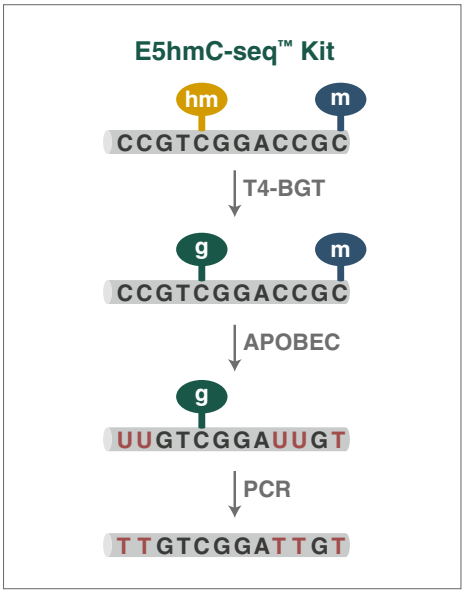
E5hmC-seq exhibits high CpG coverage across a range of inputs



200–0.1 ng of human brain genomic DNA was sheared to 350 bp (Covaris ME220) and E5hmC-seq libraries were prepared and sequenced on an Illumina NovaSeq 6000 (2 x 150 bases). Approximately 1.9 billion CpG specific file a cumulative coverage plot was generated for CpG sites covered using E5hmC-seq libraries across all inputs. The T2T genome covers a maximum of 67.8 million CpGs when the top and bottom strands are counted independently. E5hmC-seq covered over 56 million CpG sites for 0.5 ng to 200 ng inputs and roughly 48 million CpG sites for 0.1 ng input libraries.

Advantages

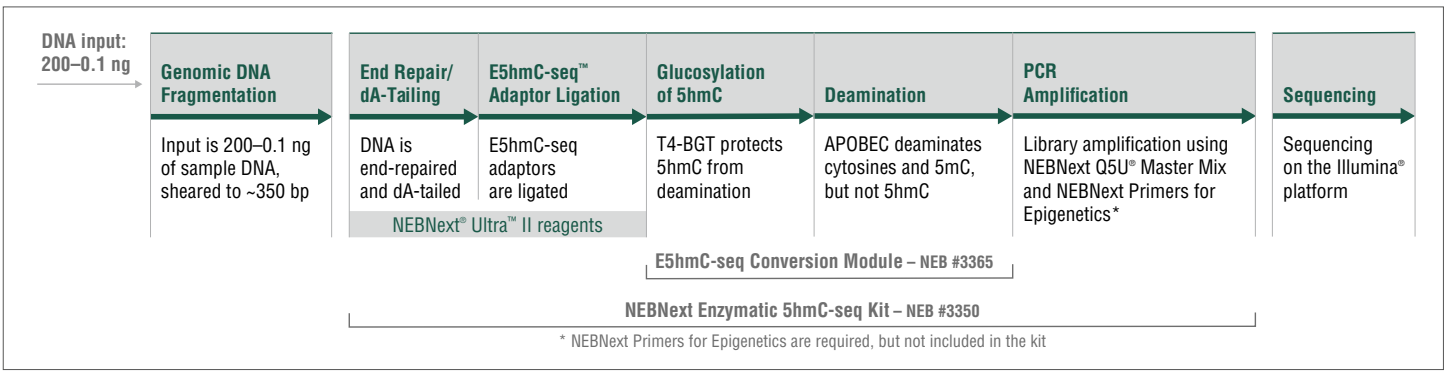
- Enzyme-based workflow enables high yields and high-quality data
- 100 pg – 200 ng inputs
- Minimal GC bias
- E5hmC-seq and EM-seq data can be combined
- Conversion module also available separately



E5hmC-seq conversion method

To enable specific 5hmC detection, 5hmC is first glucosylated using T4-BGT. 5mC and unmodified cytosine are then deaminated by APOBEC to thymine and uracil, respectively, while the protected 5hmC is unconverted. During Illumina sequencing 5hmCs are represented as cytosine, while cytosine and 5mCs are represented as thymine.

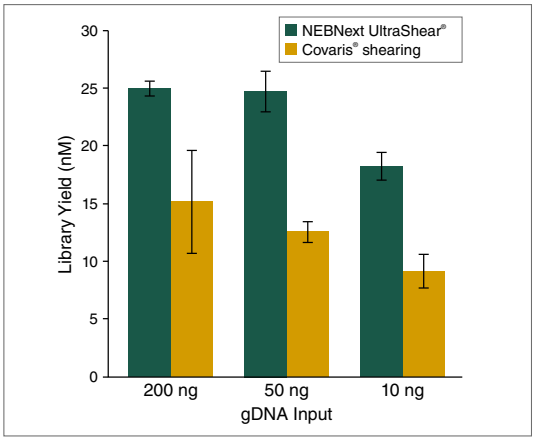
NEBNext Enzymatic 5hmC-seq (E5hmC-seq) workflow



NEBNext UltraShear

Enzymatic fragmentation of DNA as part of the library prep workflow provides many advantages compared to mechanical shearing. However, specialized fragmentation reagents are required for enzymatic shearing of challenging samples such as FFPE DNA, and in order to maintain methylation marks on samples for methylome analysis, including for use with NEBNext Enzymatic Methyl-seq (EM-seq).

NEBNext UltraShear is a mix of enzymes that has been designed and optimized to fragment these sample types upstream of library preparation. This improves library yields and diversity, and allows retention of methylation marks.



NEBNext UltraShear increases EM-seq library yields

200 ng, 50 ng and 10 ng of NA12878 DNA spiked with control DNA (CpG methylated pUC19 DNA and unmethylated lambda DNA) were fragmented by either NEBNext UltraShear (20 minutes at 37°C) or Covaris ME220 (350 bp protocol) followed by EM-seq library preparation. Library yields were quantified using Agilent TapeStation with the High Sensitivity D1000 ScreenTape. EM-seq libraries fragmented by NEBNext UltraShear have higher yields than Covaris for the same number of PCR cycles for each input (200 ng = 4 cycles; 50 ng = 6 cycles; 10 ng = 8 cycles).

Advantages

- Compatible with methylation analysis workflows, including NEBNext Enzymatic Methyl-seq (EM-seq)
- Compatible with FFPE DNA
- Fast workflow with minimal hands-on time
- For methylation analysis, improves CpG coverage and sequencing metrics
- For FFPE DNA, increases usable reads and coverage uniformity

NEBNext LV Unique Dual Index Primers

NEBNext LV Unique Dual Index Primers have been selected and paired for optimal multiplex index incorporation during PCR. Optimized for low-volume (LV) NEBNext workflows, including EM-seq v2 and E5hmC-seq, these primers enable multiplexing and have been designed to minimize index hopping. They are supplied as pairs of premixed i5 and i7 8-base index primers in a 96-well plate (Sets 1, 2, 3, 4, 5) or in 24 wells of a plate (Sets 2A and 2B) that has a pierceable foil seal for easy, single use.

The index sequences from the NEBNext LV Unique Dual Index Primers Sets are compatible with and can be combined with the other NEBNext LV Unique Dual Index Primers Sets to multiplex up to 480 reactions. Note: NEBNext LV Unique Dual Index Primers Set 2A and Set 2B are 24-index subsets of Set 2; therefore, we do not recommend combining Set 2A and/or 2B with Set 2.

Advantages

- Optimized for low-volume (LV) NEBNext library prep workflows
- Addresses the "barcode hopping" issue seen with some Illumina sequencing instruments
- Increases sample identification specificity with unique dual index primer pairs

Index Primers	NEB #	SIZE
NEBNext LV Unique Dual Index Primers Set 2A, 2B	E3390S, E3392S	24 rxns
NEBNext LV Unique Dual Index Primers Set 1/2/3/4/5	E3400S, E3402S, E3404S, E3406S, E3408S	96 rxns

NEBNext Enzymatic Methyl-seq (EM-Seq) The superior alternative to bisulfite sequencing

The identification of cytosine modifications within genomes, especially 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC), is important as they are known to have an impact on gene expression. Generally, low levels of methylation near transcription start sites are associated with higher transcription levels, while genes with regulatory regions containing high levels of cytosine modification are expressed at lower levels.

The ability to analyze a complete methylome is important for studying diseases, including those associated with cancer, metabolic disorders and autoimmune diseases.

Bisulfite Sequencing

For methylome mapping bisulfite sequencing has been developed (1). While the preparation of bisulfite libraries is relatively straightforward, the libraries have uneven genome coverage and therefore suffer from incomplete representation of cytosine methylation across genomes. This uneven coverage is the result of DNA damage and fragmentation, which is caused by the extreme temperatures and pH during bisulfite conversion. Sequenced bisulfite libraries typically have skewed GC bias plots, with a general under-representation of G- and C-containing dinucleotides and over-representation of AA-, AT- and TA-containing dinucleotides, as compared to a non-converted genome (2).

Advantages

- Superior sensitivity of detection of 5mC and 5hmC
- Greater mapping efficiency
- More uniform GC coverage
- Detect more CpGs with fewer sequence reads
- Uniform dinucleotide distribution
- Larger library insert sizes
- High-efficiency library preparation
- Allows for lower input amounts with a range of inputs from 10–200 ng
- Conversion module also available separately

References
1. Harris R.A., et al. (2010) Nat Biotechnol. 28, 1097–1105.
2. Olova, N., et al. (2018) Genome Biology, 19: 33.

CONCLUSION

Bisulfite sequencing, while commonly used, is sub-optimal in detecting 5mC and 5hmC – large amounts of DNA are needed, DNA can be damaged, and sequences are biased towards AT-rich regions. Other methods that couple chemical or enzymatic treatment with bisulfite sequencing also share similar limitations. EM-seq provides a non-bisulfite method that comprehensively addresses the limitations of bisulfite sequencing and represents a new opportunity for more complete methylome analysis. EM-seq libraries are not damaged and have longer inserts, higher PCR yields with fewer PCR cycles, and lack biases associated with GC content. More CpGs are identified with greater coverage depth using EM-seq, as compared to WGBS. These advantages all contribute to EM-seq having more usable sequencing data when comparing the same number of reads for EM-seq and WGBS, which ultimately reduces sequencing costs. EM-seq is the only commercially-available alternative to bisulfite sequencing that provides an effective method for accurate and comprehensive detection of 5mC and 5hmC across the genome, and offers a new, more accurate alternative for studying disease states.

"(…) it enables us to determine in a precise and DNA sparing way the cytosine methylation status even at low integrity DNA. If bisulfite conversion were the only approach to apply, we would definitely fail to generate relevant results. The cool, biochemical approach (…) opens new avenues to explorations of methylation at intact long DNA fragments."

~ Dr. Vladimir Benes, Head Genomics Core Facility at EMBL Heidelberg

"(…) As an ISO 17025 accredited laboratory, we need reliable and reproducible kits and protocols. After thorough benchmarking of NEBNext Enzymatic Methyl-Seq v2 interrogating 5mC, the standard bisulfite conversion method lost its reference status for our methylation analyses. NEBNext EM-seq v2 and the NEBNext E5hmC-seq Kit for detecting 5hmC specifically, are now our methods of choice to study both modifications."

~ Dr. Marta Gut, Head of the Sequencing Unit at Centro Nacional de Análisis Genómico (CNAG) in Barcelona, Spain

GERMANY & AUSTRIA
New England Biolabs GmbH
Brüningstr. 50, Geb B852
65926 Frankfurt/Main, Germany
Tel: +49/(0)69/305-23140
Fax: +49/(0)69/305-23149

Free Call: 0800/246 5227 (Germany)
Free Call: 00800/246 52277 (Austria)
info.de@neb.com

www.neb-online.de

FRANCE
New England Biolabs France SAS
Genopole Campus 1, Bâtiment 6
5 rue Henri Desbruères
91030 Evry cedex, France
Tel.: 0800 100 632 (Customer Service)

Tel.: 0800 100 633 (Technical Service)
info.fr@neb.com

www.neb-online.fr

HEADQUARTERS:
USA
New England Biolabs, Inc.
Telephone: (978) 927-5054

Toll Free (USA Orders): 1-800-632-5227
Toll Free (USA Tech): 1-800-632-7799
Fax: (978) 921-1350
info@neb.com

www.neb.com

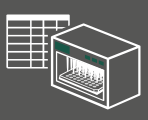
Ordering Information

EM-SEQ	NEB #	SIZE
NEBNext Enzymatic Methyl-seq v2 Kit	E8015S/L	24/96 rxns
NEBNext Enzymatic Methyl-seq Conversion Module	E7125S/L	24/96 rxns
NEBNext Enzymatic Methyl-seq v2 Conversion Module	E8020S/L	24/96 rxns
NEBNext Q5U Master Mix	M0597S/L	50/250 rxns

E5hmC-SEQ	NEB #	SIZE
NEBNext Enzymatic 5hmC-seq Kit	E3350S/L	24/96 rxns
NEBNext Enzymatic 5hmC-seq Conversion Module	E3365S/L	24/96 rxns

UltraShear	NEB #	SIZE
NEBNext UltraShear	M7634S/L	24/96 rxns

Index Primers	NEB #	SIZE
NEBNext LV Unique Dual Index Primers Set 2A, 2B	E3390S, E3392S	24 rxns
NEBNext LV Unique Dual Index Primers Set 1/2/3/4/5	E3400S, E3402S, E3404S, E3406S, E3408S	96 rxns



For automated solutions of EM-seq, please contact or visit
www.neb.com/automation

For easy calculation of values associated with read coverage in NGS protocols, visit
readcoveragecalculator.neb.com

For assistance in selecting barcode combinations when pooling NEBNext Multiplex Oligos for Illumina, visit
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CZECH REPUBLIC:
BioTech a.s.
info@ibiotech.cz
www.ibiotech.cz

DENMARK:
BioNordika Denmark A/S
info@bionordika.dk
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FINLAND:
BioNordika Oy
info@bionordika.fi
www.bionordika.fi

GEORGIA:
Oxgen-Import, LLC
info@oxgenimport.ge
www.oxgenimport.ge

GREECE:
BIOLINE SCIENTIFIC
info@bioline.gr
www.bioline.gr

HUNGARY:
Kvalitex Kft.
info@kvalitex.hu
www.kvalitex.hu

ITALY:
EUROCLONE S.P.A.
info@euroclone.it
www.euroclonegroup.it

ISRAEL:
Ornat BioChemicals & Lab Equipment
ornatbio@ornat.co.il
www.ornat.co.il

KAZAKHSTAN:
INOS, LLC
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LITHUANIA:
Nanodiagnostika, Ltd.
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NORWAY:
BioNordika AS
info@bionordika.no
www.bionordika.no

POLAND:
Lab-JOT Ltd. Sp.z o.o. Sp.k.
biuro@labjot.com
www.labjot.com

PORTUGAL:
Werfen
dac-pt@werfen.com
www.pt.werfen.com

ROMANIA:
BioZyme
contact@biozyme.ro
www.biozyme.ro

SERBIA:
Alfa Genetics d.o.o.
office@alfagenetics.rs
www.alfagenetics.rs

SPAIN:
Werfen
customerservice-es@werfen.com
www.es.werfen.com

SLOVAK REPUBLIC:
BioTech s.r.o.
info@ibiotech.sk
www.ibiotech.sk

SLOVENIA:
Mikro+Polo d.o.o.
info@mikro-polo.si
www.mikro-polo.si

SOUTH AFRICA:
Inqaba Biotechnical Industries (Pty) Ltd
info@inqababiotec.co.za
www.inqababiotec.co.za

SWEDEN, ESTONIA, LATVIA, LITHUANIA:
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www.bionordika.se

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