INSTRUCTION MANUAL

NEBNext® Enzymatic Methyl-seq Conversion Module

NEB #E7125S/L

24/96 reactions

Version 5.0_3/25

Table of Contents

The Conversion Module Includes

The volumes provided are sufficient for preparation of up to 24 reactions (NEB #E7125S) and 96 reactions (NEB #E7125L). Reagents should be stored at –20°C. Colored bullets represent the color of the cap of the tube containing the reagent.

- (lilac) Control DNA CpG methylated pUC19
- (lilac) Control DNA Unmethylated Lambda
- (white) Elution Buffer
- (yellow) TET2 Reaction Buffer
- (yellow) TET2 Reaction Buffer Supplement
- (yellow) Oxidation Supplement
- (yellow) DTT
- (yellow) Oxidation Enhancer
- (yellow) TET2
- (yellow) Fe(II) Solution
- (yellow) Stop Reagent
- (orange) APOBEC
- (orange) APOBEC Reaction Buffer
- (orange) BSA

Required Materials Not Included

- NEBNext UltraShear® (M7634) or Covaris® instrument and the required tubes or other fragmentation equipment
- PCR strip tubes or 96-well plates
- Cleanup beads: SPRIselect™ Reagent Kit (Beckman Coulter®, Inc. #B23317), AMPure® XP beads (Beckman Coulter, Inc. #A63881) or preferred bead manufacturer
- Hi-Di™ Formamide (Thermo Fisher Scientific® #4401457), Formamide (Sigma #F9037-100 ml), or 0.05 N NaOH. Formamide is preferred. If using NaOH, please see FAQ associated with NEB #E8015.
- 80% Ethanol
- 10 mM Tris-HCl pH 7.5 or 8.0 or low TE (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA)
- Nuclease-free Water
- Magnetic rack/stand, such as NEBNext Magnetic Separation Rack (NEB #S1515)
- Metal cooling block, such as Diversified Biotech® (#CHAM-1000)
- PCR machine

Overview

The Enzymatic Methyl-seq Conversion Module (EM-seq) contains the components needed to enzymatically modify and enable detection of 5-methylcytosines (5mC) and 5-hydroxymethylcytosines (5hmC).

Figure 1. Overview of Sodium Bisulfite Conversion and EM-seq.

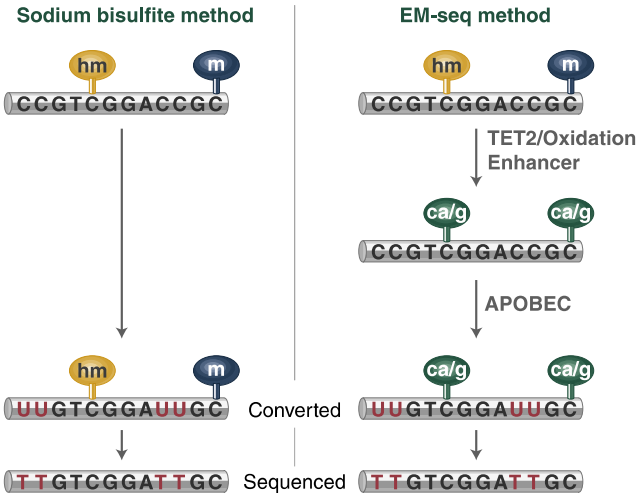

Figure 1 shows a comparison of the sodium bisulfite and EM-seq conversion methods. Sodium bisulfite treatment of DNA results in the deamination of cytosines into uracils, however the modified forms of cytosine (5mC and 5hmC) are not deaminated. Therefore, the preference of bisulfite to chemically deaminate cytosines enables the methylation status of cytosines to be determined. When bisulfite treated DNA is PCR amplified, uracils are replaced by thymines and the 5mC/5hmC are replaced by cytosines. Once sequenced, unmethylated cytosines are represented by thymines and 5mC and 5hmC are represented by cytosines. By comparing sequences to non-converted genomes the appropriate methylation status can be assessed.

The Enzymatic Methyl-seq Conversion Module is a two-step enzymatic conversion process to detect modified cytosines. The first step uses TET2 and an oxidation enhancer to protect modified cytosines from downstream deamination. TET2 enzymatically oxidizes 5mC and 5hmC through a cascade reaction into 5-carboxycytosine [5-methylcytosine (5mC) ⇒5-hydroxymethylcytosine (5hmC) ⇒ 5-formylcytosine (5fC) ⇒ 5-carboxycytosine (5caC)]. This protects 5mC and 5hmC from deamination. 5hmC can also be protected from deamination by glucosylation to form 5gmC using the oxidation enhancer. The second enzymatic step uses APOBEC to deaminate cytosine but does not convert 5caC and 5gmC.

The workflow described in the NEBNext Enzymatic Methyl-seq Conversion Module is user-friendly and enables methylation detection from inputs ranging between 10 ng–200 ng. EM-seq converted DNA is more intact than bisulfite-converted DNA, resulting in libraries with longer sequencing reads, reduced GC bias and more even genome coverage.

Each module component must pass rigorous quality control standards, and for each new lot the entire set of reagents is functionally validated together with Ultra II DNA Library Prep Kit (NEB #E7645), Multiplex Oligos for Enzymatic Methyl-seq (NEB #E7140), and Q5U™ Master Mix (NEB #M0597) to construct EM-seq indexed libraries and sequence on an Illumina® sequencing platform.

For larger volume requirements, customized and bulk packaging is available by purchasing through the Custom Solutions department at NEB. Please contact Custom@neb.com for further information.

Protocol for EM-seq Conversion Module

Symbols

This is a point where you can safely stop the protocol and store the samples prior to proceeding to the next step in the protocol.

This caution sign signifies a step in the protocol that has two paths leading to the same end point.

Colored bullets indicate the cap color of the reagent to be added.

Important Guidelines

Reagent Handling

Remove only the kit reagents needed for a specific reaction in the EM-seq protocol. For example, for “Protection of 5-Methylcytosines and 5-Hydroxymethylcytosines” remove the yellow capped tubes. Frozen kit components can be thawed at room temperature. Once thawed, place immediately on ice.

Please note that some components may form a precipitate, for example, ∘ (yellow) DTT. Precipitates should disappear once the tubes are at room temperature. For the precipitate to fully disappear it may be necessary to vortex the ∘ (yellow) DTT tube. All other components (stored at -20°C) should be stored on ice until used.

All components should be mixed before use. Typically, tubes containing enzymes can be mixed by flicking the tube and other buffers/ reagents can be quickly vortexed. All tubes should be centrifuged briefly after mixing and stored on ice until used.

Master mixes can be made for most steps in EM-seq unless otherwise stated in the protocol. If using a master mix, good practice is to add the component in the order specified in the protocol. Typically, enzymes are added last. Master mixes should be quickly vortexed then briefly centrifuged and stored on ice before use.

Care should be taken when handling some of the reagents to ensure the correct volumes are dispensed. For example, the Sample Purification Beads are viscous. It is difficult to accurately pipette these components. Please take care to transfer the correct volumes.

The • (orange) APOBEC Reaction Buffer can form bubbles when mixed. Centrifuging the • (orange) APOBEC Reaction Buffer can reduce their appearance making it easier to accurately dispense the buffer.

Once reactions are set up, mix either using a quick vortex or by pipetting up and down. Please note that if you choose to mix using a pipette, then we encourage you to use an appropriately sized pipette set to a volume that would ensure adequate mixing of the sample. For example, during the Oxidation Reaction set up, 5 µl of diluted ∘ (yellow) Fe(II) Solution is added, use a 200 µl pipette set to 40 µl to mix – do not mix with a pipette set to 5 µl. Briefly centrifuge the mixed reactions before incubating the reactions.

Another example where adequate mixing is required is during Sample Purification Bead clean-ups. Here, please use a 200 µl pipette set to an appropriate volume to ensure mixing. For the bead clean up after Oxidation, a pipette set to 80 µl would be sufficient whereas for the clean-up after APOBEC deamination a 160 µl pipette volume would be more appropriate.

After each NEBNext Sample Purification Bead clean-up please ensure that the samples are centrifuged to ensure that the sample is at the bottom of the tube prior to the next reaction in the protocol. If the samples were stored at -20°C, they can be thawed at room temp or on ice prior to centrifugation.

There are safe stop points in the protocol where reactions can be stored at -20°C. If a sample has been stored at -20°C then it should be thawed and briefly centrifuged before use in the next step in the protocol.

Starting Material: 10–200 ng fragmented double stranded DNA

1.1. DNA Preparation

1.1.1. Sample DNA and Control DNA

DNA going into the first enzymatic reaction (protection of 5mC and 5hmC) reaction needs to be fragmented as applicable for the downstream application.

For sequencing on an Illumina platform, refer to the Enzymatic Methyl-seq Kit Manual (NEB #E7120) for usage recommendations. For other downstream applications and sequencing platforms, please refer to manufacturer's guidelines.

The following table is a guide for the amount of • (lilac) Control DNA Unmethylated Lambda and • (lilac) Control DNA CpG methylated pUC19 to be added to samples prior to EM-seq library construction to evaluate conversion efficiencies.

Table 1.1 Dilutions of control DNAs for a range of genomic DNA inputs.

Sample DNA Input Amount	Control DNA Dilution Recommendations
10 ng	1:100
200 ng	1:50

The above dilutions are useful to perform a QC of conversion before deep sequencing using approximately 10 million paired-end reads. This read depth is sufficient to achieve a minimum of 5,000 paired-end reads mapping to • (lilac) unmethylated Lambda DNA and 500 paired-end reads mapping to • (lilac) CpG methylated pUC19. This level of coverage is needed for accurate conversion estimates.

Different sequencing depths may be needed depending on the application, and therefore different strategies should be employed when deciding how much control DNA should be added. For example, some applications may only need 2 million paired-end reads whereas others may require 50 million paired-end reads or even 500 million paired end reads.

The dilutions recommended in Table 1.1 will provide sufficient coverage of controls for libraries sequenced to 10 million paired-end reads and above. Dilution of controls needs to be optimized by the user if sequencing lower than 10 million paired-end reads to obtain minimum coverage for • (lilac) unmethylated lambda (5,000 paired end reads) and • (lilac) CpG methylated pUC19 (500 paired-end reads). Number of reads mapping to • (lilac) unmethylated lambda and • (lilac) CpG methylated pUC19 will be in the range of 0.5 to 1% with the suggested dilutions. Users should be aware that deep sequencing using the dilutions recommended in Table 1.1 can result in more than the minimum required • (lilac) unmethylated lambda and • (lilac) CpG methylated pUC19 reads. Ultimately, dilutions of the control DNAs should be optimized by the user.

For example, samples going into Illumina library preparation can be enzymatically fragmented using NEBNext UltraShear (NEB #M7634) or mechanically sheared to an average size of ~ 350 bp. Control DNAs should be added prior to fragmentation to provide a means to qualify the final sequencing data as specified below. Samples must be fragmented in a buffer containing 10 mM Tris-HCl pH 7.5 or 8.0. If EDTA is present in the buffer or to adjust for volume going into the Protection Reaction, sheared samples must be cleaned up using beads (~2X bead ratio) or column to remove EDTA and eluted in 28 µl of water or 10 mM Tris-HCl pH 7.5 or 8.0 to go into the Protection Reaction.

NOTE: Do not fragment input DNA in 0.1X TE (1 mM Tris-HCl pH 8.0, 0.1 mM EDTA) or water.

Table 1.2 An example setup for combining sample DNA and control DNAs prior to fragmentation.

COMPONENT	VOLUME
Sample DNA	48 µl
• (lilac) Control DNA Unmethylated Lambda (see Table 1.1.)	1 µl
• (lilac) Control DNA CpG methylated pUC19 (see Table 1.1.)	1 µl
Total Volume	**50 µl**

Post fragmentation

DNA without sequencing adaptors ligated: DNA must be in 28 µl of water or 10 mM Tris-HCl pH 7.5 or 8.0 to go into Oxidation Reaction.

1.2. Protection of 5-Methylcytosines and 5-Hydroxymethylcytosines

1.2.1. Prepare TET2 Buffer. Use Option A if you have #E7125S/E7125G (24 reactions/G size) and Option B if you have NEB #E7125L (96 reactions).

Note: The TET2 Reaction Buffer Supplement is a powder. Centrifuge before use to ensure it is at the bottom of the tube.

1.2.1A. Add 100 µl of ⚬ (yellow) TET2 Reaction Buffer to one tube of ⚬ (yellow) TET2 Reaction Buffer Supplement and mix well (for the 24-reaction/G size kit). Write date on tube.

1.2.1.B. Add 400 µl of ⚬ (yellow) TET2 Reaction Buffer to one tube of ⚬ (yellow) TET2 Reaction Buffer Supplement and mix well (for the 96-reaction kit). Write date on tube.

Note: The reconstituted buffer should be stored at -20°C and discarded after 4 months.

1.2.2. On ice, add the following components directly to fragmented DNA:

COMPONENT	VOLUME
Fragmented DNA DNA (Step 1.1.1.)	28 µl
⚬ (yellow) TET2 Reaction Buffer (TET2 Reaction Buffer Supplement reconstituted in TET2 Reaction Buffer)	10 µl
⚬ (yellow) Oxidation Supplement	1 µl
⚬ (yellow) DTT	1 µl
⚬ (yellow) Oxidation Enhancer	1 µl
⚬ (yellow) TET2	4 µl
Total Volume	**45 µl**

Mix thoroughly by vortexing 1–2 seconds or by pipetting up and down at least 10 times and centrifuge briefly. For multiple reactions, a master mix of the reaction components can be prepared before addition to the sample DNA.

5mC/5hmC oxidation is initiated by the addition of the Fe(II) Solution to the reaction in the next step.

1.2.3. Dilute the ⚬ (yellow) 500 mM Fe(II) Solution by adding 1 µl to 1,249 µl of water. Mix well by vortexing.

Note: The ⚬ (yellow) 500 mM Fe(II) Solution color can vary between colorless to yellow, this is normal. Use the diluted solution immediately, do not store it. Discard after use.

On ice, combine the diluted Fe(II) Solution and reaction mixture (from Step 1.2.2.) as described below:

COMPONENT	VOLUME
Reaction mixture (from Step 1.2.2.)	45 µl
Diluted Fe(II) Solution (from Step 1.2.3.)	5 µl
Total Volume	**50 µl**

Mix thoroughly by vortexing 1–2 seconds or by pipetting up and down at least 10 times and centrifuge briefly.

1.2.4. Place in a thermal cycler with the heated lid set to ≥ 45°C or on, and run the following program:
1 hour at 37°C
Hold at 4°C

1.2.5. Transfer the samples to ice and add 1 µl of ⚬ (yellow) Stop Reagent.

COMPONENT	VOLUME
Protected DNA (Step 1.2.4.)	50 µl
⚬ (yellow) Stop Reagent	1 µl
Total Volume	**51 µl**

Mix thoroughly by vortexing 1–2 seconds or by pipetting up and down at least 10 times and centrifuge briefly.

1.2.6. Place in a thermal cycler with the heated lid set to ≥ 45°C or on, and run the following program:
30 minutes at 37°C
Hold at 4°C

Safe Stopping Point: Samples can be stored overnight at either 4°C in the thermal cycler or at -20°C in the freezer.

1.3. Clean-Up of TET2 Converted DNA

Note: The ratios recommended for SPRIselect/AMPure XP beads in this manual have been experimentally optimized for every step; this is critical since buffer compositions differ between steps and across protocols e.g., post ligation recommendations will not apply to samples post PCR. Please adhere to these guidelines and not those recommended by other sources or for other kits.

1.3.1. Vortex Sample Purification Beads to resuspend. SPRIselect or AMPure XP Beads can be used. If using AMPure XP Beads, allow the beads to warm to room temperature for at least 30 minutes before use.

1.3.2. Add 90 µl of resuspended NEBNext Sample Purification Beads to each sample. Mix well by pipetting up and down at least 10 times. Be careful to expel all of the liquid out of the tip during the last mix.

1.3.3. Incubate samples on bench top for at least 5 minutes at room temperature.

1.3.4. Place the tubes against an appropriate magnetic stand to separate the beads from the supernatant.

1.3.5. After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets **(Caution: do not discard the beads)**.

1.3.6. Add 200 µl of freshly prepared 80% ethanol to the tubes while on the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

1.3.7. Repeat the wash once for a total of two washes.

1.3.8. Remove all visible liquid after the second wash using a p10 pipette tip.

1.3.9. Air dry the beads for up to 2 minutes while the tubes are on the magnetic stand with the lid open.

Caution: Do not over-dry the beads. This may result in lower recovery of DNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.

1.3.10. Remove the tubes from the magnetic stand. Elute the DNA target from the beads by adding 17 µl of ⚬ (white) Elution Buffer.

1.3.11. Mix well by pipetting up and down 10 times. Incubate for at least 1 minute at room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube before placing back on the magnetic stand.

1.3.12. Place the tube on the magnetic stand. After 3 minutes (or whenever the solution is clear), transfer 16 µl of the supernatant to a new PCR tube.

Caution: Carrying even a small amount of beads forward can lead to inefficient deamination.

Safe Stopping Point: Samples can be stored overnight at -20°C.

1.4. Denaturation of DNA

The DNA can be denatured using either Formamide or 0.1 N Sodium Hydroxide.
Use **Option A** for denaturing using Formamide and **Option B** for denaturing using 0.1 N Sodium hydroxide.

1.4A: Formamide (Recommended)

1.4A.1. Pre-heat thermal cycler to 85°C with the heated lid set to ≥ 105°C or on.

1.4A.2. Add 4 µl Formamide to the 16 µl of protected DNA (from Step 1.3.12.). Mix thoroughly by vortexing 1–2 seconds or by pipetting up and down at least 10 times and centrifuge briefly.

1.4A.3. Incubate at 85°C for 10 minutes in the pre-heated thermal cycler.

1.4A.4. **Critical Step:** Immediately place on metal cooling block or ice. It is important to allow the sample to fully cool (at least 2 minutes) before proceeding to Section 1.5.

1.4B: Sodium Hydroxide

Optional, See FAQ about preparing NaOH

1.4B.1. Prepare freshly diluted 0.1 N NaOH.

1.4B.2. Pre-heat thermal cycler to 50°C with the heated lid set to ≥ 60°C or on.

1.4B.3. Add 4 µl 0.1 N NaOH to the 16 µl of protected DNA (Step 1.3.12.). Mix thoroughly by vortexing 1–2 seconds or by pipetting up and down at least 10 times and centrifuge briefly.

1.4B.4. Incubate at 50°C for 10 minutes in the pre-heated thermal cycler.

1.4B.5. **Critical Step:** Immediately place on metal cooling block or ice. It is important to allow the sample to fully cool (at least 2 minutes) before proceeding immediately to Section 1.5.

1.5. Deamination of Cytosines

1.5.1. On ice, add the following components to the denatured DNA.

COMPONENT	VOLUME
Denatured DNA (Step 1A.5. or 1B.5.)	20 µl
Nuclease-free water	68 µl
• (orange) APOBEC Reaction Buffer	10 µl
• (orange) BSA	1 µl
• (orange) APOBEC	1 µl
Total volume	**100 µl**

For multiple reactions, a master mix of the reaction components can be prepared before addition to the denatured DNA.

1.5.2. Mix thoroughly by vortexing 1–2 seconds or by pipetting up and down at least 10 times and centrifuge briefly.

1.5.3. Place in a thermal cycler, and run the following program with the heated lid set to ≥ 45°C or on:
3 hours at 37°C
Hold at 4°C

Safe Stopping Point: Samples can be stored overnight at either 4°C in the thermal cycler or at -20°C in the freezer.

1.6. Clean-Up of Deaminated DNA

Note: The ratios recommended for SPRIselect/AMPure XP beads in this manual have been experimentally optimized for every step; this is critical since buffer compositions differ between steps and across protocols e.g., post ligation recommendations will not apply to samples post PCR. Please adhere to these guidelines and not those recommended by other sources or for other kits.

Caution: The Sample Purification Beads behave differently during the APOBEC clean-up. Process only as many samples that will allow you to complete the clean-up without drying out the beads. Do not overdry the beads as they become very difficult to resuspend.

Note: Depending on the downstream application, you may be able to skip the clean-up.

1.6.1. Vortex Sample Purification Beads to resuspend. SPRIselect or AMPure XP Beads can be used. If using AMPure XP Beads, allow the beads to warm to room temperature for at least 30 minutes before use.

1.6.2. Add 100 µl of resuspended NEBNext Sample Purification Beads to each sample. Mix well by pipetting up and down at least 10 times. Be careful to expel all of the liquid out of the tip during the last mix.

1.6.3. Incubate samples on bench top for at least 5 minutes at room temperature.

1.6.4. Place the tubes against an appropriate magnetic stand to separate the beads from the supernatant.

1.6.5. After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets **(Caution: do not discard the beads)**.

1.6.6. Add 200 µl of freshly prepared 80% ethanol to the tubes while on the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

1.6.7. Repeat the ethanol wash once for a total of two washes.

1.6.8. Remove all visible liquid after the second wash using a p10 pipette tip.

1.6.9. Air dry the beads for up to 60 seconds while the tubes are on the magnetic stand with the lid open.

Critical Step: Do not over-dry the beads. This may result in lower recovery of DNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry. Please also see Why, at some stages of the EM-seq protocol, do the NEBNext Sample Purification Beads behave differently when cleaning up the sample? | NEB

1.6.10. Remove the tubes from the magnetic stand. Elute the DNA target from the beads by adding 21 µl of ° (white) Elution Buffer or appropriate buffer for downstream application.

1.6.11. Mix well by pipetting up and down 10 times. Incubate for at least 1 minute at room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube before placing back on the magnetic stand.

1.6.12. Place the tube on the magnetic stand. After 3 minutes (or whenever the solution is clear), transfer 20 µl of the supernatant to a new PCR tube.

Note: Please see NEB #E7120 for the PCR amplification protocol for downstream sequencing on an Illumina platform.

Safe Stopping Point: Samples can be stored overnight at -20°C.

Kit Components

NEB #E7125S Table of Components

NEB #	PRODUCT	VOLUME
E7122A	Control DNA CpG methylated pUC19	0.024 ml
E7123A	Control DNA Unmethylated Lambda	0.024 ml
E7124A	Elution Buffer	2.1 ml
E7126A	TET2 Reaction Buffer	0.3 ml
E7127A	TET2 Reaction Buffer Supplement (x 3)	powder
E7128A	Oxidation Supplement	0.024 ml
E7139AA	DTT	0.5 ml
E7129A	Oxidation Enhancer	0.024 ml
E7130A	TET2	0.096 ml
E7131A	Fe(II) Solution	0.024 ml
E7132A	Stop Reagent	0.024 ml
E7133A	APOBEC	0.024 ml
E7134A	APOBEC Reaction Buffer	0.24 ml
E7135A	BSA	0.024 ml

NEB #E7125L Table of Components

NEB #	PRODUCT	VOLUME
E7122AA	Control DNA CpG methylated pUC19	0.096 ml
E7123AA	Control DNA Unmethylated Lambda	0.096 ml
E7124AA	Elution Buffer	9 ml
E7126AA	TET2 Reaction Buffer	1.2 ml
E7127AA	TET2 Reaction Buffer Supplement (x 3)	powder
E7128AA	Oxidation Supplement	0.096 ml
E7139AA	DTT	0.5 ml
E7129AA	Oxidation Enhancer	0.096 ml
E7130AA	TET2	0.384 ml
E7131AA	Fe(II) Solution	0.096 ml
E7132AA	Stop Reagent	0.096 ml
E7133AA	APOBEC	0.096 ml
E7134AA	APOBEC Reaction Buffer	0.96 ml
E7135AA	BSA	0.096 ml

Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	3/19
2.0	N/A	5/19
3.0	Add DTT to module and update protocol to include DTT	2/20
3.1	Step 2.2 updated component description. Updated Protocol	3/20
4.0	Updated footnote, legal disclaimer and formatting of tables	5/23
5.0	Updated protocols and legal disclaimer. Also added new NEB logo.	3/25