SHORT PROTOCOL No. 02 I November 2014

Automated Illumina® TruSeq® Stranded mRNA library construction with the ep*Motion*® 5075t/TMX

Introduction

For the MiSeq® and HiSeq® next generation sequencing (NGS) systems, Illumina offers a broad variety of sample preparation kits. These kits are needed to convert either DNA or RNA samples into sequencing ready libraries, a procedure that includes many steps and is costly. RNA sequencing requires additional steps – either the depletion of unwanted ribosomal RNA or the positive selection of mRNA from total RNA samples. RNA sequencing is being used for e.g. transcriptome analysis, differential gene expression studies or detection/characterization of splice variants.
Due to the complexity of the library construction methods, automation is regarded as highly useful. A manual preparation of 8 samples with the Illumina TruSeq Stranded mRNA Kit requires one well trained FTE for one full day. Typical labs applying these kits are sequencing core facilities of larger institutes or companies. Additionally also labs that do not own their own NGS equipment might be interested in preparing their libraries as well as they might have higher throughputs and have to/want to avoid extra costs for library prep services.

This protocol describes the configuration and preprogrammed methods for the automated construction of 8/16 or 24 sequencing ready libraries from 100 – 1000 ng total RNA with the Illumina TruSeq Stranded mRNA kit. The overall hands on time is less than 1 hour, the total run time of the entire procedure is ~11.5 hours for 24 samples. In some labs the final post PCR cleanup might have to be performed in a separate room (separation of pre and post PCR under certain circumstances), in this case only the PCR setup step of the last sub-method can be done on the ep*Motion*.

Material and Methods

Required equipment

> ep*Motion* 5075 TMX or ep*Motion* 5075t
> additional thermal module (Position C2)
> Gripper
> TS50 pipetting tool
> TS300 pipetting tool
> TM50-8 pipetting tool
> TM300-8 pipetting tool
> 4x thermoadapter for PCR plates, 96-well
> Reservoir rack
> 3x RR Module TC Safe Lock
> PCR Cycler, e.g. Eppendorf Mastercycler® Pro S
> Alpaqua® LE magnet plate (low elution volume magnet, Alpaqua order no. A000350)
➡ this magnet is the only one known to work with the low elution volumes required in some steps of the procedure - don't use an other one!

Required consumables

> epT.I.P.S.® Motion 50 µL Filter
> epT.I.P.S. Motion 300 µL Filter
> Eppendorf twin.tec® PCR plates, 96-well, semi skirted
> Eppendorf twin.tec PCR plates, 96-well, skirted (for the Index Adapters)
> Eppendorf Safe-Lock Tube 1.5 mL
> Eppendorf Safe-Lock Tube 2.0 mL
> ep*Motion* Reservoir 30 mL
> Eppendorf 400 mL reservoir
> Agencourt® AMPure® XP beads (Beckman Coulter®, order nos. A63880, A63881, A63882)
> 80 % Ethanol
> RNase free water
> mineral oil, PCR/molecular biology grade (Sigma-Aldrich®, order no. M5904-500ML)
> SuperScript® II Reverse Transcriptase (Life Technologies®, order no. 18064-014)
> Illumina TruSeq Stranded mRNA kit

Methods

Method Name	approx. Runtime (24 samples)
XXYYZZ-01-TS-SmRNA.dws	5.5hrs
XXYYZZ-02-TS-SmRNA.dws	4hrs
XXYYZZ-03-TS-SmRNA.dws	2hrs, including external PCR

(XX = year, YY = month, ZZ = day)

This approach is programmed to provide as much automation as possible, thus only up to 24 samples can be processed. Please only process 8/16 or 24 samples, sample numbers non divisible by 8 are not supported. The entire workflow – from 100 – 1000 ng total RNA sample input to sequencing ready libraries – is divided into three ep*Motion* methods (or sub-methods, see above). Each of the methods ends at a "Safe Stopping Point", allowing to store the intermediate products at -20 °C to -15 °C for up to 7 days, as stated in the Kit's user guide. In the default setup only the third sub-method requires a user intervention to perform the enrichment PCR in an external cycler.

To avoid dead volumes, all Illumina Kit reagents are programmed either in 1.5 mL or 2 mL Tubes, with the exception of the Bead Washing Buffer which as well as Agencourt Magnetic Beads and Ethanol for the washes is provided in 30 mL reservoirs to allow 8 channel pipetting. All liquid waste is collected in a 400 mL reservoir in B0. As most of the used volumes are very low, all reagents must be checked for foam, air bubbles etc. to ensure best performance prior to starting the runs. For some of the reagents, the beads and the mineral oil, it is mandatory to let them reach ambient temperature to ensure proper function and pipetting due to changes in viscosity. During the procedure no cooling of the reagents is required. All steps of the procedure are performed in 96-well twin.tec PCR plates, for the multiple heat incubation steps above 37 °C, samples are overlaid with oil to avoid evaporation and allow temperature incubations on the epMotion. Only the enrichment PCR needs to be carried out in an external PCR cycler.

The methods were developed on the ep*Motion* 5075 TMX, but can also be transferred/imported to the new ep*Motion* 5075t.

I**mportant:** The output plate containing the samples that need to be processed in the subsequent sub-method will always be placed on the C2 position (Temp) set to 10 °C at the end of the individual methods. Final libraries will be found in columns 10-12 of the plate labeled PCR. The volume is 30 µL:

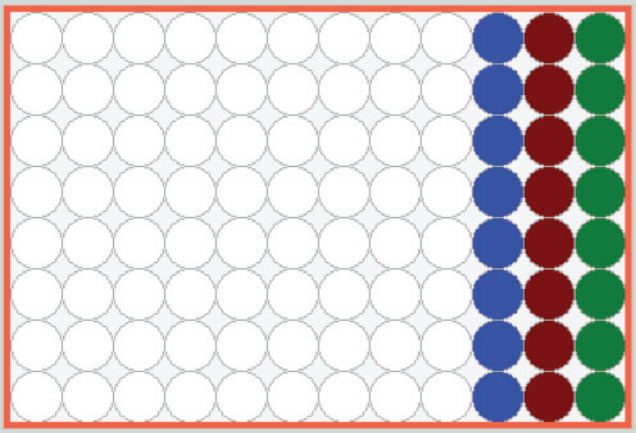

Figure 1: Position of the final libraries in the plate labeled PCR after completion of the entire procedure.

Sub-method 01

Start with 100 – 1000 ng total RNA in a volume of 50 µL per sample. 8/16 or 24 samples have to be provided in the first three columns (Wells A1-H3) of a 96-well twin.tec semi skirted PCR plate (RBP+CDP), placed on the TMX position. The method ends with clean cDNA in the first three columns (A1-H3) of a second 96-well twin.tec semi skirted PCR plate (CDP+ALP). This plate needs to be used in the sub-method 02.

Worktable Layout

Position	Item	Position	Item
A2	50 µL Filtertips	B3	300 µL Filtertips
A3	50 µL Filtertips	B4	300 µL Filtertips
A4 (TMX)	Thermoadapter PCR 96 + PCR plate with RNA samples (labeled RBP+CDP)	C1	Thermoadapter PCR 96 + empty PCR plate (labeled CDP+ALP)
B0	400 mL tub for liquid waste	C2 (Temp)	Thermoadapter PCR 96
B1	300 µL Filtertips	C3	Reservoir rack with 3x RR Module Safe Lock + 3 x 30 mL reservoir for reagents
B2	300 µL Filtertips	C4	Alpaqua LE magnet plate

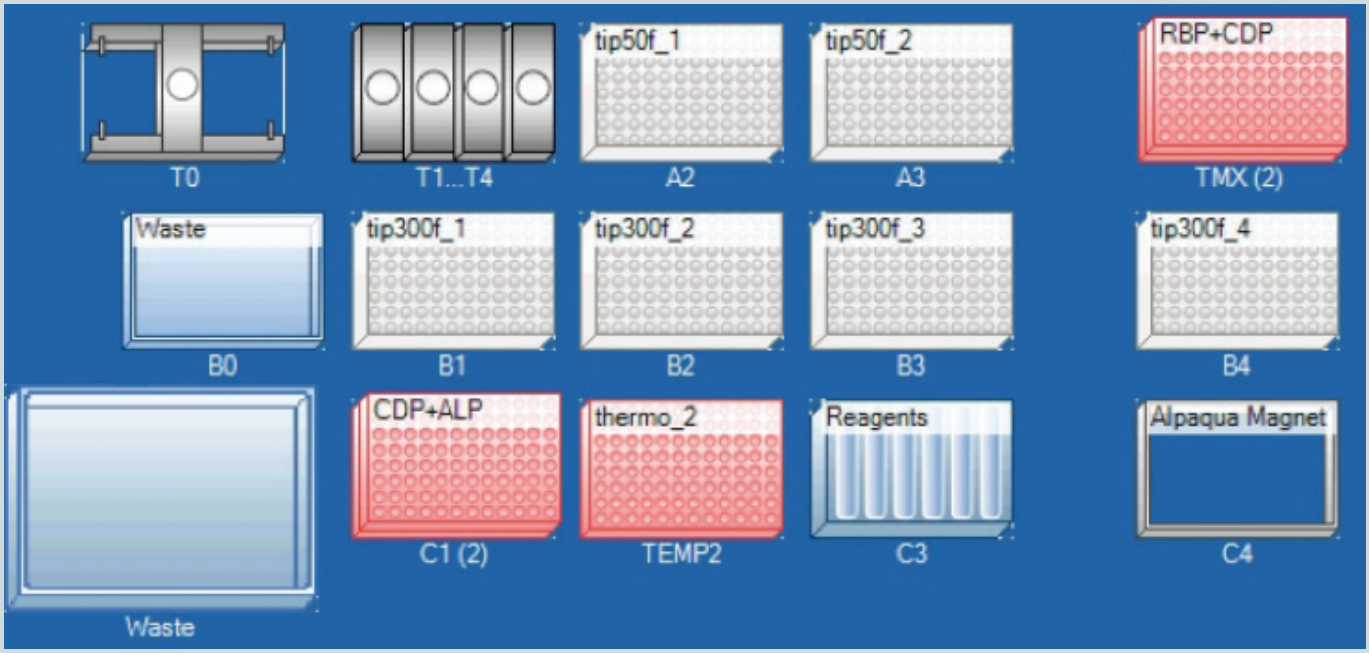

Figure 2: ep*Motion* worktable layout for method 1

Reservoir rack layout

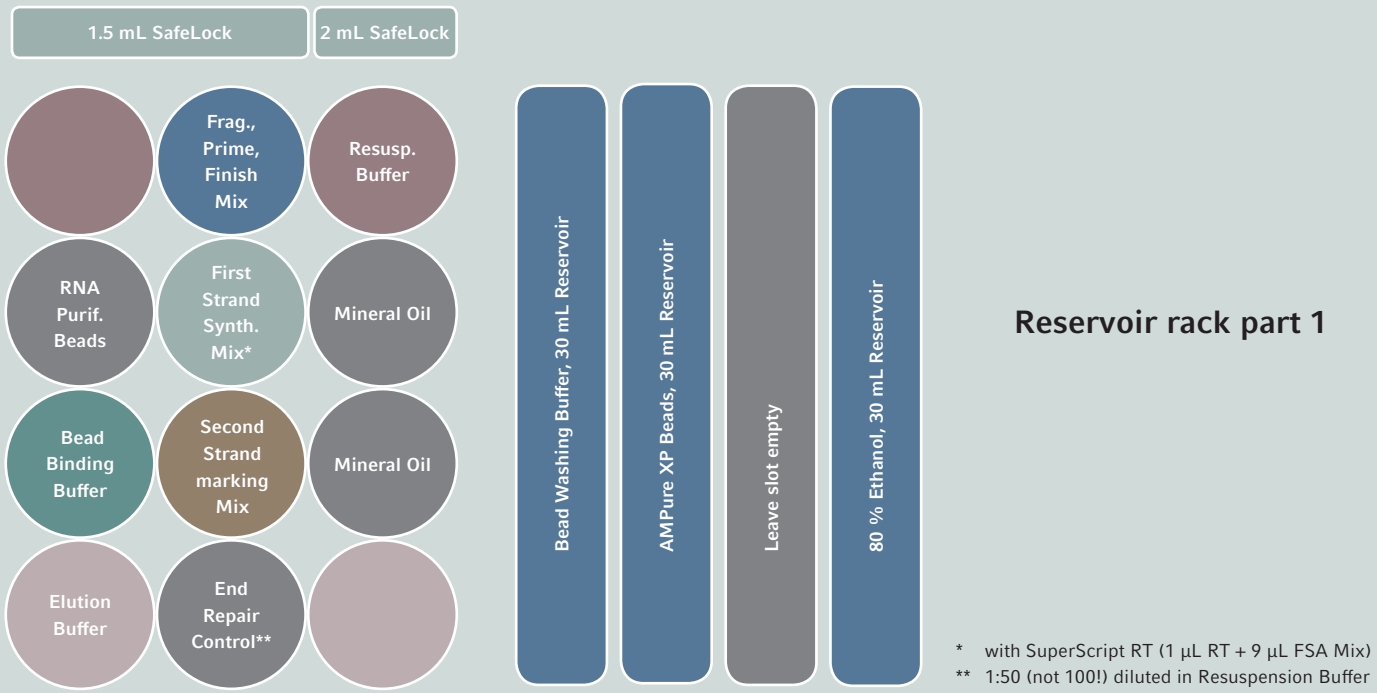

Figure 3: Reservoir rack layout for method 1

Attention: If kit included controls are not going to be used, use plain resuspension buffer in the according reagent position.

Sub-method 02

Start with the PCR plate labeled CDP+ALP containing the cDNAs from sub-method 01 in Positions A1 – H3, placed on position B4. The method ends with A-tailed and Index Adapter ligated clean cDNA in a PCR plate labeled PCR. Depending on the sample number, sequencing setup, pooling scheme etc. the number, combination and labware of the Index Adapters (position B3) needs to be modified, also review/adjust steps 26 and following + the worktable in the method. If the default setup is being used, a user intervention to refill 50 µL tips is required if > 16 samples are processed.

Worktable Layout

Position	Item
A2	50 µL Filtertips
A3	50 µL Filtertips
A4 (TMX)	Thermoadapter PCR 96
B0	400 mL tub for liquid waste
B1	300 µL Filtertips
B2	300 µL Filtertips
B3	skirted PCR plate with Index Adapters ➡ review method programming
B4	Thermoadapter PCR 96 + PCR plate with cDNA (CDP+ALP) from sub-method 01

Position	Item
C1	Thermoadapter PCR 96 + empty PCR plate (labeled PCR)
C2 (Temp)	Thermoadapter PCR 96
C3	Reservoir rack with 3x RR Module SafeLock + 2 x 30 mL reservoir (pos. 5 & 7)
C4	Alpaqua LE magnet plate

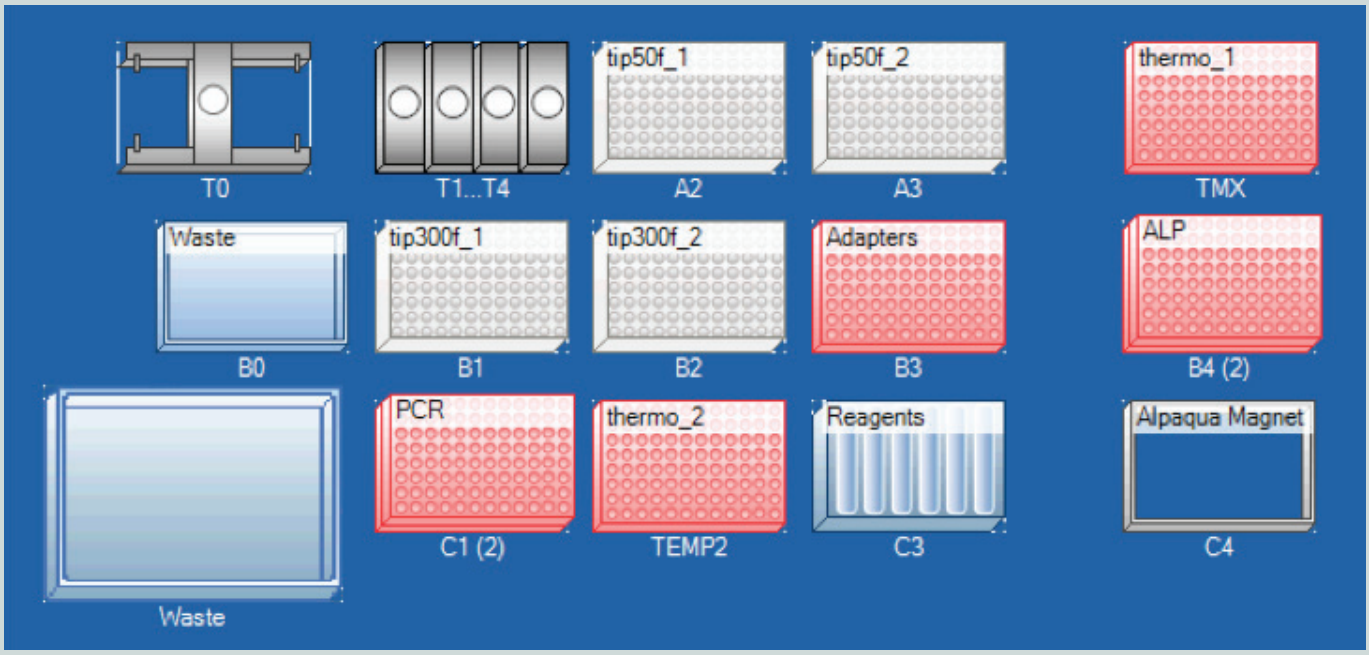

Figure 4: ep*Motion* worktable layout for method 2

Reservoir rack layout

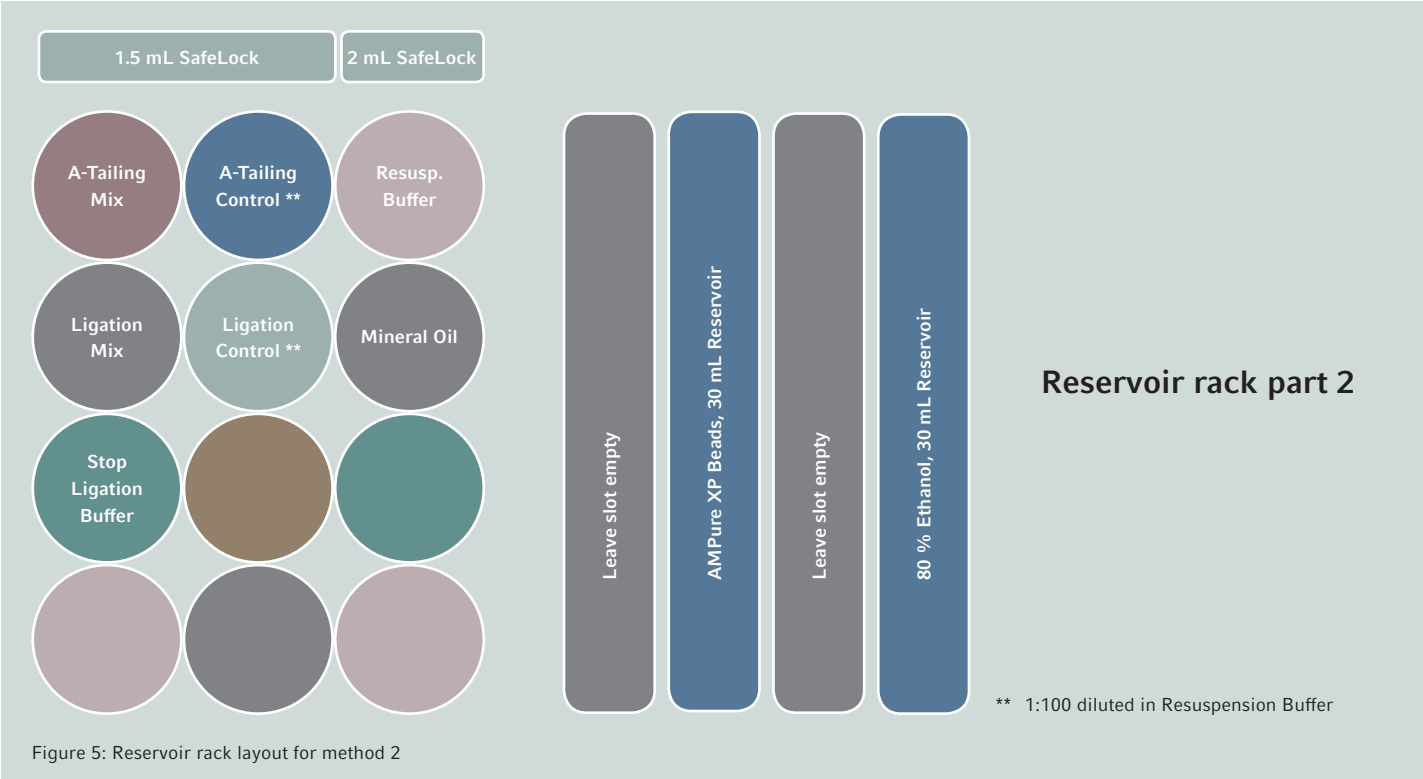

Figure 5: Reservoir rack layout for method 2

Attention: If kit included controls are not going to be used, use plain resuspension buffer in the according reagent position.

Sub-method 03

Start with 20 µL A-tailed and Index Adapter ligated samples in plate labeled PCR from sub-method 02 on TMX position. Only the PCR setup will be pipetted, which takes a couple of minutes, then a user intervention occurs where the PCR plate needs to be sealed and cycled in a PCR cycler to enrich the libraries. Reopen the plate after PCR and return to the TMX position prior to continuing the method.

Worktable Layout

Position	Item
A2	50 µL Filtertips
A3	empty
A4 (TMX)	Thermoadapter PCR 96 + PCR plate (PCR) with samples from sub-method 02
B0	400 mL tub for liquid waste
B1	300 µL Filtertips
B2	300 µL Filtertips
B3	empty
B4	Thermoadapter PCR 96

Position	Item
C1	Thermoadapter PCR 96
C2 (Temp)	Thermoadapter PCR 96
C3	Reservoir rack with 3x RR Module SafeLock + 2x 30 mL Reservoir (pos.5 & 7)
C4	Alpaqua LE magnet plate

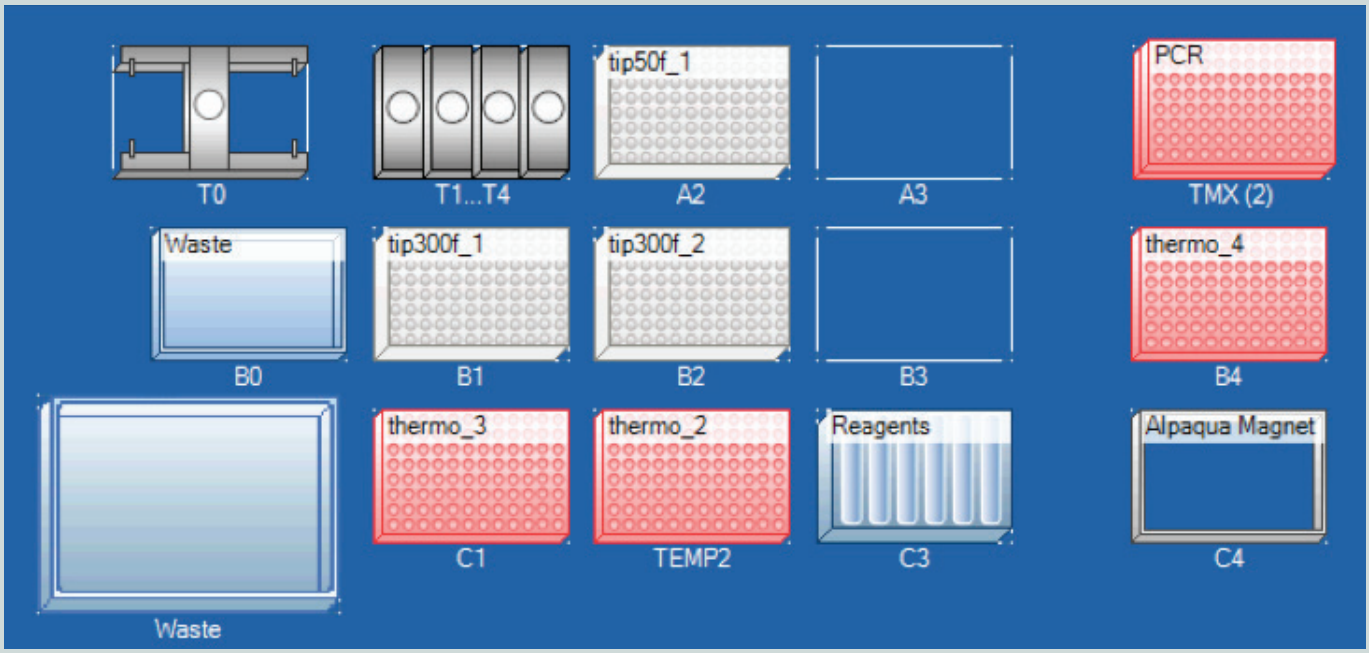

Figure 6: ep*Motion* worktable layout for method 3

Reservoir rack layout

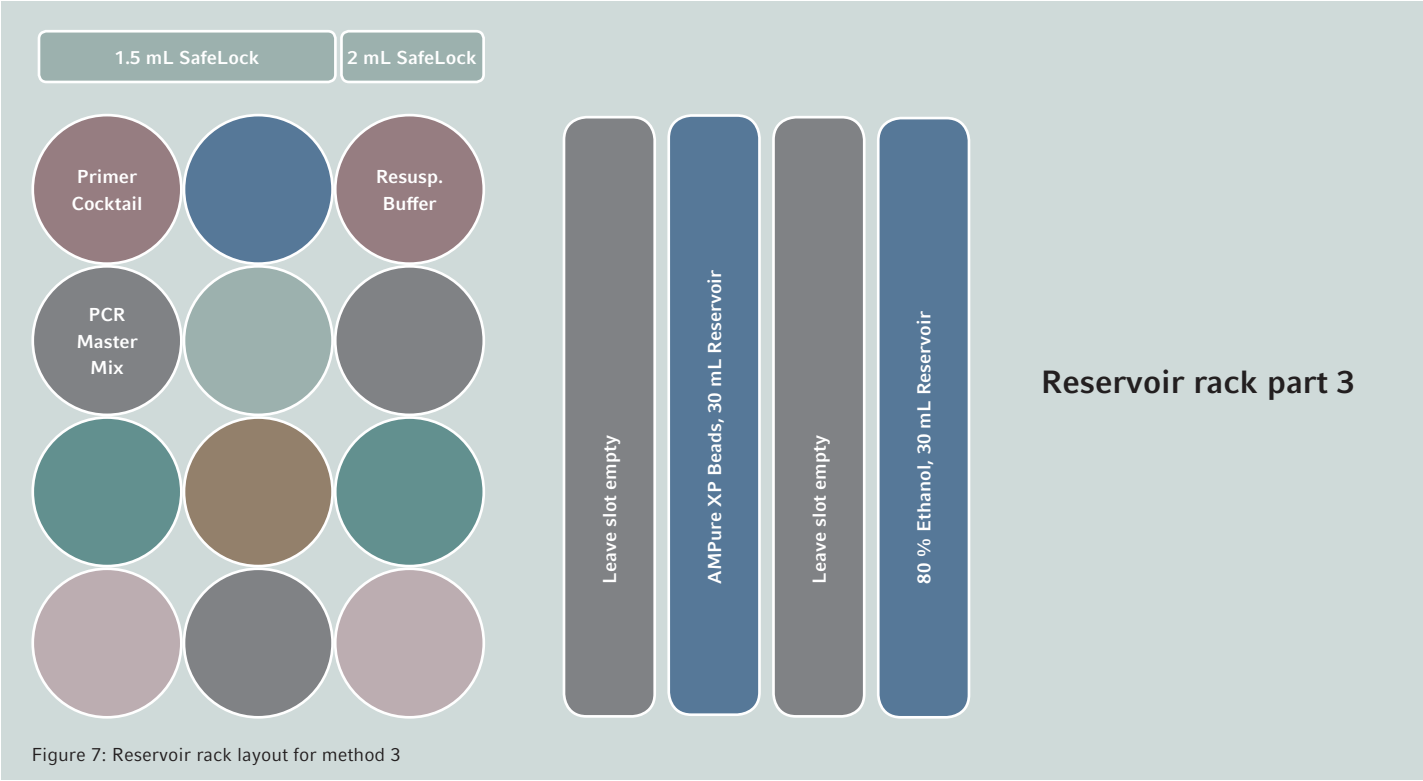

Figure 7: Reservoir rack layout for method 3

After all three sub-methods have been completed, the final libraries are in the plate labeled PCR on position C2 (Temp) at 10 °C.

Results

Typically the final libraries will be QC'ed on an Agilent® 2100 BioAnalyzer® or similar to assess the fragment size distribution (ideally with a peak around 260 bp). Then either a qPCR approach or a fluorescence based measurement will be done to quantify the libraries. Typical BioAnalyzer results from the ep*Motion* method look like this:

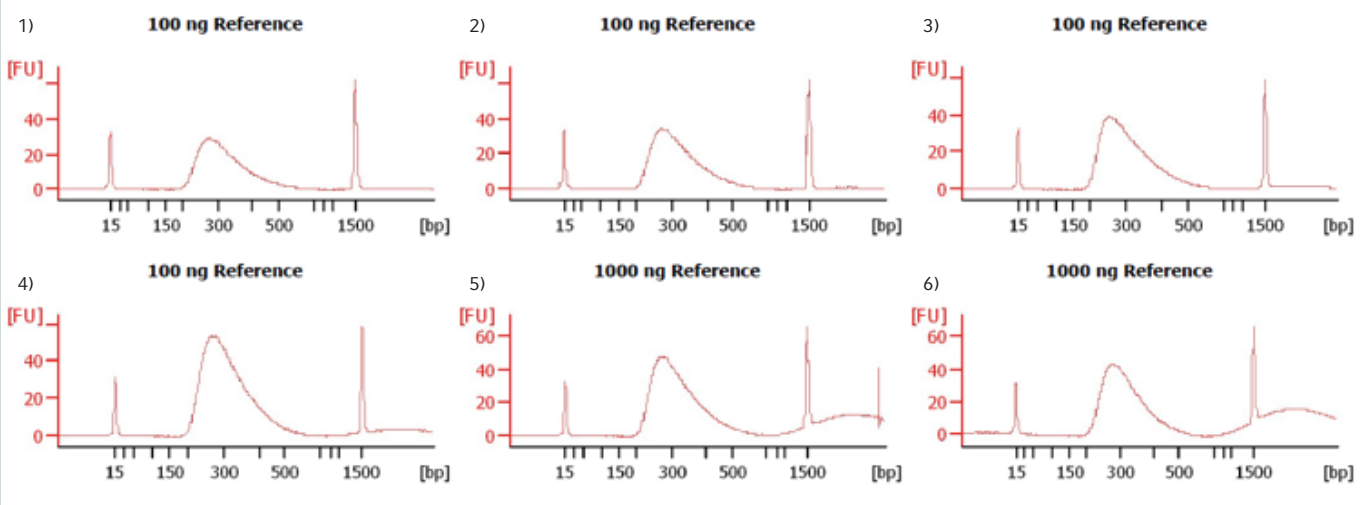

Figure 8: typical BioAnalyzer Traces for sequencing ready libraries generated with the epMotion.

The first 4 images are epMotion libraries created from 100 ng, the last two from 1000 ng total RNA (Universal Human Reference, Agilent). The extra peak above 1500 bp in the 1000 ng sample is caused by the high amount of input RNA and is normal. Typical Qubit® (PicoGreen®) readings of additional customer libraries created from 100 ng or 500 ng input total RNA resulted in 30-40 ng/µL, these values need to be transformed into molarities, as for the sequencing Picomolar amounts of library are going to be sequenced.

Ordering Information

Description	Order no. international
ep*Motion*® 5075t	5075 000.302
Thermal module	5075 757.001
TS 50 dispensing tool	5280 000.010
TS 300 dispensing tool	5280 000.037
TM50-8 dispensing tool	5280 000.215
TM300-8 dispensing tool	5280 000.231
Gripper	5282 000.018
Thermoadapter PCR 96	5075 787.008
Reservoir rack	5075 754.002
Reservoir rack module TC Safe-Lock	5075 799.081
epT.I.P.S.® Motion, 50 µL, filtered	0030 014.413
epT.I.P.S.® Motion, 300 µL, filtered	0030 014.456
Reservoir 30 mL	0030 126.505
400 mL reservoir	5075 751.364
Eppendorf twin.tec® PCR Plate 96, semi-skirted	0030 128.575
Eppendorf twin.tec® PCR Plate 96, skirted	0030 128.648
Eppendorf Safe-Lock Tubes, 1.5 mL	0030 120.086
Eppendorf Safe-Lock Tubes, 2.0 mL	0030 120.094

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