Rapid Massive Analysis of cDNA Ends (rapid MACE)

Library Preparation Kit – User Guide

Table of Contents

1 Overview ... 1

1.1 Introduction ... 1

1.2 TrueQuant Technology ... 2

1.3 Library Preparation ... 2

2 Kit Contents* ... 3

3 Equipment and Consumables ... 4

4 Protocol ... 5

4.1 RNA Fragmentation and Reverse Transcription ... 5

4.2 Test PCR Amplification ... 7

4.3 Optional prePCR Pooling ... 9

4.4 PCR Amplification ... 10

4.5 SPRI bead clean-up of amplified cDNA ... 11

4.6 Quality Control and Sequencing ... 13

5 Bioinformatics ... 15

Appendix A - Additional PCR Amplification ... 16

Appendix B - Additional SPRI bead purification of the amplified library pool ... 17

Appendix C - Index sequences ... 18

1 Overview

1.1 Introduction

Rapid Massive analysis of cDNA ends (MACE) is an improved version of 3' end RNA sequencing that allows for accurate and reproducible transcriptome profiling including PCR bias elimination. In contrast to RNA-Seq, which yields variable numbers of reads per transcript, MACE generates one read for each polyadenylated RNA molecule (Fig. 1). The resulting reduction in complexity of the profiled transcriptomes strongly decreases the required sequencing depth for reliable quantification of lowly abundant transcripts such as transcription factors and receptors. Equally important, the focus on 3' ends strongly improves the coverage of this highly diverse and regulatory important site and allows for examining allele-specific gene expression and alternative polyadenylation.

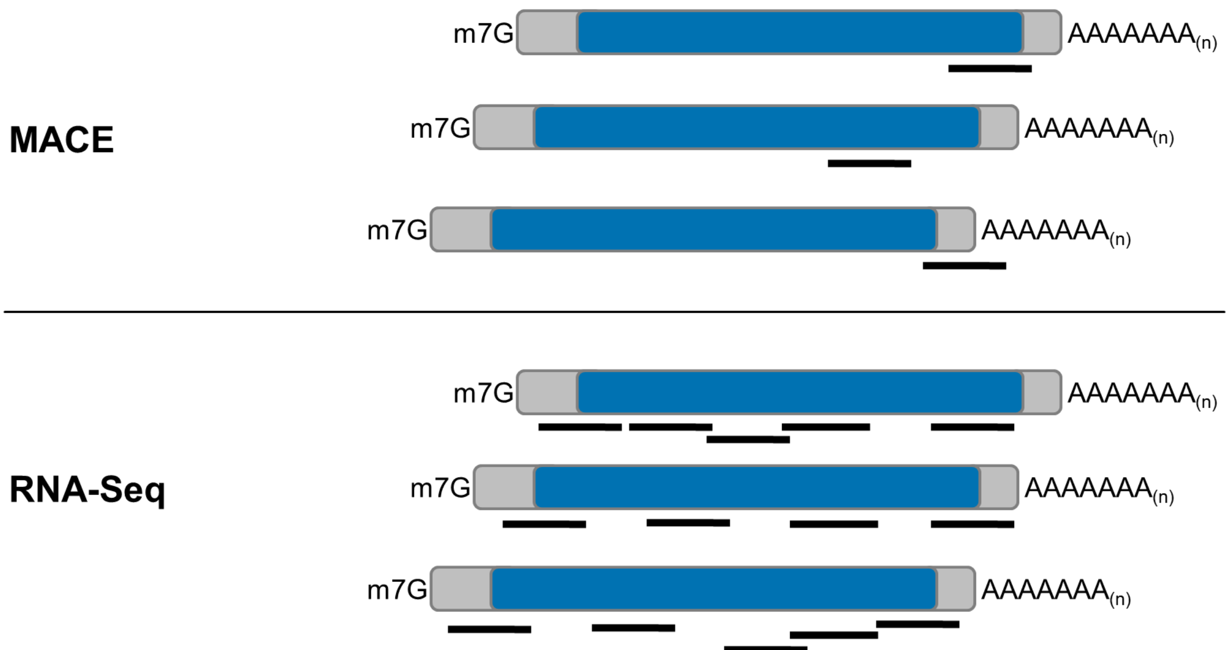

Fig 1.: Illustration of mapping patterns from sequenced reads prepared by MACE and RNA-Seq. The distribution of sequencing reads (black lines) is shown for three copies of the same protein-coding transcript (ORFs are shown in blue and UTRs in gray). MACE generates one read derived from the last hundreds of nucleotides from each polyadenylated transcript, while RNA-Seq yields numerous reads per transcript that originate across the entire length of a given transcript. The read count for MACE precisely equals the copy number of a profiled transcript, resulting in unbiased and highly quantitative gene expression profiling. As with RNA-Seq, overlapping MACE reads from different copies of the same transcript can be used for assembly of longer contigs, which can cover up to 1,500 nucleotides of the 3' end from a given transcript. Due to the greatly increased coverage of 3' ends compared to RNA-Seq, MACE additionally allows for profiling of transcriptional termination sites (TTS) with high accuracy. Alternative polyadenylation (APA) events can be directly deduced by filtering of poly-A-tail positive reads. Moreover, the high coverage of 3' ends facilitates discovery of single-point mutations (SNP) as well as allele-specific gene expression.

1.2 TrueQuant Technology

The present kit involves our patented TrueQuant Technology that eliminates reads that originate from PCR-derived derived copies of the original template transcript molecule. This is achieved by incorporation of oligo(dN)s, termed TrueQuant adapters or unique molecular identifiers (UMI) to the template molecules prior to PCR amplification. Preferential PCR amplification inevitably occurs when template molecules of different length and GC content are present and generates bias that rapidly increases with every PCR cycle. Following sequencing of the PCR-amplified libraries, original template molecules are identified in-silico by the unique sequence combinations of the TrueQuant adapters and template molecules.

1.3 Library Preparation

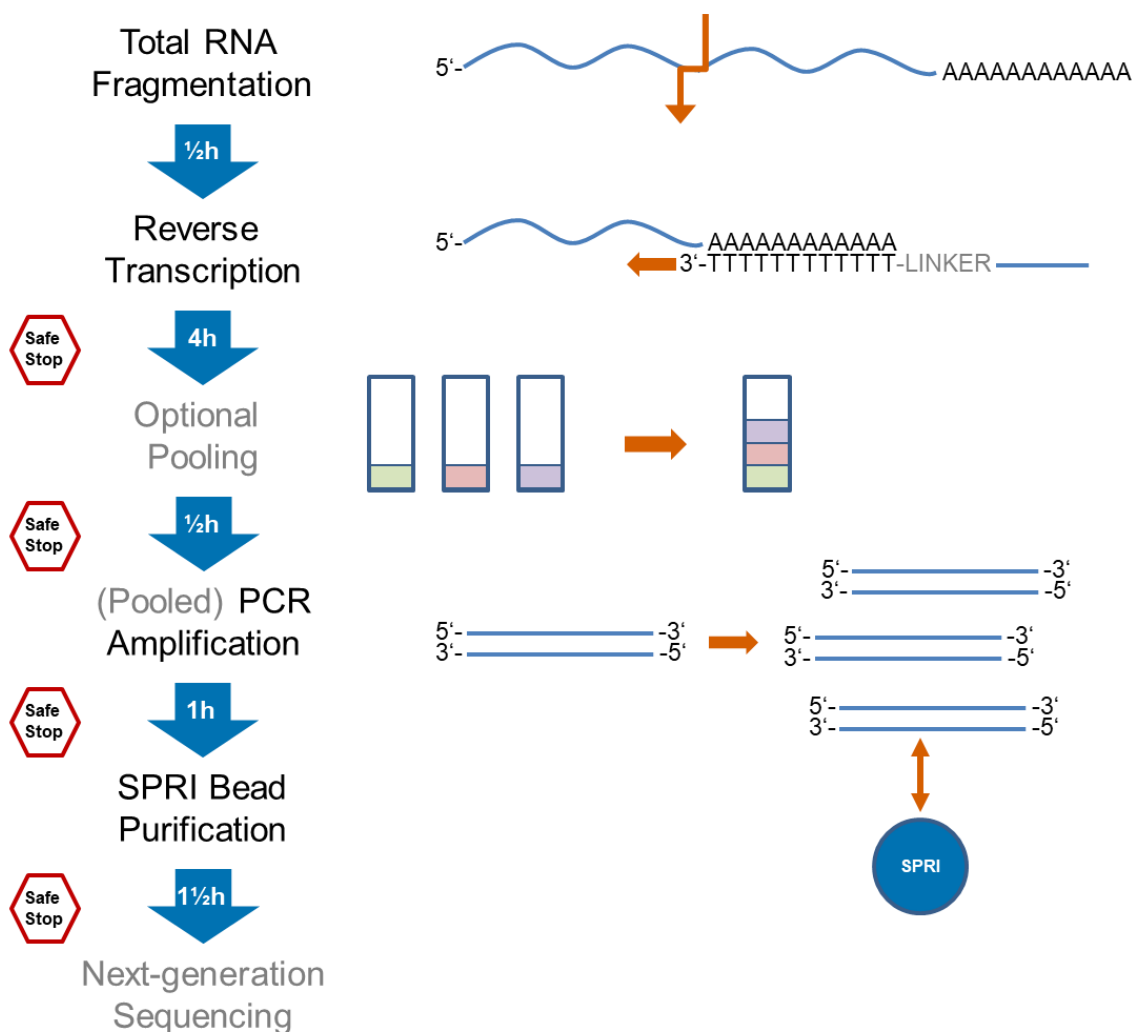

Fig. 2: Library preparation workflow for rapidMACE. Starting with DNase-treated and quality-controlled total RNA, the complete protocol can be carried out in a single day. The workflow starts with individual fragmentation and reverse transcription of each sample, followed by (optionally pooled) PCR amplification. The final library is ready for sequencing on any of Illumina's NextSeq, NovaSeq, HiSeq, MiSeq, MiniSeq, or iSeq platforms.

2 Kit Contents*

Category	Contents
For all reactions	Nuclease-free Water
	TE Buffer
Reverse Transcription	RT Buffer
	RT Enhancer
	RT Enzyme
	PrePCR Enzyme
	RT Primer ID N701 RT Primer ID N702 RT Primer ID N703 RT Primer ID N704 RT Primer ID N705 RT Primer ID N706 RT Primer ID N707 RT Primer ID N710
PCR Amplification	PCR Buffer
	PCR Enzyme
	6-plex Kit: PCR Primer ID N502 24-plex Kit: PCR Primer ID N502 PCR Primer ID N503 PCR Primer ID N505 96-plex Kit: PCR Primer ID N502 PCR Primer ID N503 PCR Primer ID N505 PCR Primer ID N506 PCR Primer ID N507 PCR Primer ID N508 PCR Primer ID N510 PCR Primer ID N511 PCR Primer ID N513 PCR Primer ID N515 PCR Primer ID N516 PCR Primer ID N517 PCR Primer ID N518 PCR Primer ID N520 PCR Primer ID N521 PCR Primer ID N522

*** Store all reagents at -20 °C, and briefly spin down the contents of the tubes prior to first use.**

3 Equipment and Consumables

Necessary Equipment
Automated microfluidic electrophoresis station (only one of the following): • Bioanalyzer or TapeStation (Agilent) • LabChip GX or GXII (PerkinElmer) • QIAxcel Advanced System (Qiagen) • Fragment Analyzer (Advanced Analytical Technologies)
Qubit (Thermo Fisher Scientific) or Quantus (Promega) or similar Fluorometer
Magnetic stand (suitable for 1.5 ml tubes)
Thermocycler (suitable for 0.2 ml tubes)
Benchtop microcentrifuge for 0.2-1.5 ml tubes
Vortex mixer
Calibrated single-channel pipettes

Customer supplied consumables and reagents	
SPRI Beads	Agencourt AMPure XP or Agencourt SPRIselect (Beckman Coulter)
	Select-a-Size DNA Clean & Concentrator MagBead Kit (Zymo Research)
	NucleoMag NGS Clean-up and Size Select (Macherey-Nagel)
Intercalating qPCR Dye	EvaGreen (Biotium)
	SYBR Green (Molecular Probes)
	dsGreen (Lumiprobe)
Fluorometric RNA Quantitation	RNA High Sensitivity Assay (Qubit)
	QuantiFluor RNA Assay (Quantus)
Fluorometric DNA Quantitation	dsDNA High Sensitivity Assay (Qubit)
	QuantiFluor dsDNA Assay (Quantus)
1.5 ml Tubes (low binding, nuclease-free)	General laboratory supplier
0.2 ml Tubes (nuclease-free)	General laboratory supplier
80 % Ethanol	General laboratory supplier
Pipette Tips (with aerosol barriers, nuclease-free)	General laboratory supplier

4 Protocol

- Before starting make sure that all total RNA isolates have been DNase-treated on-column or preferably in solution. Assess the RNA quality (RIN or RQN or similar) of each isolate with an automated microfluidic electrophoresis station (RNA assay).

- Determine the concentration of all isolates by fluorometric quantitation (RNA assay). The recommended quantity for reverse transcription per sample is 100 ng of total RNA.

4.1 RNA Fragmentation and Reverse Transcription

- Set up the following PCR program on a thermocycler with heated lid set at ≥ 100 °C:

Temperature	Duration	Ramp Rate
94° C	8 min	Standard
70° C	1 min	Standard
42° C	Hold until addition of **RT Master Mix**	0.1 °C per second
42° C	15 min	Standard
42° C	Hold until addition of **RT Enhancer**	Standard
42° C	120 min	Standard
70° C	10 min	Standard
37 °C	Hold until addition of **PrePCR Enzyme**	Standard
37 °C	60 min	Standard
80 °C	20 min	Standard
4° C	Forever	Standard

- On ice, transfer up to 8 µl of each RNA isolate to a new nuclease-free 0.2 ml tube and fill up the volume to 8 µl with nuclease-free water (supplied with the Kit) if necessary. Subsequently, add the corresponding RT Primer ID as indicated below[1].

	Volume per Sample
RNA Sample	8 µl
RT Primer ID	2 µl
Total Volume	10 µl

- Mix carefully by vortexing or pipetting and briefly spin down. Place back on ice.

[1] Make sure to assign an individual RT Primer ID to each RNA isolate, so that each ID is represented only once subsequent to pooling of the reverse-transcribed cDNAs.

- Prepare the RT Master Mix at room temperature as indicated below. Include sufficient spare volume to account for pipetting inaccuracies.

	Volume per Sample (x)	RT Master Mix (x times volume)
RT Buffer	8.8 µl	
RT Enzyme	1.2 µl	
Total Volume	10 µl	

- Mix the RT Master Mix by pipetting or vortexing and briefly spin down.
- Place the individual 0.2 ml tubes (with sample RNA and RT Primer ID) in the PCR cycler and start the program. Be attentive to add the RT Master Mix approximately 15-20 minutes after starting the program.
- As soon as the cycler reaches 42 °C, add 10 µl of the RT Master Mix to each of the tubes in the cycler as indicated below.

	Volume per Sample
Reaction Mix	10 µl
RT Master Mix	10 µl
Total Volume	20 µl

- Mix carefully by pipetting or vortexing and briefly spin down. Immediately continue with the PCR program.
- Following 15 minutes of incubation at 42 °C, add 1 µl of RT Enhancer to each of the sample tubes as indicated below.

	Volume per Sample
Reaction Mix	20 µl
RT Enhancer	1 µl
Total Volume	21 µl

- Mix carefully by pipetting or vortexing and briefly spin down. Immediately continue with the PCR program.
- Following 10 minutes of incubation at 70 °C, add 1 µl of PrePCR Enzyme Mix to each of the sample tubes as indicated below. Mix carefully by pipetting or vortexing and briefly spin down. Immediately proceed with the PCR program.

	Volume per Sample
Reaction Mix	21 µl
PrePCR Enzyme	1 µl
Total Volume	22 µl

4.2 Test PCR Amplification

Test PCR amplification ensures that the cDNA pool is amplified with the lowest possible cycle number for sequencing[2]. For that purpose, a small fraction of reverse-transcribed cDNA is used as template for quantitative real-time PCR (qPCR). Set up the following program on a qPCR cycler (without adding a melt curve):

Temperature	Duration	Ramp Rate	Cycles
98° C	45 sec	Standard	1
98° C	20 sec	2 °C per second	35
65 °C	20 sec		
72 °C	60 sec		

- Prepare test PCR amplification for each sample in preparation as indicated below.

	Volume per cDNA Pool	
Reverse-transcribed cDNA	2 µl	
SYBR Green I (for a final conc. of 0.1x) or dsGreen (for a final conc. of 1x) or EvaGreen (for a final conc. of 1x)	x µl	Not supplied with Kit
PCR Buffer	13.25 µl	
PCR Enzyme	0.5 µl	
PCR Primer ID N5xx	1.25 µl	
Nuclease-free water	8 – x µl	
Total Volume	25 µl	

- Mix carefully by vortexing or pipetting and briefly spin down.

- Transfer the 25 µl reaction to a qPCR-compatible 0.2 ml tube or plate.

- Incubate the 0.2 ml tube or plate using the qPCR program described above. Independently of the applied dye, record the SYBR channel without additional ROX.

- After completion of the run, go to the amplification plot displaying the normalized fluorescence per cycle (not the difference in normalized fluorescence per cycle that is typically denoted with an additional delta) and choose the linear (not logarithmic) scale (Fig. 3).

[2] This step is mandatory for all samples that are pooled before final PCR amplification. For individual PCR amplification of each sample, test PCR amplification can be done with random sampling of similar input RNA (similar quality and same tissue type). For preparation of previously assessed samples this step can be omitted.

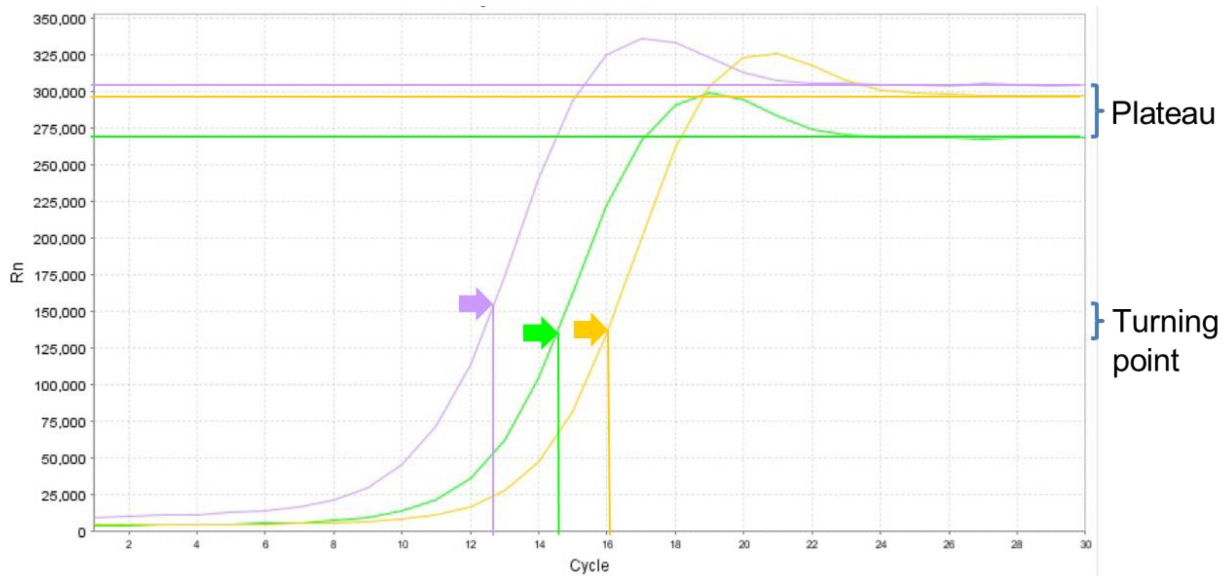

Fig. 3: Quantitative PCR plots of 3 distinct cDNAs (purple, green, and yellow). The optimal cycle number for PCR amplification of the respective cDNA equals the cycle number of the turning point minus 2 cycles. The turning point for each cDNA pool (approximated as half of the fluorescence level of the plateau) is indicated by an arrow along with the respective plateau. In case of doubt, we recommend to round up to the higher cycle number to ensure sufficient amounts of amplified cDNA for sequencing. The yellow amplification curve reaches the plateau at ~290,000 fluorescent units, and thus the turning point is located at ~145,000, which corresponds to 16 cycles. The optimal cycle number for amplification consequently equals 14 cycles. The plateau of the green amplification curve equals ~270,000 and the turning point ~135,000 fluorescent units, which corresponds to roughly 15 cycles. Therefore, 13 cycles represents the optimal cycle number for amplification of this cDNA. In case of the purple amplification curve, the plateau is located at ~310,000 and the turning point at ~155,000 fluorescent units. With approximately 13 cycles the cycle number of this turning point indicates an optimal cycle number for amplification of 11 cycles.

- Locate the plateau of the amplification plot and determine the corresponding normalized fluorescence value. Divide this value by a factor of two to **approximate the turning point of amplification** and determine the corresponding cycle number (in case of doubt round up to the higher cycle number).

- **Subtract two cycles from the determined cycle number of the turning point** to obtain the optimal number of cycles for amplification of the respective cDNA.

4.3 Optional prePCR Pooling

- The present Kit allows for individual amplification of each sample in preparation. However, the samples can alternatively be pooled prior to PCR amplification. This is especially useful for low-input and/or high throughput library preparations.

- In the latter case, make sure to only pool samples that were reverse-transcribed with a unique RT Primer ID, so that each ID is represented not more than once in the final pool[3]. The present Kit allows for pooling of up to 8 samples. For sequencing of more samples, the cDNAs have to be combined into pools of 8 samples, and subsequently indexed with a unique PCR Primer ID. Pooling of RT Primer IDs that are used for more than a single sample will result in ambiguous demultiplexing of the sequenced reads.

- In case of doubt, pool up to 8 samples using equal volumes of cDNA. For that purpose, combine 5 µl of each reverse-transcribed sample in a single 1.5 ml low-binding tube.

- For optimization of the sequencing depth of each sample, pooling can be further adjusted. 24-plex and 96-plex preparations can be grouped into pools with similar Ct values (please ensure that each RT Primer ID is only represented once per pool). Within each pool, outliers can be normalized by adjusting the volume: instead of adding 5 µl of a sample that exhibits a higher or lower Ct value, use 2.5 µl (if the Ct value is more than 1 cycle lower than the average of all other samples) or 10 µl (if the Ct value is more than 1 cycle higher than the average of the other samples).

- Distribute the pools into multiple PCR amplification reactions using the same template. Do not add more than 10 µl pooled cDNA template to each reaction. Fill up the template volume of the last PCR reaction to 10 µl using nuclease-free water (supplied with the Kit).

- Subsequent to pooling of the different samples, the combined sample pool and all remaining cDNAs can be safely stored at -20 °C.

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[3] Please note that it is mandatory to use specific ID combinations for Illumina sequencing of low-plexity pools, i.e. for sequencing of less than 6 samples. For additional information please refer to the low-plexity pooling guidelines from Illumina for the sequencing platform of your choice.

4.4 PCR Amplification

- Set up the following PCR program on a thermocycler with heated lid set at ≥ 100 °C:

Temperature	Duration	Ramp Rate	Cycles
98° C	45 sec	Standard	1
98° C	20 sec	2 °C per second	Check test amplification
65 °C	20 sec		
72 °C	60 sec		
72 °C	5 min	Standard	1
4 °C	Infinite	Standard	∞

- Transfer 10 µl of template cDNA to a new 0.2 ml tube and add 13.25 µl of PCR Buffer, 1.25 µl of one of the PCR Primer IDs, and finally 0.5 µl of PCR Enzyme as indicated below. Make sure to only use a single PCR Primer ID for each pool that is amplified.

	Volume per cDNA Pool
(Pooled) cDNA	10 µl
PCR Buffer	13.25 µl
PCR Primer ID N5xx	1.25 µl
PCR Enzyme	0.5 µl
Total Volume	25 µl

- Mix carefully by vortexing or pipetting and briefly spin down.

- Incubate the 0.2 ml tube in a PCR cycler as indicated at the top of this page.

4.5 SPRI bead clean-up of amplified cDNA

- Verify that the necessary equipment and reagents are available in sufficient amounts:
  - 100 µl SPRI beads for each PCR reaction
  - 400 µl 80% ethanol (molecular biology grade) for each PCR reaction
  - 1.5 ml low-binding tubes
  - Magnetic stand

- If samples were pooled prior to PCR, combine all PCR reactions that were split into more than one 25 µl PCR reaction in a 1.5 ml low-binding tube (be careful to only mix identical PCR Primer IDs). Adjust the volume of SPRI beads accordingly for a ratio of 1 to 0.9 (e.g. 90 µl SPRI beads for 100 µl PCR reaction after pooling).

- Remove the SPRI select beads from storage, vortex the tube thoroughly (min. 30 seconds), aliquot an appropriate amount, and let stand for at least 30 minutes before proceeding with the protocol.

- Add the according amount of SPRI beads to each tube and mix thoroughly by pipetting 10 times up and down or vortexing (approx. 10 seconds).

- Incubate for 2 minutes at room temperature.

- Place on a magnetic stand until the liquid becomes clear (up to 5 minutes).

- Remove and discard the supernatant.

- Add 180 µl of 80% EtOH without disturbing the beads. Do not remove the tubes from the magnetic stand.

- Incubate for 30 seconds on the magnet, then remove and discard the supernatant.

- Repeat the last two steps for a total of two washes with 180 µl of 80% EtOH.

- Briefly centrifuge the tube to collect all the remaining EtOH on the bottom of the tube and place it back on the magnetic stand.

- Remove remaining ethanol with a 10 µl pipette tip and air-dry the beads for a couple of minutes on the magnetic stand (cap must be open).

- Take the tube from the magnetic stand, add 50 µl of TE buffer (supplied with the Kit), and mix thoroughly by pipetting 10 times up and down or vortexing.

- Incubate for 2 minutes at room temperature.
- Place the tube on the magnetic stand for 2-5 min (until the liquid becomes clear) and transfer the eluate to a new 1.5 ml low-binding tube.

  Repeat the whole purification with the freshly eluted 50 µl volume:

- Add 45 µl SPRI beads to each reaction and mix thoroughly by pipetting 10 times up and down or vortexing (approx. 10 seconds).
- Incubate for 2 minutes at room temperature.
- Place on a magnetic stand until the liquid becomes clear (up to 5 minutes).
- Remove and discard the supernatant.
- Add 180 µl of 80% EtOH without disturbing the beads. Do not remove the tubes from the magnetic stand.
- Incubate for 30 seconds on the magnet, then remove and discard the supernatant.
- Repeat the last two steps for a total of two washes with 180 µl of 80% EtOH.
- Briefly centrifuge the tube to collect all the remaining EtOH on the bottom of the tube and place it back on the magnetic stand.
- Remove remaining ethanol with a 10 µl pipette tip and air-dry the beads for a couple of minutes on the magnetic stand (cap must be open).
- Take the tube from the magnetic stand, add 20 µl of TE buffer (supplied with the Kit), and mix thoroughly by pipetting 10 times up and down or vortexing.
- Incubate for 2 minutes at room temperature.
- Place the tube on the magnetic stand for 2-5 minutes (until the liquid becomes clear) and transfer the eluate to a new 1.5 ml low-binding tube. Make sure not to transfer any beads. In case that beads entered the pipette tip, push the entire volume back into the tube within the magnetic stand. Try again, after a short incubation (< 1 minute) using a new pipette tip.

4.6 Quality Control and Sequencing

- Assess the concentration of the library pool by fluorometric quantitation (DNA Assay) using 2 µl of the 20 µl elution volume. In case that the amplified cDNA library is not sufficiently concentrated for sequencing, use a vacuum concentrator (SpeedVac) to evaporate the excess volume. If the total amount of amplified cDNA library is insufficient, repeat PCR amplification as indicated in Appendix A.

- Check the size distribution of the cDNA library with an automated microfluidic electrophoresis station using 1-2 µl of the 20 µl elution volume and compare it to the examples given in Fig. 4. In case that additional peaks below 150 base pairs are visible, repeat the SPRI bead purification as indicated in Appendix B.

- In case that both quality and quantity of the library meet the necessary specifications, proceed with sequencing.

- Sequencing should be performed with single-end reads of 50-100 base pairs and dual-indexing reads. This ensures accurate demultiplexing of multiple libraries on a single sequencing lane. Fragments are sequenced in 5’ to 3’ direction, i.e. towards the poly-A-tail of the original mRNA. However, paired-end sequencing is also possible if the library pool is to be combined with other library preparation techniques such as RNA-Seq for sequencing. In this case, paired-end reads can contain poly-A tails at the beginning, which might affect the sequencing quality of ‘Read 2’ due to low complexity.

- The present version of the MACE Kit incorporates 8 base pair TrueQuant adapters that are sequenced together with the ‘index 1 read’ (i7 index). For that purpose, sequencing of the i7 index has to be manually adjusted to 16 cycles instead of 8 cycles. Additionally, these cycles have to be subtracted from sequencing of ‘read 1’. Do not change settings for sequencing of the ‘index 2 read’ (i5 index).

  For example, use 16 sequencing cycles for ‘index read 1’, 8 sequencing cycles for ‘index read 2’, and 68 sequencing cycles for ‘read 1’ for a 75 base pair sequencing Kit from Illumina. During bioinformatic analysis, reads with identical TrueQuant barcodes and cDNA inserts are discarded except for one, which is subsequently counted as a single read for quantification and normalization of the libraries.

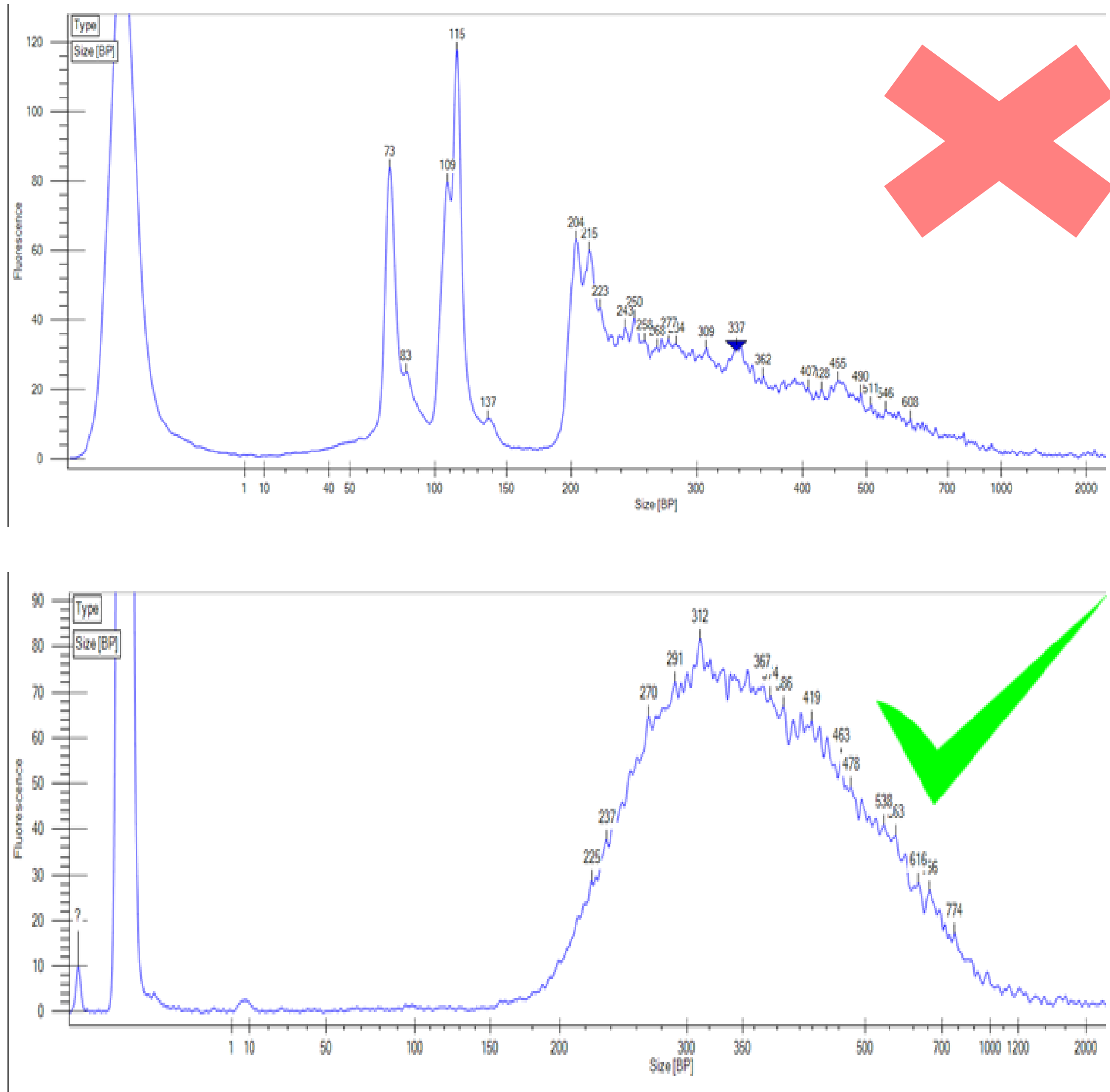

Fig. 4: Reference electropherogram of a library pool before (top) and after (bottom) final purification in step 4.7. The amplified library pool should ideally comprise a range from approximately 180 up to 800 (and more) base pairs, while the main peak is located around 350 base pairs. Additional peaks around 70 and/or 110 base pairs represent PCR primers and primer-dimers that negatively affect sequencing performance.

5 Bioinformatics

Analysis of the raw sequencing data can be done on our servers. We developed an optimized analysis pipeline for MACE libraries that takes care of all the necessary processing steps (Fig. 5), and which outputs the data via our easily accessible web interface.

To analyze your data, login at http://tools.genxpro.net and upload the .bcl or .fastq files via the Data Uploader to our servers.

As soon as the analysis is complete you will be notified by email. The ready-to-use results are available in the Data Center (also accessible with the above mentioned link) in form of Excel-compatible and tab-delimited tables as well as plots of your data and Gene Ontology enrichment analyses.

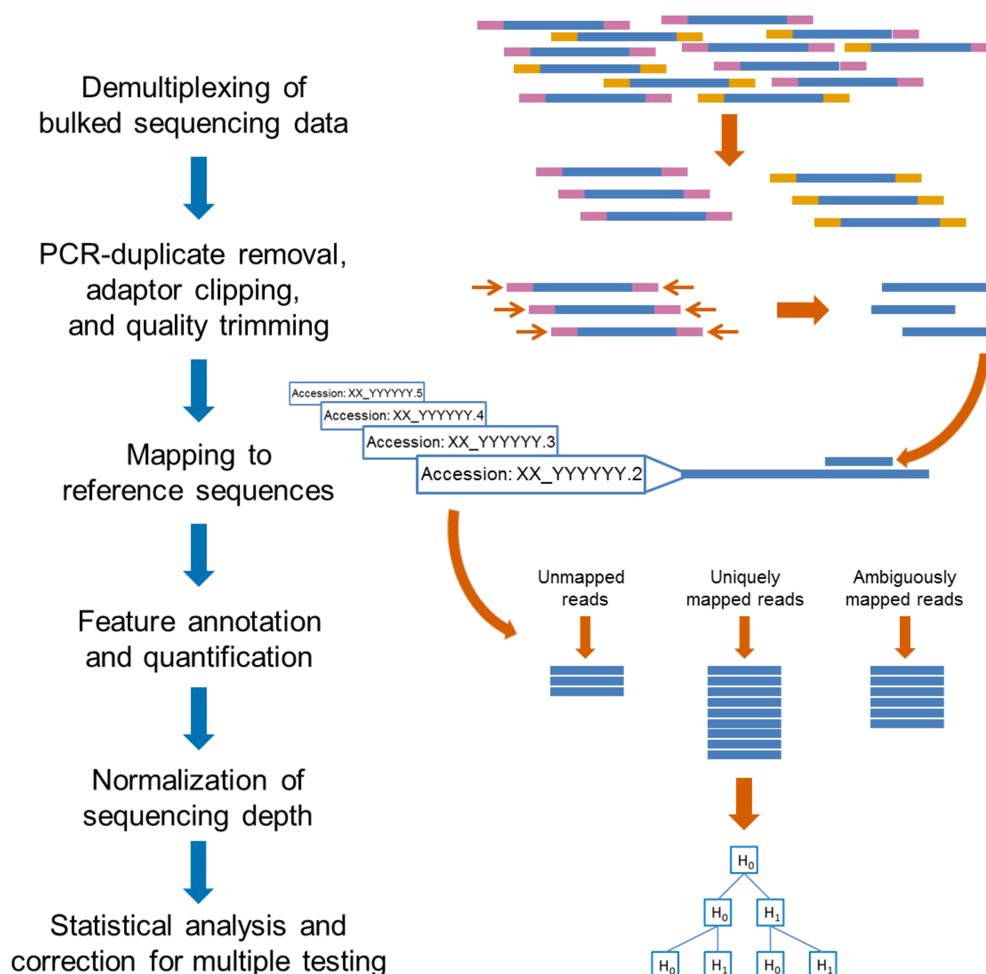

Fig. 5: Raw sequencing data is demultiplexed according to distinct barcodes and the sorted reads are trimmed for adapter-free, high-quality sequences. The pre-processed reads are subsequently mapped to the designated reference with Bowtie 2 and annotated with corresponding features. Following quantification, the read numbers of unambiguously mapped reads are normalized to account for different sequencing depths. Statistical analysis takes into account both the normalized and original read numbers. Statistical significance of differentially expressed genes is additionally adjusted for multiple testing.

Appendix A - Additional PCR Amplification

The ready-to-sequence library can be re-amplified with additional cycles (depending on the amount that is lacking for sequencing).

- Transfer 10 µl of the remainder of the amplified library to a new 0.2 ml tube and fill up the volume to 10 µl using nuclease-free water (supplied with the Kit) if necessary.

- Add 13.25 µl of PCR Buffer, 1.25 µl of PCR Primer ID, and 0.5 µl of PCR Enzyme as indicated below. Make sure to **use the very same PCR Primer ID** that was used in the previous amplification.

	Volume per cDNA Pool
Purified cDNA with water	10 µl
PCR Buffer	13.25 µl
PCR Primer ID N5xx	1.25 µl
PCR Enzyme	0.5 µl
Total Volume	25 µl

- Mix carefully by vortexing or pipetting and briefly spin down.

- Incubate the 0.2 ml tube in a PCR cycler as indicated below.

Temperature	Duration	Ramp Rate	No. of cycles
98° C	45 sec	Standard	1
98° C	20 sec	2 °C per second	2 to 6
65 °C	20 sec		
72 °C	60 sec		
72 °C	5 min	Standard	1
4 °C	Infinite	Standard	∞

- Following re-amplification**, proceed with step 4.6** of the protocol to purify the sequencing library again.

Appendix B - Additional SPRI bead purification of the amplified library pool

- Verify that the necessary equipment and reagents are available in sufficient amounts:
  - 50 µl SPRI beads for each pool
  - 400 µl 80% ethanol (molecular biology grade) for each pool
  - 1.5 ml low-binding tubes
  - Magnetic stand

- Remove SPRI beads from storage (4 °C) and equilibrate to room temperature. All SPRI bead clean-up steps are performed at room temperature. Mix the beads thoroughly by vortexing for 30 seconds before use.
- Fill up the volume of the amplified sequencing library to 50 µl with nuclease-free water (supplied with the Kit).
- Add 42.5 µl SPRI beads to the amplified library pool (0.85-fold volume of beads).
- Pipette the entire volume up and down 10 times or vortex to mix thoroughly.
- Incubate for 2 minutes at room temperature.
- Place the tube on the magnetic stand for up to 5 minutes (until the liquid becomes clear).
- Carefully remove and discard the supernatant. Take care not to disturb the beads.
- Do not remove the tube from the magnetic stand and add 180 µl of 80% ethanol without disturbing the beads.
- Incubate 30 seconds, then carefully remove and discard the supernatant. Take care not to disturb the beads.
- Repeat the last two steps for a total of two washes.
- Briefly centrifuge the tube to collect the remaining ethanol at the bottom of the tube and place it back on the magnetic stand.
- Remove the remaining ethanol with a 10 µl pipette tip and air-dry the beads for up to 5 minutes on the magnetic stand (leave the cap open).
- Take the tube from the magnetic stand and add 20 µl TE buffer (supplied with the Kit). Pipette the entire volume up and down 10 times or vortex to mix thoroughly. Ensure that the beads are completely rehydrated and resuspended.
- Incubate for 2 minutes at room temperature.
- Place the tube on the magnetic stand for 2-5 minutes (until the liquid becomes completely clear) and transfer the supernatant to a new 1.5ml low-binding tube. Make sure not to transfer any beads. In case that beads entered the pipette tip, push the entire volume back into the tube within the magnetic stand. Try again, after a short incubation (< 1 minute) using a new pipette tip.

- **Proceed with step 4.6** of the protocol in order to check the quality and concentration of the sequencing library again.

Appendix C - Index sequences

Rapid MACE allows for dual-indexing with 8 base pair indices. The index read 1 (i.e. p7 index) comprises up to 8 indices, and index read 2 (i.e. p5 index) up to 16 indices. The corresponding index for each of the IDs is listed in the following table.

RT Primer ID	Index Read 1 Sequence (sample and molecular barcode)
N701	TAAGGCGANNNNNNNN
N702	CGTACTAGNNNNNNNN
N703	AGGCAGAANNNNNNNN
N704	TCCTGAGCNNNNNNNN
N705	GGACTCCTNNNNNNNN
N706	TAGGCATGNNNNNNNN
N707	CTCTCTACNNNNNNNN
N710	CGAGGCTGNNNNNNNN

PCR Primer ID	Index Read 2 Sequence (second sample barcode)
N502	ATAGAGAG
N503	AGAGGATA
N505	CTCCTTAC
N506	TATGCAGT
N507	TACTCCTT
N508	AGGCTTAG
N510	ATTAGACG
N511	CGGAGAGA
N513	CTAGTCGA
N515	AGCTAGAA
N516	ACTCTAGG
N517	TCTTACGC
N518	CTTAATAG
N520	ATAGCCTT
N521	TAAGGCTC
N522	TCGCATAA

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