Rapid Massive Analysis of 3' cDNA Ends (MACE)
with Unique Molecular Identifiers (UMIs)
and Unique Dual Indicies (UDIs)

User Guide

Library Preparation Kit for Illumina Sequencing

GenXPro GmbH
info@genxpro.de
www.genxpro.de
+49(0)69-95739710

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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 (Figure 1). The resulting reduction in complexity of the profiled transcriptomes strongly decreases the required sequencing depth for reliable gene quantification including of lowly abundant transcripts such as transcription factors and receptors. 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.

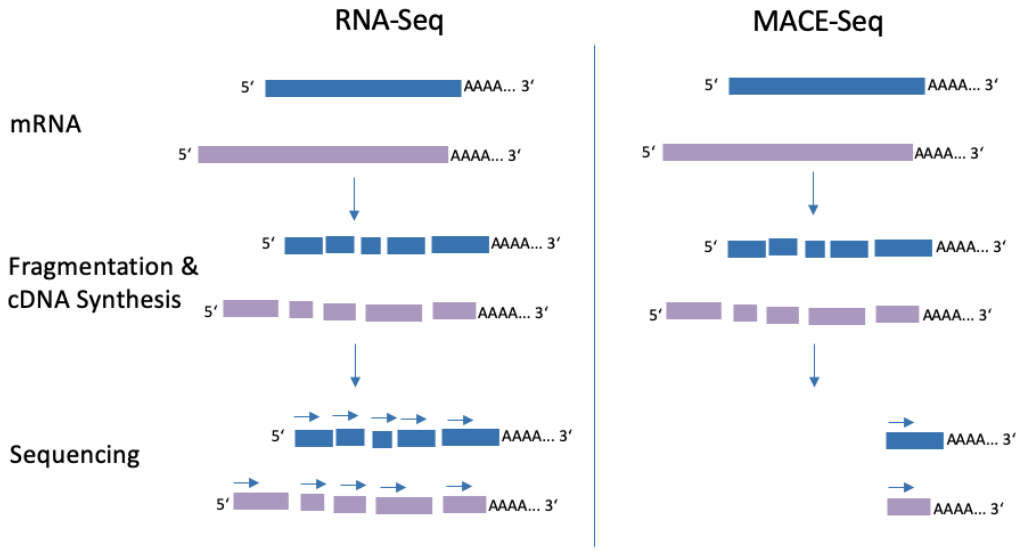

Figure 1: Comparison of profile patterns from libraries prepared by MACE and RNA-Seq. MACE generates one read derived from the last hundreds of nucleotides from each polyadenylated transcript, while RNA-Seq yields numerous reads per transcript that originate across the entire length of a given transcript. The read count for MACE equals the copy number of a profiled transcript, Overlapping MACE reads from different copies of the same transcript can be assembled to longer contigs, for example for non-model organisms. ,. 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 unique molecular identifiers (UMIs) 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 Unique Dual Index

The Unique Dual Index (UDI) concept for library preparation involves the incorporation of unique barcodes (indices) flanking each end of the cDNA tag, to uniquely label each sample twice (Figure 2). This approach addresses the challenges of sample misidentification by index hopping, where the barcode from one sample can be erroneously transferred to another. Together with our patened TrueQuant technology, the Unique Dual Index ensures reliable and precise sample tracking throughout the sequencing workflow.

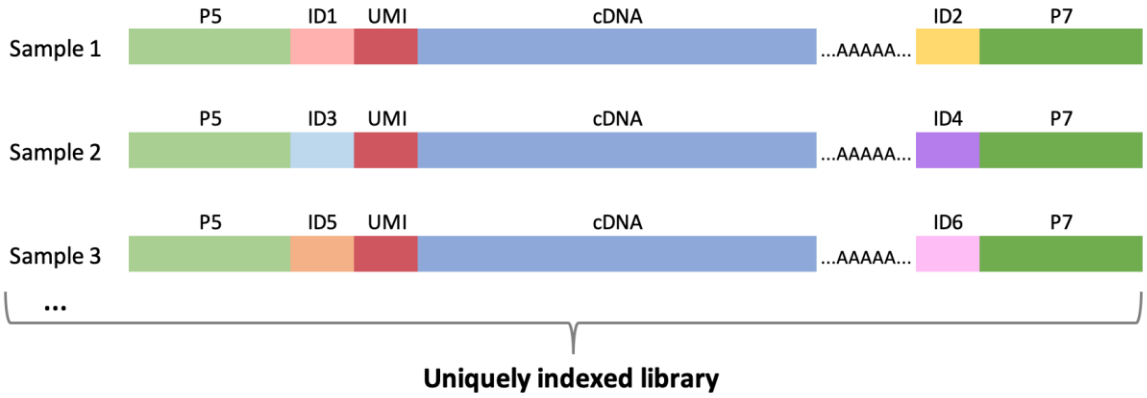

Figure 2: Schematic representation of the uniquely indexed library prepared by Rapid MACE with Unique Dual Index Kit. Each of the library samples incorporates two unique IDs, ensuring the accurate sample tracking.

1.4 Library Preparation Workflow Chart

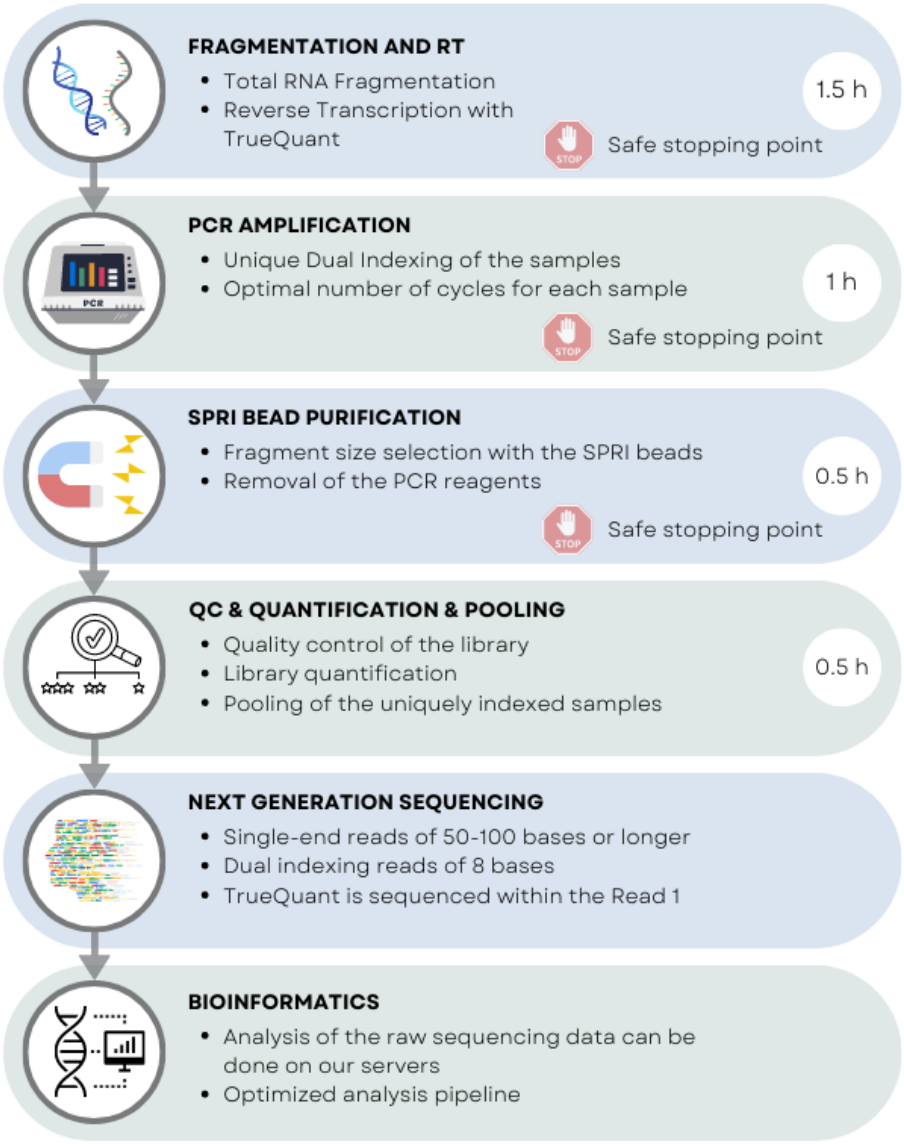

Figure 3: Library preparation workflow for rapid MACE with UDIs. Starting with total RNA, the complete protocol can be carried out in a few hours. The workflow starts with individual fragmentation and reverse transcription of each sample, followed by indexing-PCR, amplification and SPRI beads purification. After pooling, the indexed libraries can be sequenced on any of Illumina's platforms.

2 Kit Contents[1]

For all reactions	Nuclease-free Water
	TE Buffer
Reverse Transcription	Universal RT Primer
	RT Buffer
	RT Enzyme
	PrePCR Enzyme
	PrePCR Enhancer
PCR Amplification	PCR Buffer
	PCR Enzyme
	PCR Primer ID Mix (1-96)[2]
qPCR Amplification (optional)[3]	qPCR Buffer Mix
	qPCR Enzyme

[1] Store all reagents at -20 °C. Briefly spin down the contents of the tubes prior to first use.
[2] The number of the PCR Primer ID Mixes supplied varies depending on the Kit size.
[3] qPCR Supplementary Kit can be extra provided by request.

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)
Fluorometric RNA Quantification	RNA High Sensitivity Assay (Qubit)
	QuantiFluor RNA Assay (Quantus)
Fluorometric DNA Quantification	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
85 % Ethanol	General laboratory supplier
Pipette Filter Tips (nuclease-free)	General laboratory supplier
Universal Human Reference RNA (UHRR)	Optional positive control (Agilent)

4 Protocol

- All total RNA isolates should be DNase-treated on-column or preferably in solution before starting library preparation. Make sure that the RNA is dissolved in pure water.

- Assess the RNA quality (RIN / RQN / or similar) of each isolate with an automated microfluidic electrophoresis station and determine the concentration of all isolates by fluorometric quantification (RNA assay). The recommended quantity for reverse transcription is 10-100 ng[4] of total RNA from each isolate (and optional positive control).

4.1 RNA Fragmentation and Reverse Transcription

- Set up the following RT program on a thermocycler as described in Table 1.

Temperature	Duration	Ramp Rate
Lid: 110 °C	-	Standard
94 °C	8 min	Standard
70 °C	1 min	Standard
Lid: 70 °C	-	Standard
42 °C	Hold until addition of RT Master Mix	0.1 °C per second
42 °C	60 min	Standard
70 °C	15 min	Standard
37 °C	Hold until addition of PrePCR Enzyme	Standard
37 °C	15 min	Standard
80 °C	3 min	Standard
4 °C	Forever	Standard

Table 1. RT program

[4] Input range could be broadened from 0.03 ng to 250 ng of total RNA.

- On ice, transfer up to 8 µl of each RNA sample 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 Universal RT Primer as indicated in Table 2.

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

Table 2.

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

- Prepare the RT MasterMix at room temperature as indicated in Table 3. Include sufficient spare volume to account for pipetting inaccuracies.

	Volume per Sample (x)	Example for x = 6 samples (x times volume + 10% spare volume)
RT Buffer	8.75 µl	57.75 µl
RT Enzyme	1.25 µl	8.25 µl
Total Volume	10 µl	66 µl

Table 3. RT MasterMix

- Mix the RT MasterMix by pipetting or vortexing and briefly spin down.

- Place the individual 0.2 ml tubes (with the RNA sample and Universal RT Primer) in the thermocycler and start the RT program.

- When the thermocycler reaches 42 °C, place the prepared RT MasterMix in the thermocycler for 1 min for the pre-warming.

- Add 10 µl of the pre-warmed RT MasterMix to each of the sample tubes in the thermocycler after the pre-warming as indicated in Table 4. Pipette the RT MasterMix to the sample tubes without removing them from the termocycler.

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

Table 4.

- Mix carefully by pipetting or vortexing and briefly spin down. Immediately proceed with the incubation at 42 °C for 1 hour (included in the RT program).

- Following 15 minutes of incubation at 70 °C (RT inactivation), add 1 µl of the PrePCR Enzyme to each of the sample tubes as indicated in Table 5.

	Volume per Sample
Reaction Mix	20 µl
PrePCR Enzyme	1 µl
Total Volume	21 µl

Table 5.

- Mix carefully by pipetting or vortexing and briefly spin down. Immediately proceed with the incubation at 37 °C for 15 min.

- Following 3 minutes of incubation at 80 °C (PrePCR inactivation), take the sample tubes out of the thermocycler and add 2 µl of the PrePCR Enhancer to each of the sample tubes as indicated in Table 6. Mix carefully by pipetting or vortexing and briefly spin down.

	Volume per Sample
Reaction Mix	21 µl
PrePCR Enhancer	2 µl
Total Volume	23 µl

Table 6.

Safe stopping point: store your sample cDNA at -20 °C, or proceed directly with the PCR Amplification, or perform the qPCR Amplification first (optional) as described I Appendix A.

4.2 PCR Amplification

- Set up the PCR program on a thermocycler as described in Table 7:

Temperature	Duration	Ramp Rate	Cycles
Lid: 110 °C	-	Standard	-
98 °C	30 sec	Standard	1
98 °C	20 sec	2 °C per second	N*
65 °C	20 sec		
72 °C	60 sec		
72 °C	5 min	Standard	1
4 °C	Infinite	Standard	∞

Table 7.

* The number of cycles for PCR amplification may vary depending on sample specifics. We recommend to use our qPCR Amplification Supplementary Kit for precise determination of the PCR cycle number (Appendix A).

- Carefully vortex and spin down the tubes with the PCR Primer ID Mix.

- Prepare the PCR MasterMix for each sample in preparation as indicated in Table 8. Include sufficient spare volume to account for pipetting inaccuracies. Mix carefully by pipetting or vortexing and briefly spin down.

	Volume per Sample (x)	Example for x = 6 samples (x times volume + 10% spare volume)
PCR Buffer	38 µl	250.8 µl
PCR Enzyme	1 µl	6.6 µl
Total Volume	39 µl	257.4 µl

Table 8.

- Add 39 µl of the PCR MasterMix, 3 µl of the PCR Primer ID Mix X per each sample to a new 0.2 ml tube, and transfer 8 µl of sample cDNA as indicated in Table 9 (next page). Make sure to use only a unique PCR Primer ID Mix for each sample that is amplified.

	Volume per Sample
PCR Master Mix	39 µl
PCR Primer ID Mix X	3 µl
Sample cDNA	8 µl
Total Volume	50 µl

Table 9.

- Mix carefully by vortexing or pipetting and briefly spin down.
- Incubate the 0.2 ml tube in a thermocycler as described in Table 7.

Safe stopping point: store your amplified cDNA at -20 °C or proceed directly with the SPRI bead clean-up of the amplified cDNA.

4.3 SPRI bead clean-up of the amplified cDNA

- Verify that the necessary equipment and reagents are available in sufficient amounts:
  - 87.5 µl SPRI beads for each PCR reaction
  - 720 µl 80% ethanol (molecular biology grade) for each PCR reaction
  - 1.5 ml low-binding tubes
  - Magnetic stand
- 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 45 µl of SPRI beads to each tube with the 50 µl amplified library (0.9-fold volume of beads) and mix thoroughly by pipetting 10 times up and down or vortexing (approx. 10 seconds).
- Incubate for 2 minutes at room temperature.
- Place the tubes 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.

- Add 180 µl of 85% ethanol without disturbing the beads. Do not remove the tubes from the magnetic stand.
- 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 with 180 µl of 85% ethanol.
- Briefly centrifuge the tube to collect all the remaining ethanol on 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 tubes 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. Ensure that the beads are completely rehydrated and resuspended.
- Incubate for 2 minutes at room temperature.
- Place the tubes on the magnetic stand for 2-5 minutes (until the liquid becomes completely 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.

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

- Add 42.5 µl SPRI beads to each tube with the freshly eluted 50 µl library (0.85-fold volume of beads) and mix thoroughly by pipetting 10 times up and down or vortexing (approx. 10 seconds).
- Incubate for 2 minutes at room temperature.
- Place the tubes 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.
- Add 180 µl of 85% ethanol without disturbing the beads. Do not remove the tubes from the magnetic stand.
- 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 with 200 µl of 85% ethanol.
- Briefly centrifuge the tubes to collect all the remaining ethanol on 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 tubes 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. Ensure that the beads are completely rehydrated and resuspended.
- Incubate for 2 minutes at room temperature.
- Place the tubes on the magnetic stand for 2-5 minutes (until the liquid becomes completely 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.

4.4 Quality Control and Sequencing

- Assess the concentration of the library pool by fluorometric quantification (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. An extra PCR Supplementary Kit for the additional PCR amplification (Appendix B) can be also provided by request.

- 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 Figure 4. In case that additional peaks below 150 base pairs are visible, repeat the SPRI bead purification as indicated in Appendix C.

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

- Calculate molar DNA concentration, based on the size of the DNA library as determined by the electrophoresis trace. Normalize all libraries to a DNA concentration of 5 nM. Aliquot 5 µl of diluted DNA from each library and mix aliquots for pooling all libraries with unique indices. Proceed with the sequencing.

- Sequencing should be performed with single-end reads of 50-100 bases or longer and dual-indexing reads of 8 bases. The TrueQuant is sequenced within the Read 1 (the first 8 bases). 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.

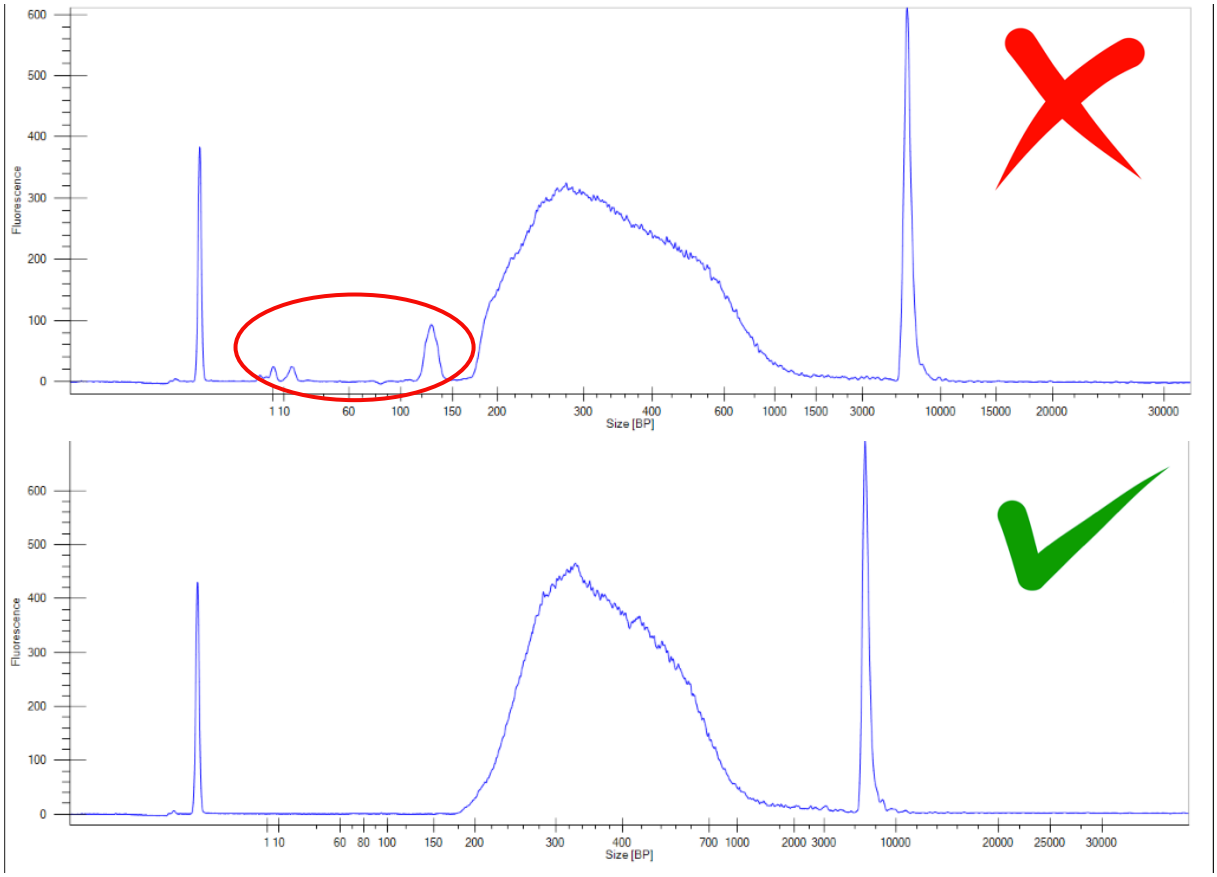

Figure 4: Reference electropherogram of a library pool before (top) and after (bottom) the final purification. The amplified library pool should comprise a range from approximately 200 up to 800 (and more) base pairs, while the main peak is located around 350 base pairs. Additional peaks below 150 base pairs represent PCR primers and primer-dimers that negatively affect the sequencing performance.

5 Bioinformatics

The raw data can optionally be analysed on our cloud-service tool for NGS data analyses. Our optimized analysis pipeline for MACE libraries takes care of all the necessary processing steps (Figure 5) and includes data preprocessing (demultiplexing, clean, filter, and quality-check of the data); alignment to the reference genome of choice, transcript- or gene quantification and differential gene expression analyses of predefined groups of samples. The results tables, qualitative and quantitative data as well as numerous scatter plots and PCA are readily accessible via our web-interface.

To analyze your data you may login at 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.

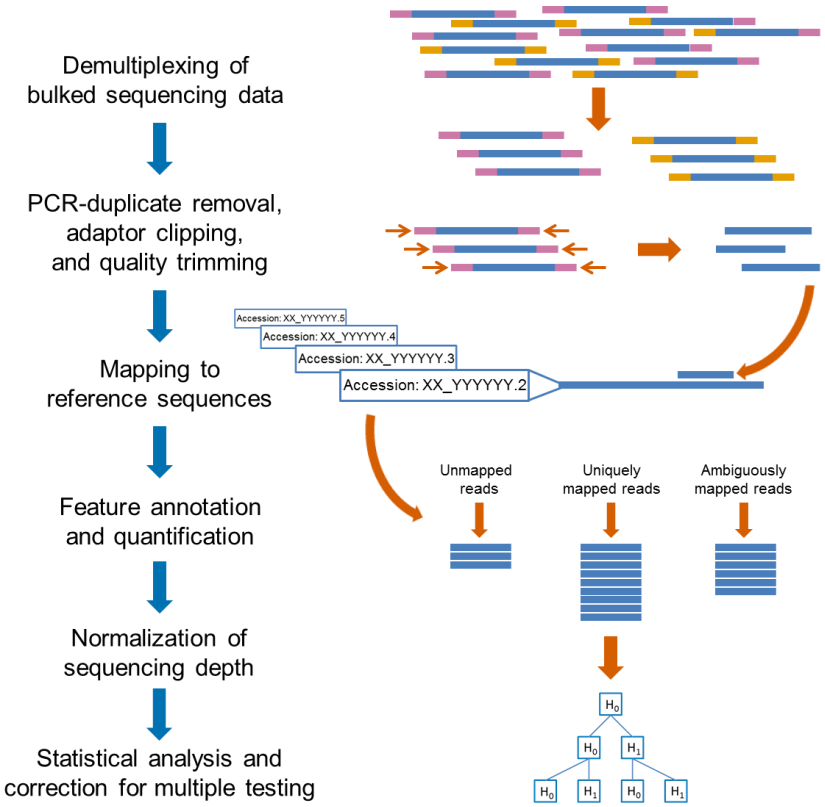

Figure 5: Raw sequencing data is demultiplexed; the reads are trimmed to obtain high-quality sequences, mapped to the designated reference genome or transcriptome and annotated with corresponding features. Following quantification, the read numbers of unambiguously mapped reads are normalized to account for different sequencing depths or overrepresented transcripts. Statistical significance of differentially expressed genes is determined and additionally adjusted for multiple testing. Furhter information is available at https://genxpro.net/bioinformatics.

Appendix A – qPCR Amplification

For the precise determination of the cycle number for each sample in preparation we recommend to use our qPCR Supplementary Kit. qPCR amplification ensures that the sample cDNA is amplified with the lowest possible cycle number for sequencing. For that purpose, a small fraction of reverse-transcribed cDNA is used as a template for quantitative real-time PCR (qPCR).

- Set up the program on a qPCR cycler (without adding a melt curve) as described in Table 10.

Temperature	Duration	Ramp Rate	Cycles
Lid: 110 °C	-	Standard	-
98 °C	30 sec	Standard	1
98° C	20 sec	2 °C per second	30
65 °C	20 sec		
72 °C	60 sec		

Table 10.

- Prepare the qPCR MasterMix for each sample in preparation as indicated in Table 11. Note that the qPCR Buffer Mix is light sensitive, make sure it is not exposed to light for too long while pipetting.

	Volume per Sample (x)	Example for x = 6 samples (x times volume + 10% spare volume)
qPCR Buffer Mix	17.6 µl	116.16 µl
qPCR Enzyme	0.4 µl	2.64 µl
Total Volume	18 µl	118.8 µl

Table 11.

- Mix carefully by pipetting or vortexing and briefly spin down.
- Add 18 µl of the qPCR MasterMix and 2 µl of sample cDNA to a new 0.2 ml tube as indicated in Table 12.

	Volume per Sample
Sample cDNA	2 µl
qPCR MasterMix	18 µl
Total Volume	20 µl

Table 12.

- Incubate the 0.2 ml tube or plate using the qPCR program described in Table 10.

- 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 (Figure 6).

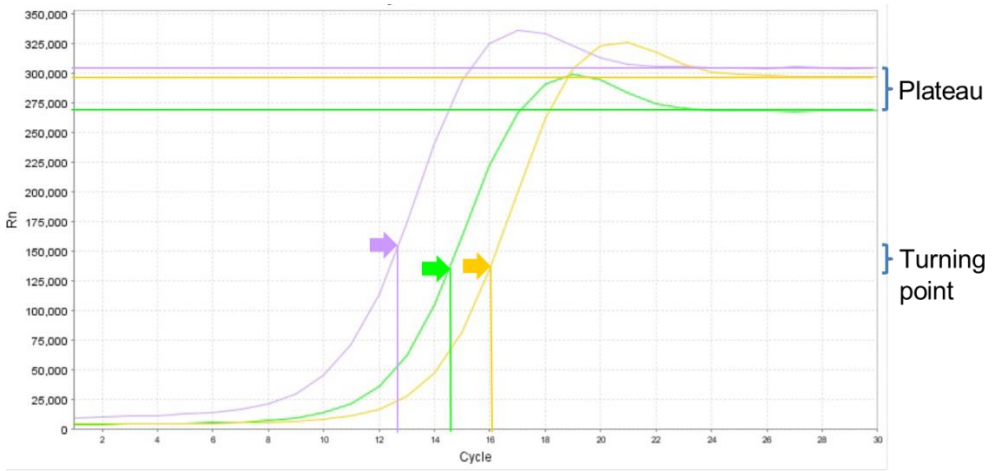

Figure 6: Quantitative PCR plots of 3 distinct cDNAs (purple, green, and yellow). The optimal cycle number for PCR amplification of the respective cDNA equals the cycle number of the turning point minus 2 cycles. The turning point for each cDNA pool (approximated as half of the fluorescence level of 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.

Appendix B – Additional PCR Amplification

If the total amount of amplified cDNA library is insufficient, the ready-to-sequence library can be re-amplified using our PCR Supplementary Kit.

- Set up the PCR program on a thermocycler as described in Table 13:

Temperature	Duration	Ramp Rate	Cycles
Lid: 110 °C	-	Standard	-
98 °C	30 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	∞

Table 13.

- Prepare the PCR MasterMix for each sample as indicated in Table 14. Include sufficient spare volume to account for pipetting inaccuracies. Mix carefully by pipetting or vortexing and briefly spin down.

	Volume per Sample (x)	Example for x = 6 samples (x times volume + 10% spare volume)
PCR Buffer	38 µl	250.8 µl
PCR Enzyme	1 µl	6.6 µl
Total Volume	39 µl	257.4 µl

Table 14.

- Transfer 8 µl of the sample after the SPRI bead clean-up to a new 0.2 ml tube and fill up the volume to 8 µl using nuclease-free water (supplied with the Kit) if necessary.

- Add 39 µl of the PCR MasterMix and 3µl of the PCR Primer ID Mix X to 8 µl of sample as indicated in Table 15. Make sure to use the very same PCR Primer ID that was used in the previous amplification.

	Volume per Sample
Sample after SPRI bead clean-up	8 µl
PCR MasterMix	39 µl
PCR Primer ID Mix X	3 µl
Total Volume	50 µl

Table 15.

- Mix carefully by vortexing or pipetting and briefly spin down.
- Incubate the 0.2 ml tube in a thermocycler as described in Table 13.
- Following the re-amplification, proceed with the step 4.3 of the protocol to clean-up the sequencing library again.

Appendix C – Additional SPRI bead clean-up

- Verify that the necessary equipment and reagents are available in sufficient amounts:
    - 42.5 µl SPRI beads for each pool
    - 360 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 each tube (0.85-fold volume of beads) and mix thoroughly by pipetting 10 times up and down or vortexing (approx. 10 seconds).
- Incubate for 2 minutes at room temperature.
- Place the tubes 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.
- Add 180 µl of 85% ethanol without disturbing the beads. Do not remove the tubes from the magnetic stand.
- 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 with 180 µl of 85% ethanol.
- Briefly centrifuge the tubes to collect all the remaining ethanol on 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 tubes 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. Ensure that the beads are completely rehydrated and resuspended.
- Incubate for 2 minutes at room temperature.
- Place the tubes on the magnetic stand for 2-5 minutes (until the liquid becomes completely 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.

- Proceed with step 4.4 of the protocol in order to check the quality and concentration of the sequencing library again.

Appendix D – Index sequences

Rapid MACE allows for unique dual-indexing with 8 base indices. The corresponding Read 1 and Read 2 index sequences for each of the PCR Primer IDs are listed in the following table.

PCR Primer ID Mix	Index Read 1 sequence	Index Read 2 sequence
1	CTGATCGT	AGGCTATA
2	ACTCTCGA	GCCTCTAT
3	TGAGCTAG	AGGATAGG
4	GAGACGAT	TCAGAGCC
5	CTTGTCGA	CTTCGCCT
6	TTCCAAGG	TAAGATTA
7	CGCATGAT	ACGTCCTG
8	ACGGAACA	GTCAGTAC
9	CGGCTAAT	ATAGAGAG
10	ATCGATCG	AGAGGATA
11	GCAAGATC	CTCCTTAC
12	GCTATCCT	TATGCAGT
13	TACGCTAC	TACTCCTT
14	TGGACTCT	AGGCTTAG
15	AGAGTAGC	ATTAGACG
16	ATCCAGAG	CGGAGAGA
17	GACGATCT	CTAGTCGA
18	AACTGAGC	AGCTAGAA
19	CTTAGGAC	ACTCTAGG
20	GTGCCATA	TCTTACGC
21	GAATCCGA	CTTAATAG
22	TCGCTGTT	ATAGCCTT
23	TTCGTTGG	TAAGGCTC
24	AAGCACTG	TCGCATAA

Table 12. PCR Primer IDs with Illumina Index Read 1/2 Sequences. Note: Index Read 2 Sequence corresponds to the Reverse Complement Workflow.

Notes

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GenXPro GmbH

Altenhöferallee 3

60438 Frankfurt am Main

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