MI
LABORATORY

MOUSE α/β TCR RNA KIT

MOUSE TCR α AND β REPERTOIRES PROFILING

User Manual v.2.0

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KIT OVERVIEW

The **Mouse α/β TCR RNA Kit** is designed for unbiased amplification of TCR alpha and beta cDNA libraries using 5'RACE (**R**apid **A**mplification of **c**DNA **E**nds) with unique molecular identifiers (UMI) incorporated within template switch oligo. 5'RACE UMI technique allows for advanced PCR and sequencing error correction, exact quantification of template cDNA molecules, and accurate normalization of samples for comparisons of repertoire diversity metrics.

The kit includes a set of reagents sufficient to prepare 24 TCR alpha and 24 TCR beta cDNA libraries starting from 24 RNA samples.

The kit allows to start with RNA derived from 100 to 0.5 million sorted/purified T-cells, from peripheral blood leukocytes, or from T cell-containing tissues (see **Appendixes A** and **B** for recommended RNA isolation procedures), and produces indexed ready-to-sequence-on-Illumina® libraries. Up to 24 samples can be processed in parallel.

1st PCR amplification starts from the same cDNA sample and includes both TCR alpha and beta libraries in the same tube. 2nd PCR amplification starts from the product of 1st PCR and is performed separately for TCR alpha and beta chain repertoires (**Figure 1**).

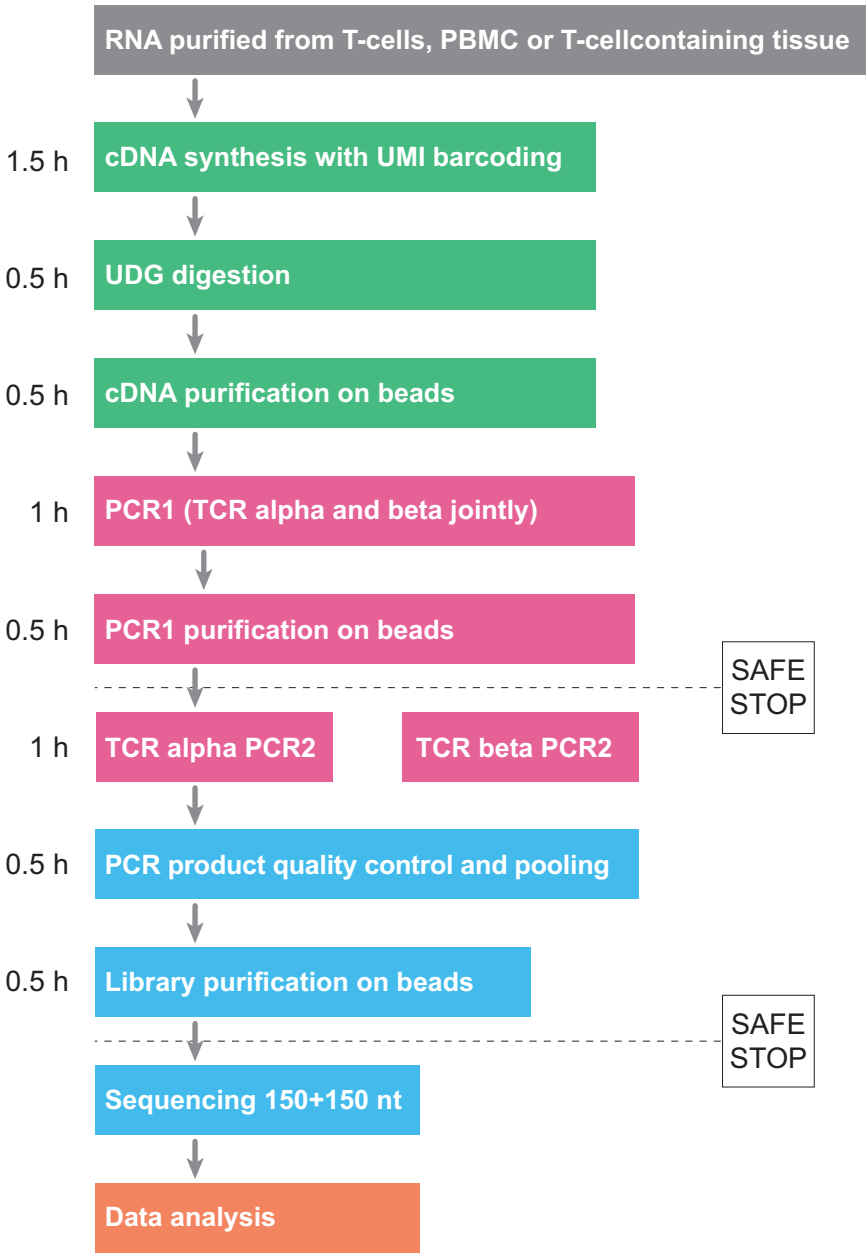

Figure 1. Mouse α/β TCR RNA kit pipeline.

KIT CONTENTS

Table 1.

Component	Water volume to add (µl)
NN oligo	60
TR mus synt	60
TR mus PCR1	60
Control RNA	50
TR-A mus PCR2	30
TR-B mus PCR2	30
Nuclease free water	–

MATERIALS REQUIRED BUT NOT INCLUDED

- ☐ SMARTScribe™ Reverse Transcriptase (Clontech, Takara, Inc. #639536)
- ☐ RNAsin® 40 U/µl (Promega, #N2515)
- ☐ dNTP mix (10mM each)
- ☐ Uracil-DNA Glycosylase (New England Biolabs, #M0280S)
- ☐ Q5® Hot Start High-Fidelity DNA Polymerase (New England Biolabs, #M0493L)
- ☐ AMPure® XP Beads (Beckman Coulter, Inc. #A63881) or SPRIselect® Reagent Kit (Beckman Coulter, Inc. #B23317)
- ☐ Thermal Cycler
- ☐ Appropriate Magnetic Rack
- ☐ Freshly prepared 80% Ethanol (1200 µl per sample)
- ☐ Nuclease free water
- ☐ Low-speed benchtop Mini-centrifuge/vortex
- ☐ Qubit® fluorometer and Qubit dsDNA HS Assay Kit
- ☐ Bioanalyzer or agarose gel electrophoresis system
- ☐ Elution Buffer /EB (10 mM Tris-HCl, pH 8.0–8.5)
- ☐ IDT for Illumina Nextera DNA Unique Dual Indexes or IDT for Illumina — DNA/RNA UD Indexes, Tagmentation (any of #20027213, #20027214, #20027215 or #20027216) (recommended)

or

- ☐ Nextera XT Index Kit (FC-131-1001) or Nextera XT Index Kit v2 (any of Set A, B, C and D, FC-131-2001, FC-131-2002, FC-131-2003, and FC-131-2004)

GENERAL RECOMMENDATIONS TO PREVENT CONTAMINATION

General recommendations to lower the risk of RNA degradation and contamination should be implemented such as using labcoats, gloves, tips with aerosol filters, certified RNAse/DNAse free reagents, performing of non-template control reaction.

To prevent cross-sample contamination library preparation should be performed in two separate workspaces (PCR boxes) located in different rooms: pre-PCR and post-PCR. The user should avoid transferring anything (reagents, pipets, racks etc.) from post-PCR to pre-PCR workspace. In the case of possible contamination, use decontaminating procedures with special reagents such as DNA-OFF™ (MP Biomedicals) and UV.

Perform cell isolation, RNA purification, cDNA synthesis and the 1st PCR preparation in the pre-PCR workspace (**Steps 1–8**). After the 1st PCR amplification transfer the tubes to the post-PCR workspace and perform all other steps (**10–19**) except for **Step 11**.

Perform UV decontamination each time before making up a PCR master-mix.

BEFORE YOU START

Add nuclease-free water to the tubes according to the **Table 1** in **Kit Content**. Mix by vortexing and spin down.

Make aliquots of NN-oligo depending on planned experimental setup. Each aliquot can be frozen/thawed no more than 4 times. Store all tubes at –20°C after dilution.

Add 50 µl of nuclease-free water to Control RNA. Incubate the tubes at room temperature for 10 min. Mix by vortexing and spin down. Store at –70°C.

STARTING MATERIAL

See **Appendixes A** and **B** for our recommendations on RNA isolation.

Verified minimal number of cells for the protocol is 100 T-cells sorted directly into Qiagen RLT® buffer, followed by RNA extraction with TRIzol® (**Appendix B**).

High RNA quality is often critical for the efficient library preparation.

Large amounts of gDNA may significantly affect cDNA synthesis when starting from large (>200,000 cells) samples. It is recommended to perform DNAse treatment and additional purification of RNA before start.

Use Qubit® fluorometer or other fluorescent based method to determine RNA concentration.

For a small amount of starting material (100–50,000 T-cells), preferably, do not use DNAse and take all the RNA extracted from the sample of interest for cDNA synthesis.

Do not use heparin coated tubes for blood collection. Heparin dramatically decreases cDNA synthesis efficiency.

PROTOCOL

NOTE: Perform steps 1–8 and step 11 in a pre-PCR box/room.

cDNA synthesis with template switch

1. In sterile reaction tube(s)/strip(s), mix the following reagents in a final reaction volume of 12 µl:

1–10 µl	RNA, up to 1000 ng per reaction*
2 µl	**TR Mus Synt**
0–9 µl	RNAse free water
12 µl	Total

* For control reaction, take 10 µl of control RNA.

2. Place the reaction tube(s)/strip(s)/plate into a thermal cycler and incubate for 2 minutes at 70°C, then keep the tubes at room temperature for at least 5 minutes while preparing Master Mix.

3. While incubating, prepare Master Mix of the following components in a final volume of 18 µl per reaction, plus 10% of the total reaction mix volume:

6 µl	cDNA Synthesis Buffer (Clontech, Takara)
3 µl	DTT (20 mM)
2 µl	NN oligo
3 µl	dNTPs (10 mM each)
1 µl	RNAsin® 40 U/µl
3 µl	SMARTScribe™ Reverse Transcriptase (Clontech, Takara)
18 µl	Total

NOTE: Add the Reverse Transcriptase to Master Mix just before use and mix gently by pipetting.

4. Add 18 µl of Master Mix to each reaction, pipette gently, spin briefly, and incubate for 60 minutes at 42°C.

5. Add 1 µl of Uracyl DNA glycosylase, gently mix by pipetting, and incubate for 30 minutes at 37°C.

NOTE: Uracyl DNA glycosylase treatment removes residual template switch oligo that is critical for the accurate labeling of starting cDNA molecules.

6. Purify obtained cDNA products using AMPure® XP Beads (Beckman Coulter) according to manufacturer protocol. Use 1:1 sample: AMPure® XP Beads ratio.

NOTE: To reduce drying time get out the tubes from the magnetic rack after second ethanol washing. Briefly spin the tubes to drop down all the remaining liquid. Place the tubes back on the magnetic rack, make sure there are no beads left in the supernatant and remove remaining liquid with a pipette.

Elute with 13 µl EB (10 mM Tris-HCl, pH 8.0–8.5) to get 12 µl of purified cDNA.

During the elution step, AMPure beads clumping may significantly reduce the cDNA yield, which is critical at the cDNA purification step. To maximize the cDNA yield, we strongly recommend to break up the clumps using pipetting and heating at 50°C for 10–30 minutes until complete dissolving.

If the supernatant volume recovered is less than 12 µl for any reason, bring the volume up to 12 µl by adding EB.

• 1st PCR amplification

NOTE: Both alpha and beta TCR chains are amplified in the same first round PCR reaction.

7. In a sterile tube, prepare PCR1 Master Mix in a final volume of 38 µl per reaction:

24.5 µl	Nuclease free water
10 µl	Q5® polymerase buffer (5x, New England Biolabs)
1 µl	dNTPs (10 mM each)
2 µl	**TR mus PCR1**
0.5 µl	Q5® Hot Start High-Fidelity DNA Polymerase (New England Biolabs)
38 µl	Total

8. Add 38 µl of PCR1 Master Mix to each of 12 µl cDNA samples. Mix gently by pipetting.

9. Perform PCR using the following parameters:

98°C	30 s	
98°C	10 s	
60°C	20 s	15–21 cycles*
72°C	40 s	
72°C	2 min	
4°C	hold	

* Choose the number of cycles depending on the amount of RNA/ T-cell numbers at the start (**Table 2**).

NOTE: Proceed in the post-PCR box/room.

10. Purify the obtained PCR products using AMPure® XP Beads (Beckman Coulter) according to manufacturer protocol. Use 1 : 0.8 sample: AMPure® XP Beads ratio. Elute with 30 µl EB (10 mM Tris HCl, pH 8.0–8.5).

SAFE STOP POINT: Purified product of the 1st PCR can be stored up to 1 month at –20°C.

• 2nd PCR amplification

NOTE: At this point, the samples must be split and processed separately for TCR alpha and beta chain libraries. Therefore, from each of the 1st PCR reactions, you will obtain two libraries in the 2nd PCR: one for TCR alpha and one for TCR beta.

11. Dilute **TR-A mus PCR2** and **TR-B mus PCR2** with nuclease-free water 1 : 9 just before use. Discard diluted aliquots after use.

12. Prepare two separate PCR2 Master Mixes in a final volume of 22 µl per reaction.

For TCR alpha

15.25 µl	Nuclease free water
5 µl	Q5® polymerase buffer (5x, New England Biolabs)
0.5 µl	dNTPs (10 mM each)
1 µl	**TR-A mus PCR2 (diluted 1 : 9)**
0.25 µl	Q5® High-Fidelity DNA Polymerase (New England Biolabs)

For TCR beta

15.25 µl	Nuclease free water
5 µl	Q5® polymerase buffer (5x, New England Biolabs)
0.5 µl	dNTPs (10 mM each)
1 µl	**TR-B mus PCR2 (diluted 1 : 9)**
0.25 µl	Q5® High-Fidelity DNA Polymerase (New England Biolabs)

13. For each reaction, add 22 µl of PCR2 Master Mix to a nuclease-free 0.2 ml tube/strip. Two separate reactions volumes are required for TCR alpha and beta chain.

14. Add 2 µl of purified 1st PCR product to each TCR alpha and TCR beta chain reaction.

15a. If using **IDT for Illumina Nextera Unique Dual Indexing primers** (recommended):
Add 1 µl of IDT for Illumina Nextera DNA UD Index to each reaction. Use unique Index for each reaction.

15b. If using **Nextera XT Indexing primers**:
Add 1 µl of Nextera N70X and 1 µl of Nextera S50X primer to each reaction. Use unique Indexing primers combination for each reaction.

16. Perform PCR using the following parameters:

98°C	30 s	
98°C	10 s	
55°C	20 s	10–16 cycles*
72°C	40 s	
72°C	2 min	
4°C	hold	

* Choose the number of cycles depending on the amount of RNA/ T-cell numbers at the start (**Table 2**).

Table 2. Approximate number of PCR cycles and sequencing reads.

Estimated number of T-cells in a sample	Amount of RNA to start cDNA synthesis	Recommended number of 1st PCR cycles	Approximate number of 2nd PCR cycles	Recommended number of sequencing reads per sample
10^2 – $5x10^2$ T-cells	all	21	14–16	10^4
$5x10^2$ – $5x10^3$ T-cells	all			$5x10^4$
$5x10^3$ – $5x10^4$ T-cells	5–50 ng	18	12–14	$5x10^5$
$5x10^4$ – 10^5 T-cells	50–100 ng		11–12	10^6
10^5 – $5x10^5$ T-cells	100–500 ng	15	12–15	$5x10^6$
–	10 µl control RNA	18	10–11	–

NOTE: The optimal number of cycles may vary for different templates, cell types, thermal cyclers, and sample amounts. We recommend that you determine the minimal number of PCR

cycles required to obtain a sufficient amount (at least 30 ng of each library) empirically for each experiment. Furthermore, we recommend that you generate parallel libraries of similar nature (e.g., ten samples of 2,000–20,000 sorted T-cells each, TCR alpha and beta chains) using the same number of PCR cycles, and mix the obtained libraries in equal volume proportions for sequencing. This allows obtaining an even coverage in terms of reads-per-cDNA. For example, the library, which started from 50,000 T-cells may produce more PCR product than one which started with 20,000 T-cells after the same number of PCR cycles. However, the former library would also carry proportionally more TCR cDNA molecules, and thus requires more sequencing reads to achieve a comparable coverage.

17. Verify quality of the obtained PCR product by analyzing an aliquot of the sample alongside a DNA ladder on the agarose gel or capillary electrophoresis (e.g. BIOANALYZER® or TapeStation®, Agilent Technologies).The library should have a peak size at approximately 650 bp (**Figure 2**).

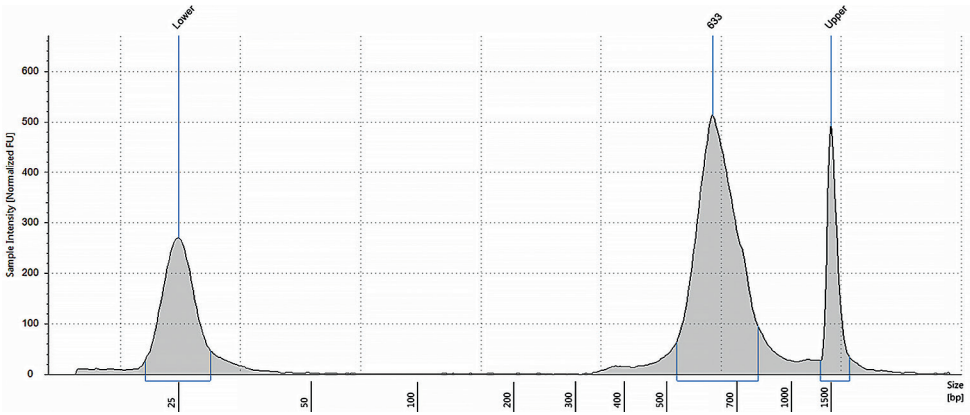

Figure 2. A typical library peak size. The shape and length distribution of a TCR library may vary depending on the repertoire composition.

18. Pool the obtained libraries (separate pools, one for TCR alpha and another one for TCR beta) by combining an equal volume portion of each individual sample from the 2nd PCR. The resulting amount of pooled PCR products should be at least 100 ng for each pool.

19. Purify up to 100 µl of pooled PCR product using AMPure® XP Beads (Beckman Coulter) according to manufacturer protocol. Use 1 : 0.8 sample: AMPure® XP Beads ratio. Elute with 30 µl EB (10 mM Tris HCl, pH 8.0–8.5).

20. Verify quality of obtained pooled libraries by analyzing the aliquot by agarose gel or capillary electrophoresis (e.g. BIOANALYZER® or TapeStation®, Agilent Technologies). In some cases non-specific amplification products shorter than 400 bp are detected along with normal TCR libraries 650-700 bp long. To remove these non-specific products repeat beads purification procedure using 1 : 0.7 sample: AMPure® XP Beads ratio.

SAFE STOP POINT: Store purified libraries at –20°C.

Sequencing recommendations

The obtained purified pooled library is ready for Illumina sequencing.

Determine library concentration according to the Illumina recommendations.

Spike with at least 25% of PhiX or another random library (e.g. RNA-Seq, Exome-Seq etc).

Analyze the resulting pooled library using at least 150+50 nt (for the high accuracy of V gene identification use 150+150 nt) paired end Illumina sequencing with standard Illumina® sequencing primers. 150+50 nt length is sufficient to cover the CDR3 region and UMI. However, the longer the reads are, the more accurate the V gene segments identification is afterwards. For comparative analysis of the obtained TCR repertoire data, use the same sequencing platform. Ideally, use the same sequencing run for the samples under comparison, or mix control and experimental samples in order to minimize batch effects.

Data analysis can be performed with MiGEC and MiXCR software for UMI group assembly and initial clonotype extraction from raw sequencing reads.

APPENDIX A: PREPARING STARTING MATERIAL

TCR cDNA libraries can be generated starting from RNA isolated from sorted/isolated T-cells, PBMCs or any tissue containing T-cells. Control for the counts of T-cells of interest in a sample is desirable for the downstream data analysis.

It is preferable to use freshly isolated cell sample. In the case of frozen samples, culture thawed cells overnight in RPMI-1640 supplemented with 10% mouse serum.

Lysed cells/tissues can be stored in RLT® buffer (QIAGEN) at –70°C for at least 6 months.

Large samples (>50,000 cells) can be also stored in TRIzol® at –70°C for up to 6 months.

For large samples (>50,000 cells): verify quantity and quality of extracted RNA, using QuBit and Agilent Bioanalyzer or gel electrophoresis. RNA Integrity Number > 7, or correct 28S rRNA:18S rRNA ratio (around 1.5–2.5 : 1) and a low number of shadow bands above and below 18S band are indicative of high quality RNA. However, it is often the case that medium quality RNA allows obtaining high quality TCR libraries.

For small samples (i.e. < 50,000 cells) it is not necessary to check quantity and quality of extracted RNA.

Degraded RNA may indicate that the samples were stored too long before processing, isolated RNA was stored at an incorrect temperature or RNAse contamination.

Carrier poly(A) RNA can be used during the RNA extraction procedure to increase the yield of isolated RNA. Carrier RNA will not interfere with primers for cDNA synthesis reaction.

Purified RNA can be stored in water or in 75% ethanol at –70°C. For small RNA amounts (less than 100 ng), it is preferable to start cDNA synthesis immediately after RNA extraction.

Further recommendations should help to choose the appropriate strategy for RNA isolation in most situations.

PBMC

Perform isolation of mononuclear cells from whole blood using Ficoll Paque density gradient centrifugation. Spin down the cells at 350g for 15 minutes, remove the supernatant.
Add at least 50 µl (or 50 µl per $2x10^5$ cells) of RLT® buffer and mix by pipetting for:

– storage at -70°C for up to 6 months, or

– RNA purification using RNeasy Micro or Mini kit (QIAGEN), or

– RNA purification using 4 volumes of TRIzol® added per 1 volume of RLT® buffer (**Appendix B**).

Alternatively, place cells in at least 300 µl (or 300 µl per 3×10^6 cells) of TRIzol® and extract RNA using TRIzol® protocol (**Appendix B**).

Large amounts of gDNA may affect cDNA synthesis. For large number of cells (> 200,000), when using a column-based RNA extraction method, DNAse treatment is recommended. gDNA eliminator columns (QIAGEN) can also be used for DNA removal.

From 100 to 50,000 sorted or purified T-cells

Sort/place directly in 50-300 µl RLT® buffer. The volume of RLT® buffer should not be diluted more than 20% during sorting. 50,000 sorted cells may carry the volume around 50 µl when using 70 µm nozzle for cell sorting. The cells are lysed immediately in the collection tube and mRNA is protected from degradation. Lysed cells can be stored in RLT® buffer at –70°C for at least 6 months.

Use RNeasy Micro or Mini kit (QIAGEN) for RNA purification. Do not use DNAse.

To obtain maximum number of cDNA molecules for small samples (100–10,000 cells), add 4 volumes of TRIzol® to RLT® cellular lysate and extract RNA using TRIzol® protocol (**Appendix B**).

More than 50,000 sorted or purified T-cells

Sort/place cells into 300 µl of PBS, then spin down the cells at 350 g for 15 minutes, remove the supernatant.

Add at least 50 µl (or 50 µl per $2x10^5$ cells) of RLT® buffer and mix by pipetting for:

– storage at –70°C for up to 6 months, or
– RNA purification using RNeasy Micro or Mini kit (QIAGEN), or
– RNA purification using 4 volumes of TRIzol® (**Appendix B**).

Alternatively, place cells in at least 300 µl (or 300 µl per 3×10^6 cells) of TRIzol® and extract RNA using TRIzol® protocol (**Appendix B**).

Large amounts of gDNA may affect cDNA synthesis. For large numbers of cells (> 200,000), when using a column-based RNA extraction method, DNAse treatment is recommended. gDNA eliminator columns (QIAGEN) can also be used for DNA removal.

T cell-containing tissue

Homogenize fresh tissue. Ideally, obtain a single cell suspension using incubation with DNAse and proteases mixes (such as Liberase™ TL from Roche). Optionally, wash cells with PBS. Immediately proceed with RNeasy Micro kit (QIAGEN) using RLT® buffer or use TRIzol® (**Appendix B**) for RNA extraction.

APPENDIX B: RNA ISOLATION USING TRIZOL®

- Lyse cell pellet in TRIzol® reagent by repetitive pipetting. Use at least 300 µl of the reagent per **100 – 3 x 10^6 cells**. For larger cell amounts increase the volume of the reagent according to the proportion of **1 ml per 10^7 cells.**

- If you have RNA in RLT buffer add 4 volumes of TRIzol® reagent and mix well by vortexing.

- Incubate the homogenized samples for 5 minutes at room temperature.
Add 1/5 volume of chloroform and mix well by vortexing. Incubate tubes at room temperature for 3 minutes.

- Centrifuge the samples at > 10,000 g for 10 minutes at 2 to 8°C, put on ice. After centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase.

- Transfer the colorless aqueous phase without disturbing the interphase to a fresh tube. Add 1 µl of co-precipitant (Pellet Paint® #70748 Merck Millipore or analog) and mix well by pipetting.

- Precipitate the RNA from the aqueous phase by mixing with equal volume of isopropyl alcohol. Incubate samples at 15 to 30°C for 10 minutes and centrifuge at > 10,000 x g for 10 minutes at 2 to 8°C. RNA with co-precipitant forms a visible pellet on the bottom of the tube.

- Remove the supernatant without disturbing the pellet.

I. If starting from < 50,000 cells

- Add 1 ml of freshly prepared 75% ethanol to the RNA pellet. Mix the sample by vortexing and centrifuge at > 10,000 x g for 5 minutes at 2 to 8°C. Remove the supernatant.

- Dry the RNA pellet completely. It is important, however, not to overdry the pellet as this will decrease its solubility. Dissolve RNA in 10 µl of RNase-free water and **immediately** proceed to cDNA synthesis.

II. If starting from > 50,000 cells

- Add 1 ml of freshly prepared 75% ethanol to the RNA pellet. Mix the sample by vortexing and centrifuge at > 10,000 x g for 5 minutes at 2 to 8°C. Remove the supernatant.

- Repeat this step.

- Dry the RNA pellet completely. It is important, however, not to overdry the pellet as this will decrease its solubility. Dissolve RNA in 10 µl of RNase-free water.

- Proceed to cDNA synthesis or store at –70°C for up to one week. For longer storage add 1/10 volume of sodium acetate (3M pH=5,5) and 3 volumes of 96% ethanol, mix well by vortexing. Store at –70°C.

TROUBLESHOOTING

Problem	Possible reason	Possible solution
Low product yield	RNA contains impurities that inhibit cDNA synthesis	In some cases, ethanol precipitation or additional column-based purification of RNA can remove impurities. If this does not help, re-isolate the RNA
	RNA is heavily degraded	Re-isolate the RNA
	PCR undercycling	Repeat the PCR amplification, using two or three more PCR cycles
	Excess of 1st strand cDNA in the first PCR reaction	Repeat the cDNA synthesis reaction. After purification (step 6) dilute 1st strand cDNA 1:5. Use 12 µl in 1st PCR reaction
Bands and background smear are very intense	PCR overcycling	Repeat the PCR amplification, using two or three fewer 2nd PCR cycles
Background smear is intense or short length fragments are visible	Low TCR RNA content in the initial RNA sample	Purify target library using AMPure XP beads or agarose gel purification