MONKEY TCR KIT

Monkey TCR α and β repertoires profiling

User Manual v.1.1

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Kit Overview

The **Monkey TCR Kit** is designed for unbiased amplification of TCR alpha and beta cDNA libraries using 5'RACE with unique molecular identifiers (UMI) incorporated within template switch oligo. 5'RACE UMI technique 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 is designed for TCR repertoire profiling of the following species:

- **Rhesus macaque (*Macaca mulatta*)**
- **Cynomolgus monkey (Crab-eating macaque, *Macaca fascicularis*)**
- **Japanese macaque (*Macaca fuscata*)**

The kit includes a set of oligonucleotides 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 500 to 0,5 million of 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 (indexing) PCR amplification starts from the product of 1st PCR and is performed separately for TCR alpha and beta chain repertoires (**Fig. 1**). Illumina Unique Dual Indexing system used in the second PCR step helps to avoid cross-sample contamination due to "barcode hopping" in course of library preparation and sequencing.

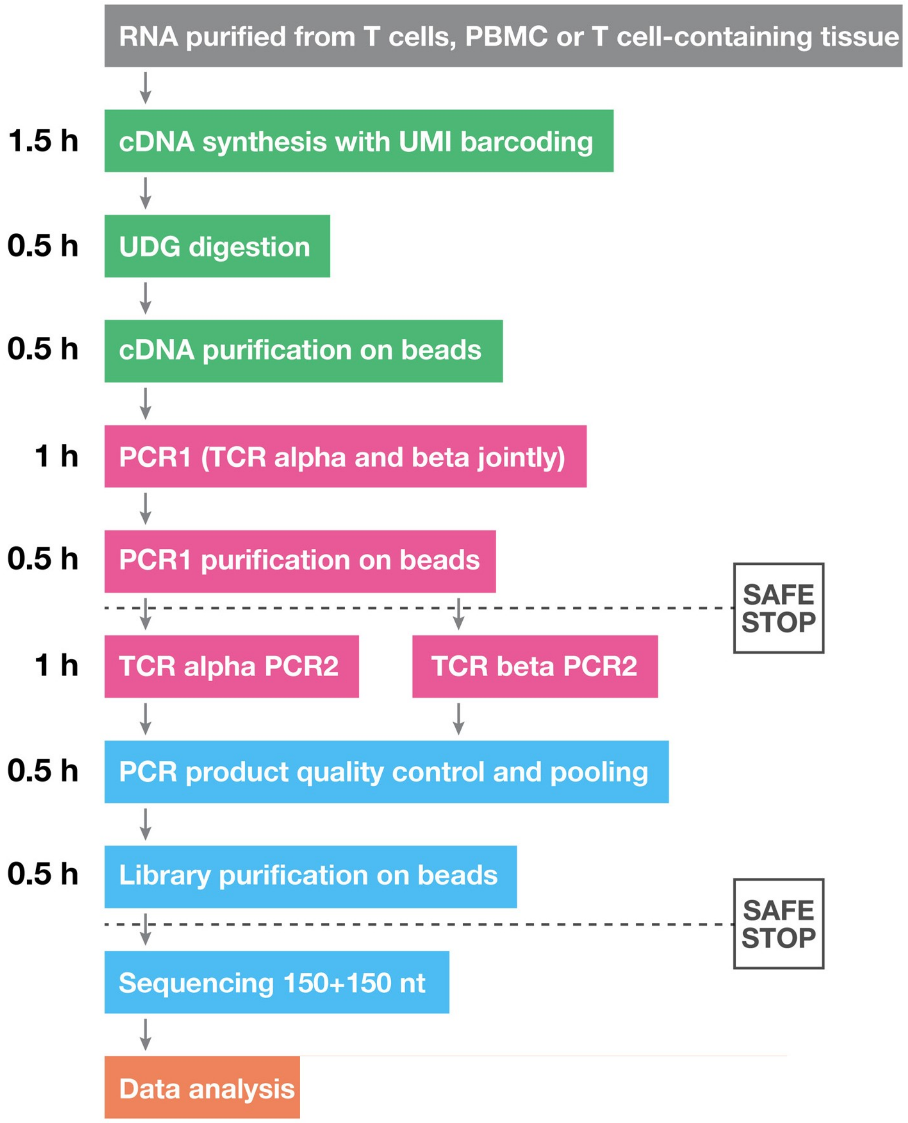

Figure 1. Monkey TCR kit pipeline.

Kit contents

Name	Description	Number of Tubes	Number of Reactions	Protocol step
NN oligo	Template switch oligo with UMI	1	24	cDNA synthesis
TR mnk Synt	Synthesis primers mix	1	24	cDNA synthesis
TR mnk PCR1	1st PCR primers mix	1	24	1st PCR
PCR2 mnk Alpha	2nd PCR primer mix	1	24	2nd PCR (TCR α)
PCR2 mnk Beta	2nd PCR primer mix	1	24	2nd PCR (TCR β)
Control RNA	Control RNA	1	5	All steps
RNAse-free water	RNAse-free water	2	-	All steps

Materials Required but Not Included

- SMARTScribe™ Reverse Transcriptase (Clontech, Takara, Inc. #639538)
- RNAsin® (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)
- Low-speed benchtop Mini-centrifuge/vortex
- Qubit® fluorometer
- Agilent Bioanalyzer, Tape Station or agarose gel electrophoresis system
- Elution Buffer (10 mM Tris-HCl, pH 8.0-8.5)
- IDT for Illumina Nextera DNA Unique Dual Indexes (any of #20027213, #20027214, #20027215 or #20027216)

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 RNAse-free water to the tubes according to the table below. Mix by vortexing and spin down.

Make aliquots of NN-oligo depending on planned experimental setup. Each aliquote can be frozen/thawed no more than 3 times. Store all tubes at -20ºC after dilution.

Add 50 µl of RNAse-free water to Control RNA. Incubate the tubes at room temperature for 10 minutes. Vortex briefly and spin down. Store Control RNA at -70 ºC.

Component	**Water volume (µl)**
NN oligo	60
TR mnk synt	60
TR mnk PCR1	60
Control RNA	50
PCR2 mnk Alpha	30
PCR2 mnk Beta	30

Starting material

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

Verified minimal number of cells for the protocol is 500 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.

For a small amount of starting material (500-50,000 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)/plate, mix the following reagents in a final reaction volume of 12 µl:

Component	**Volume, µl**
RNA, up to 500 ng per reaction*	1-10
TR Mnk Synt cDNA synthesis primer mix	2
RNAse free water	0-9
Total volume	12 µl

* For control reaction, put 10 µl of control RNA

2. Place the reaction tube(s)/strip(s)/plate into a thermal cycler and incubate for 2 minutes at 70ºC, 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:

Component	**Volume, µl**
5X First-Strand Buffer (Clontech)	6
DTT (20 mM)	3
NNoligo	2
dNTP solution (10 mM each)	3
SMARTScribe® Reverse Transcriptase (Clontech)	3
RNAsine®, 40u/µl	1
Total volume	18 µl

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 42ºC.

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

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: Do not overdry the beads! To reduce drying time get out the tubes from the magnetic separator after second ethanol washing. Briefly spin the tubes to drop down all the remaining liquid. Place the tubes back on the magnetic separator, 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 cDNA.

During the elution step, 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 50ºC for 10-30 minutes until complete dissolving.

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, plus 10% of the total reaction mix volume:

Component	**Amount, µl**
Nuclease free water	24.5
Q5® polymerase buffer (5x, New England Biolabs)	10
dNTPs (10 mM each)	1
TR mnk PCR1 primers mix	2
Q5® High-Fidelity DNA Polymerase (New England Biolabs)	0.5

Total volume 38 µl

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:

98ºC for 30 s

98ºC for 10 s
55ºC for 20 s } **18-21 cycles***
72ºC for 40 s

72ºC for 2 min
4ºC hold

* Use 21 cycles for RNA input < 50 ng and 18 cycles for RNA input > 50 ng

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 20 µl EB (10 mM Tris HCl, pH 8.0-8.5).

SAFE STOP POINT: Purified product of the 1st PCR can be stored for 1-3 months 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 **PCR2 mnk Alpha** and **PCR2 mnk Beta** with RNase free water 1:9 just before use. Discard diluted aliquots after use.

Prepare two separate PCR2 Master Mixes in a final volume of 22 µl per reaction, plus 10% of the total reaction mix volume:

For **TCR alpha**

Component	**Amount, µl**
Nuclease free water	15.25
Q5® polymerase buffer (5x, New England Biolabs)	5
dNTPs (10 mM each)	0.5
PCR2 mnk Alpha primer mix	1
Q5® High-Fidelity DNA Polymerase (New England Biolabs)	0.25

For **TCR beta**

Component	**Amount, µl**
Nuclease free water	15.25
Q5® polymerase buffer (5x, New England Biolabs)	5
dNTPs (10 mM each)	0.5
PCR2 mnk Beta primer mix	1
Q5® High-Fidelity DNA Polymerase (New England Biolabs)	0.25

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

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

14. Add 1 µl of each of IDT for Illumina Nextera DNA UDI indexing primer mix to each reaction:

15. Perform PCR using the following parameters:

98ºC for 30 s

98ºC for 10 s
60ºC for 20 s } **9-18 cycles**
72ºC for 40 s

72ºC for 2 min
4ºC 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	**Approximate amount of RNA**	**Recommended number of 1st PCR cycles**	**Approximate number of 2nd PCR cycles**	**Recommended number of 150+150 nt sequencing reads per sample**
$5x10^2$ – $5x10^3$ T cells	0.5-5 ng	21	18-21	$5x10^4$
$5x10^3$ – $5x10^4$ T cells	5–50 ng		14-17	$5x10^5$
$5x10^4$ – $5x10^5$ T cells	50–500 ng	18	11-15	$5x10^6$
$5x10^5$ – 10^6 T cells	500–1000 ng		9-12	10^7
	10 µl control RNA	18	12-13	

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.

16. Verify quality and concentration of 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 approximately 670 bp for TCR alpha and 630 for TCR beta chains (**Fig. 2**).

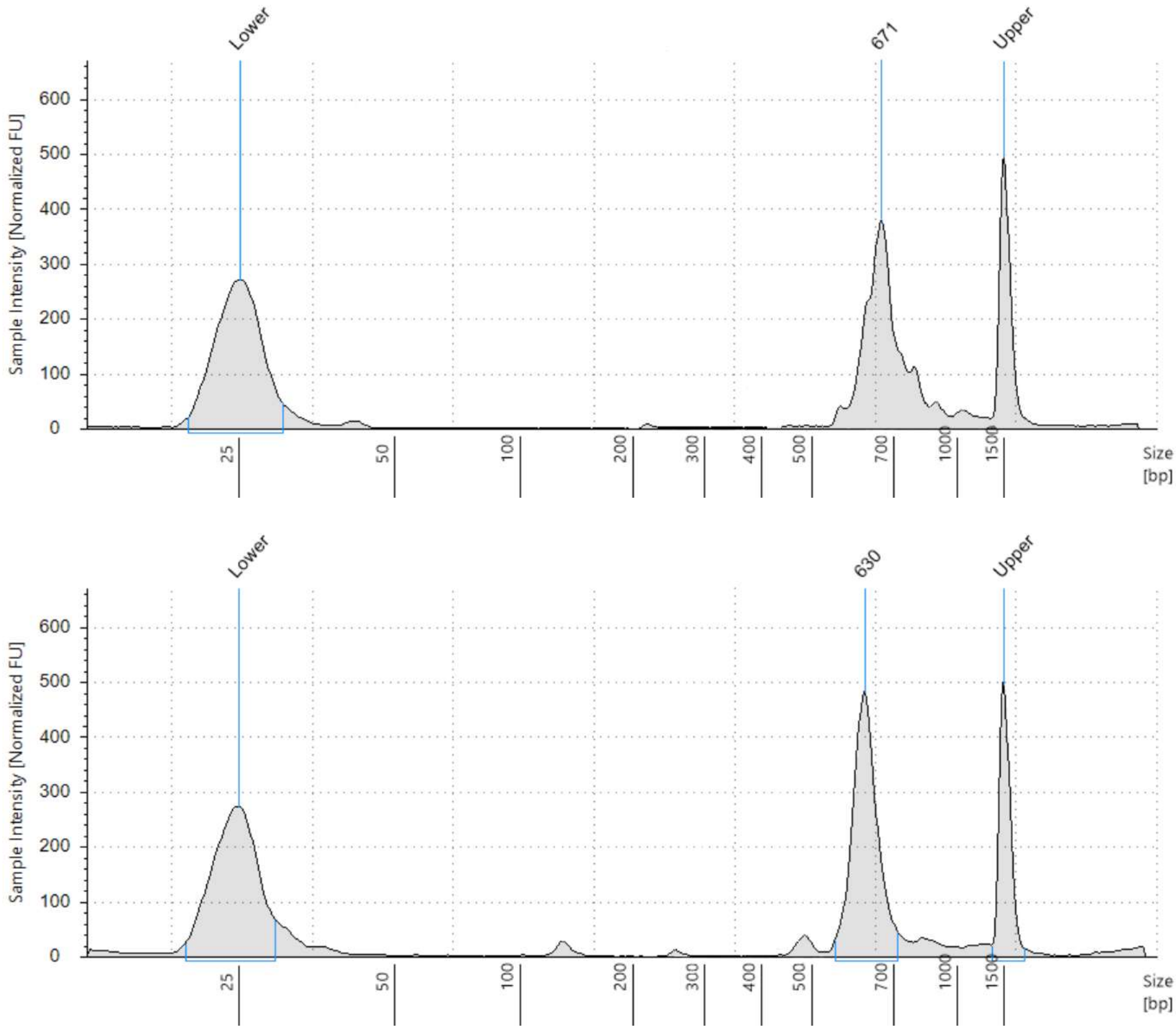

Figure 2. Typical peak size for alpha (top) and beta (bottom) TCR libraries. The shape and length distribution of TCR library may vary depending on the repertoire composition.

17. Pool all obtained libraries (both TCR alpha and 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 150 ng.

18. 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).

19. Verify quality and concentration of obtained pooled library by analyzing the aliquot by agarose gel or capillary electrophoresis (e.g. BIOANALYZER® or TapeStation®, Agilent Technologies). The resulting amount of pooled PCR products should be at least 100 ng. If fragments shorter than 400 nt are detected 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.

Use qPCR to determine library concentration according to Illumina QC recommendations.

Spike with at least 30% 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. Use double indexing read 10+10 nt. 150+50 nt length is sufficient to cover 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

Data analysis can be performed with MiXCR Pro® software that allows to make initial clonotype extraction and calculation of important repertoire characteristics, from the intuitive graphical user interface using best in class algorithms.

MiXCR Pro® is available as native package for Microsoft Windows, GNU/Linux, and macOS X. Additionally to graphical interface, software supports execution from the command line, which makes it suitable for incorporation into automated analysis pipelines and execution on remote servers.

Please download the software from: https://kit.mixcr.pro

You can find the license code for activation of MiXCR Pro® inside the box with the Kit.

The output provided by MiXCR PRO® will contain annotated TCR clonotypes and several post-analysis reports.

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 2-3 hours in RPMI-1640 supplemented with 10% fetal bovine 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 then 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.

RNA can be stored in 75% ethanol for at least 1 year at -20°C, or at least 1 week at 4°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:

1. PBMC

Perform isolation of mononuclear cells from whole blood using Ficoll Paque density gradient centrifugation. 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® added per 1 volume of RLT® buffer (**Appendix B**).

Alternatively, place cells in at least 300 µl (or 300 µl per 3 x 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.

2. From 500 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 (500-10,000 cells), add 4 volumes of TRIzol® to RLT® cellular lysate and extract RNA using TRIzol® protocol (**Appendix B**).

3. 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.

4. 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 500 – 3x10^{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