Purification of total RNA from peripheral blood mononuclear cells

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Peripheral blood is often used for in vitro studies of the human immune system or immune responses, such as inflammation. An important part of the human immune system is represented by the peripheral blood mononuclear cells (PBMC). PBMC are blood cells characterized by a round nucleus and consist mainly of lymphocytes (T cells, B cells, and NK cells), macrophages and dendritic cells. Here, we describe the analysis of lipopolysaccharide-induced transcriptional response of isolated PBMC from whole blood using the RNeasy® Mini Kit or RNeasy Micro Kit, RT² First Strand Kit, RT² SYBR® Green ROX™ qPCR Mastermix, and RT² Profiler PCR Arrays.

Introduction

PBMC are extensively used in immunology or cancer research. Of special interest, is the analysis of cytokine expression as a response of immune system activation by either microorganisms (viruses or bacteria) or immune stimulation due to vaccine or drug treatment. (1) In bacterial infection, immune response is elicited by lipopolysaccharide (LPS) and causes a dynamic response in the gene expression of PBMC. The in vitro induction of immune response using LPS is a good control system to analyze genes involved in LPS-induced transcriptional response.

PBMC can be isolated from whole blood using density-gradient media such as Ficoll-Paque®, a hydrophilic polysaccharide. Due to its physical properties, it can be used to separate whole blood through density-gradient centrifugation. After centrifugation, the blood is separated into a top layer of plasma, a middle, white layer of PBMC, and a lower fraction of polymorphonuclear cells (e.g., neutrophils and eosinophils) and erythrocytes, which are removed during the centrifugation due to their higher density.

Materials and methods

Blood was drawn into lithium-heparin tubes from healthy volunteers with informed written consent. For stimulation of blood with LPS, a stock solution of 0.1 mg/ml in dimethylsulfoxide (DMSO) was prepared and 6 µl of the stock solution was dispensed into each well of a 6-well plate. The LPS stock solution was then diluted by adding 500 µl RPMI complete medium to prevent hemolysis by the DMSO. Immediately after dilution of the stimulating reagent, 5.5 ml Li-heparin–stabilized blood was added to each well and incubated for 1 h at 100 rpm in an incubator (37°C; 90% relative humidity; 5% CO_2). PBMC were isolated using the QIAGEN Supplementary Protocol "Isolation of Peripheral Blood Mononuclear Cells (PBMC) and Purification of Total RNA from PBMC Using the RNeasy Micro or Mini Kit". In this protocol, PBMC are isolated from whole blood by density centrifugation using Ficoll-Paque as a density-gradient medium. RNA from isolated PBMC was

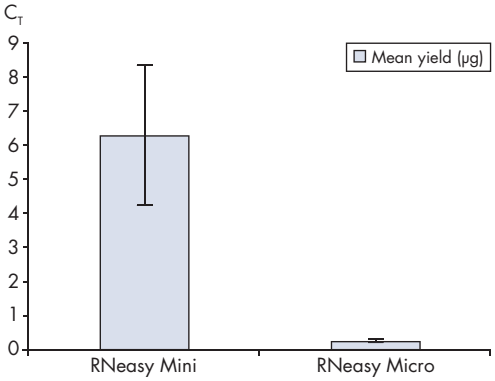

Figure 1. Total RNA obtained from 1 x 10^7 and 1 x 10^6 PBMC using either the RNeasy Mini or RNeasy Micro protocol. Average yields as determined by a NanoDrop 8000 Spectrophotometer were 6.29 µg RNA and 0.29 µg RNA.

extracted using the RNeasy Mini or RNeasy Micro Kit. Buffer RLT was supplemented with β-mercaptoethanol. The RNA yield and quality were determined using a NanoDrop® 8000 Spectrophotometer and an Agilent® 2100 Bioanalyzer. The cDNAs from control and stimulated samples were prepared using 250 ng of the purified RNA and the RT² First Strand Kit. In the next step, cDNA was mixed with RT² SYBR Green ROX qPCR Mastermix and the mixture aliquotted into the wells of an RT² RNA QC PCR Array or RT² Profiler PCR Array Human Inflammatory Cytokines & Receptors. After PCR, relative expression was determined using the data from the real-time cycler and the $\Delta\Delta C_T$ method. Differences in gene expression between control and stimulated samples were expressed as fold-changes and visualized using volcano or scatter plots.

Results: RNA quality

Total RNA was extracted from PBMC using the RNeasy Mini and Micro Kit in duplicates (for each kit). Yield was determined by using a NanoDrop 8000 Spectrophotometer (Figure 1). The average RNA yields obtained from 1 x 10^7 and 1 x 10^6 PBMC were 6.29 µg and 0.29 µg RNA with the RNeasy Mini and RNeasy Micro protocols, respectively.

To control the integrity and overall quality of the RNA eluates, samples were checked using an Agilent 2100 Bioanalyzer and by real-time PCR using the RT² RNA QC PCR Array. All samples were analyzed using an Agilent RNA 6000 Nano Kit and 1 µl of each RNA eluate. Results are shown in Figure 2.

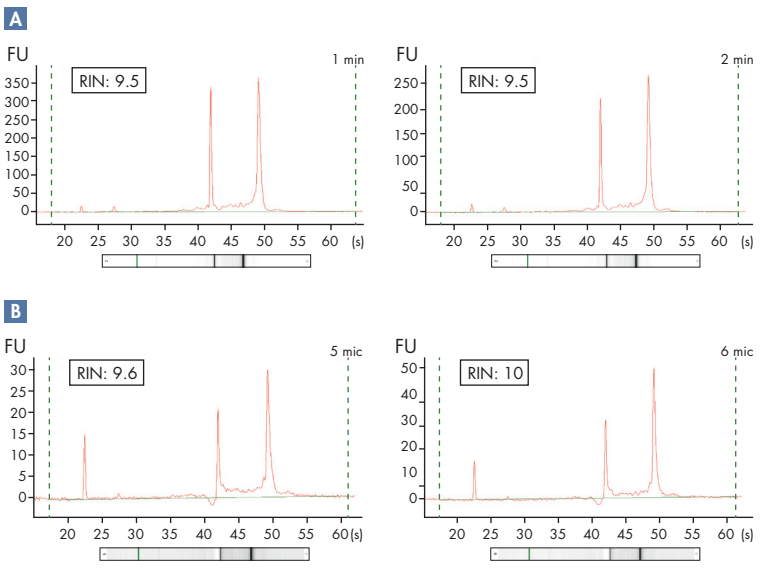

Figure 2. Analysis of RNA eluates on an Agilent 2100 Bioanalyzer. Each eluate (1 µl) was applied to an RNA Nano chip. Electropherograms from all eluates show sharp peaks for the 18S and 28S ribosomal RNA. No shoulders are visible, especially to the left of each peak, indicating high-quality RNA. **A** RNA eluates from 1 x 10^7 PBMC resulted in RIN values of 9.5 (left) and 9.5 (right). **B** RNA eluates obtained from RNA extraction starting with 1 x 10^6 PBMC gave RIN values of 9.6 (left) and 10 (right).

Agilent Bioanalyzer analysis showed high-quality RNA in all eluates. Electropherograms and gel images show sharp peaks for 18S and 28S ribosomal RNA. The RNA integrity number (RIN) values for all samples were consistently ≥9.5. Consistent RIN values across multiple samples within each experiment are desirable for reliable quality data comparisons.

The quality of the RNA eluates was then assessed using the RT² RNA QC PCR Array. The RT² RNA QC PCR Array contains controls for RNA integrity, the presence of inhibitors of reverse transcription and PCR amplification, and the presence of genomic or other DNA contamination. Results are shown in Tables 1 and 2 and Figure 3.

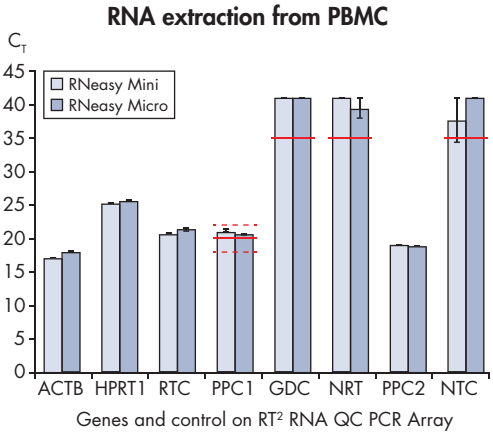

Figure 3. RNA analysis using the RT² RNA QC PCR Array. Graphical representation of the mean C_T values for the RNA eluates extracted with either the RNeasy Mini Kit (1 x 10^7 PBMC) or the RNeasy Micro Kit (1 x 10^6 PBMC). Mean C_T values derived from both samples are comparable with no significant differences. The ACTB and HPRT1 housekeeping genes do not vary significantly between samples. There is no indication of inhibition of the reverse transcription or PCR or contamination with genomic DNA. The cut-off values for PPC1, GDC, NRT, and NTC are shown by the red lines.

Table 1. Mean C_T values obtained for RNA eluates

	RNeasy Mini Kit		RNeasy Micro Kit	
Genes/controls	**Mean C_T**	**SD**	**Mean C_T**	**SD**
ACTB	17.08	0.02	17.95	0.01
HPRT1	25.25	0.11	25.60	0.06
RTC	20.67	0.06	21.37	0.19
PPC1	21.15	0.13	20.56	0.05
GDC	41.00	0.00	41.00	0.00
NRT	41.00	0.00	39.50	1.50
PPC2	19.01	0.10	18.93	0.03
NTC	37.68	3.32	41.00	0.00

β-actin (ACTB) and HPRT (HPRT) are two housekeeping genes expressed at a higher and a lower-mid level. Expression levels of both genes enable prediction of the expected threshold cycle value in future analysis. The reverse transcription control (RTC) tests the efficiency of the reverse transcription step during cDNA synthesis. The positive PCR control (PPC) is a plasmid template with an artificial sequence and primers to detect it. Two controls are characterized with (PPC1) or without experimental template (PPC2) to test for the presence of PCR inhibitors in the RNA samples. The genomic DNA control (GDC) specifically detects genomic DNA contamination in the sample eluate. The no reverse transcription control (NRT) tests for genomic DNA contamination in the RNA sample by trying to amplify a housekeeping gene directly from the RNA sample. The no-template control (NTC) tests for general DNA contamination in the PCR system introduced during plate setup.

Table 2. Verification of C_T values against cut-off values for the different controls and validity criteria

		RNeasy Mini Kit		RNeasy Micro Kit	
Controls	**QC criteria for controls**	**Value**	**Assay valid**	**Value**	**Assay valid**
RTC PPC2	Calculate $\Delta C_T = C_T^{RTC} - C_T^{PPC2} = <5$	20.67 – 19.01 = 1.66	✓	21.37 – 18.93 = 2.44	✓
PPC1	$C_T = 20 \pm 2$	21.15	✓	20.56	✓
GDC	$C_T > 35$	41	✓	41	✓
NRT	$C_T > 35$	41	✓	39.5	✓
NTC	$C_T > 35$	37.68	✓	41	✓

The mean C_T values derived from both samples (RNA eluates from RNeasy Mini [1 x 10^7 PBMC] and RNeasy Micro [1 x 10^6 PBMC]) show no significant differences. The ACTB and HPRT1 housekeeping genes do not vary significantly between the samples with the ΔC_T for both genes ≤1 (Table 1; Figure 3). No inhibition of the reverse transcription or PCR or contamination with genomic DNA was observed. All C_T values were verified against the cut-off values for the different controls to check the validity criteria; with all C_T values valid. The results demonstrate that high-quality RNA can be extracted from isolated PBMC using either the RNeasy Mini or Micro RNA extraction protocol.

Results: Immune stimulation of PBMC

We analyzed the PBMC response after induction with an immune-stimulating reagent. Whole blood was incubated with LPS and a mock control was used. PBMC were isolated using the described density-gradient method, after exposing the blood cells to the reagent. RNA was extracted from induced and control PBMC (1 x 10^7 PBMC each) as previously described. The cDNA were transcribed using 250 ng of each extracted RNA in the reverse transcription reaction. Differences in gene expression between the induced and the control sample were analyzed using the RT^2 Profiler PCR Array Human Inflammatory Cytokines & Receptors. This array profiles the expression of a focused panel with 84 genes that encode inducible cytokines, chemokines, and their receptors for genes related to the immune system.

Three arrays were used for each sample and C_T values obtained from real-time PCR were uploaded to a web-based analysis tool for further analysis. Differences in gene expression between control and stimulated samples were based on the averaged C_T values, calculated by the $\Delta\Delta C_T$ method, and expressed as fold-changes. All built-in controls (RTC, PPC, and GDC) on the array passed the validity criteria. Gene expression levels were normalized against the housekeeping genes GAPDH (glyceraldehyde-3-phospahte dehydrogenase) and RPLP0 (large ribosomal protein P0).

Table 3 shows all genes with 2-fold or greater change in gene expression. In total, 17 genes were upregulated in the LPS-induced sample (Group 1), whereas 1 gene was downregulated.

Table 3. Induction of chemokine and cytokine genes after stimulation with LPS

Group 1 (LPS-induced) vs. control group	Genes		
	Gene symbol	Fold regulation	P-value
Upregulated	BMP2	3.8	0.0063
	CCL20	46.9	0.0241
	CCR8	2.7	0.0207
	CXCL1	6.8	0.0228
	CXCL2	7.9	0.0195
	CXCL3	20.8	0.0201
	CXCL5	2.3	0.0278
	CXCL6	6.7	0.0318
	CXCL9	2.2	0.0242
	CXCR2	3.2	0.0498
	IL1A	66.7	0.0160
	IL1B	22.7	0.0117
	IL1RN	19.3	0.0163
	IL27	5.8	0.0014
	IL7	9.3	0.0085
	IL8	3.9	0.0359
	NAMPT	2.3	0.0414
Downregulated	VEGFA	–2.3	0.0133

All genes are listed with a difference in gene expression (up- or downregulated) after stimulation with LPS and a 2-fold or greater change in gene expression.

A difference in gene expression at $p \leq 0.05$ was considered as statistically significant.

In addition, the differences in gene expression obtained for all 84 genes were visualized using either a volcano or a scatter plot to give an overview over all 84 genes on the array (Figure 4).

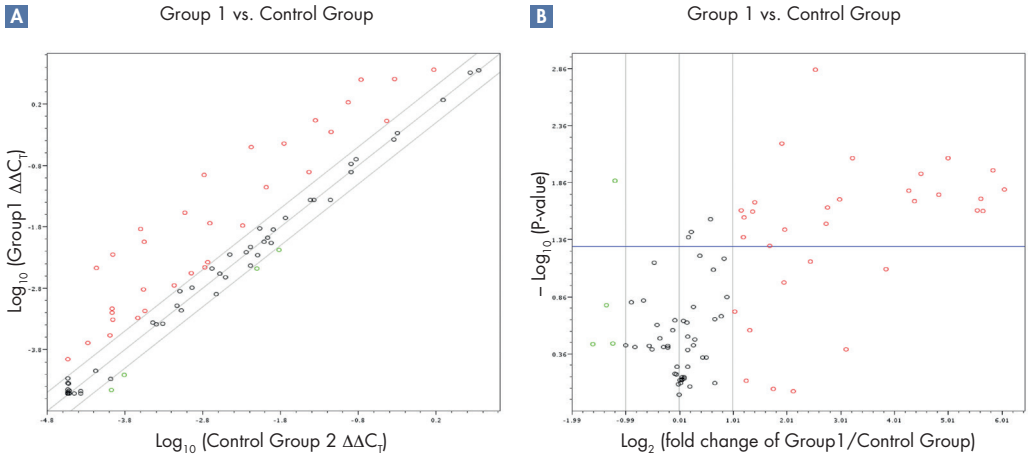

Figure 4. Effect of LPS stimulation on PBMC from whole blood. **A** Differences in gene expression after stimulation with LPS are visualized in a scatter plot. Upregulated genes are indicated by a red circle and downregulated genes with a green circle. The diagonal lines indicate the boundary of 2-fold change in gene expression. **B** In the volcano plot, the fold-changes are visualized relative to p-value. All circles above the blue line indicate genes with a 2-fold or greater change in gene expression and a p-value ≤0.05. Upregulated genes are indicated by a red circle and downregulated genes with a green circle. Significance for the obtained fold-regulation was accepted at p ≤0.05. **Group 1**: LPS-stimulated sample; **Control group**: sample without LPS stimulation.

The upregulation of IL1B and IL8 in LPS-treated PBMC is described in the literature (2). However, the stimulation with LPS induces the co-expression of certain cytokines and chemokines, as well as their receptors. Physical interactions, such as protein–protein interactions, are also reported. For 10 of the significantly regulated genes, the relationships after LPS stimulation are visualized in a network (Figure 5). Seven genes underlie a co-expression whereas 9 genes show physical interactions. The network was created using Gene Network Central Pro. GNCPro™ integrates biological knowledge (e.g., from publicly available data) and graphically displays the interactions among a group of genes.

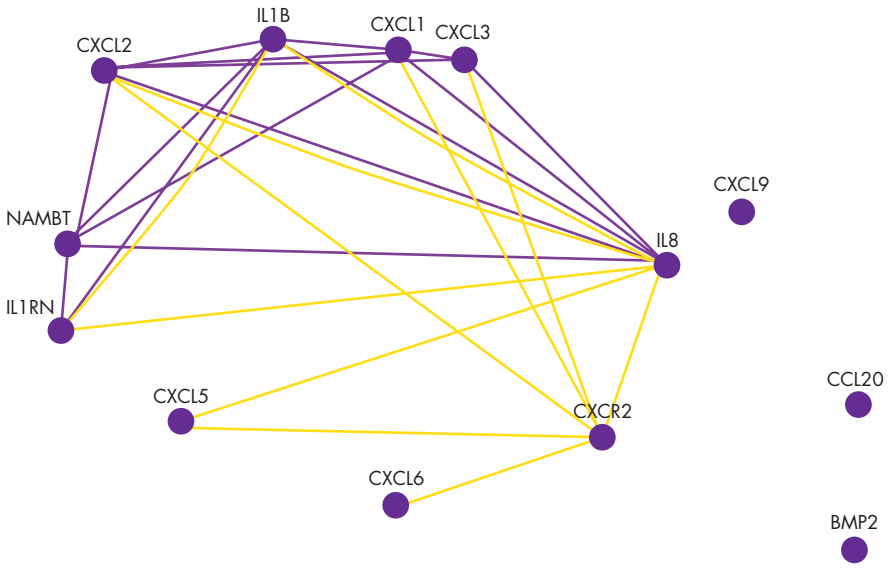

Figure 5. Relationship of regulated genes after LPS stimulation of whole blood and PBMC isolation. Relationships between genes that were regulated after LPS stimulation are graphically represented using GNCPro. Co-expression of genes is indicated by purple lines and physical interactions are indicated by yellow lines.

Conclusions

- This study revealed that isolated PBMC are well suited for in vitro experiments to study a dynamic and systematic immune response after treatment of whole blood with LPS. High-quality RNA can be extracted from isolated PBMC.
- LPS-induced transcriptional change in gene expression can be profiled with a real-time PCR-based approach; using a collection of 84 genes that are involved in regulating inflammation and immune response after bacterial infection.
- PBMC represent a good model system for the response to virus- and bacteria-infected cells, as well as transforming tumor cells or in testing the efficacy of new drugs.

Ordering Information

Product	Contents	Cat. no.
RNeasy Mini Kit (50)*	50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free Reagents and Buffers	74104
RNeasy Plus Mini Kit (50)*	For 50 minipreps: RNeasy Mini Spin Columns, gDNA Eliminator Spin Columns, Collection Tubes, RNase-Free Water and Buffers	74134
RNeasy Micro Kit (50)*	50 RNeasy MinElute Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free DNase I, Carrier RNA, RNase-free Reagents and Buffers	74004
RNeasy Plus Micro Kit (50)	For 50 micropreps: RNeasy MinElute Spin Columns, gDNA Eliminator Spin Columns, Collection Tubes, Carrier RNA, RNase-Free Water and Buffers	74034
RT² First Strand Kit (12)	RT² First Strand Kit	330401
RT² SYBR Green ROX qPCR Mastermix (12)*	RT² qPCR SYBR Green/ROX Mastermix	330522
RT² RNA QC PCR Array	RT² Profiler QC Array	Varies
RT² Profiler PCR Array Human Inflammatory Cytokines & Receptors	RT² Profiler PCR Array	PAHS-011Z

* Other kit sizes are available; please inquire.

References

1. Kierstead, L.S., Dubey, S., Meyer, B., et al. (2007) Enhanced rates and magnitude of immune responses detected against an HIV vaccine: effect of using an optimized process for isolating PBMC. AIDS Res. Hum. Retroviruses. **23**, 86.
2. Schildberger, A., Rossmanith, E., Eichhorn, T., Strassl, K., Weber, V. (2013) Monocytes, peripheral blood mononuclear cells, and THP-1 cells exhibit different cytokine expression patterns following stimulation with lipopolysaccharide. Mediators Inflamm. **2013**, 697972.

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