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APPLICATION NOTE #1

Laser Capture Microdissection (LCM) for the Analysis of Macrophage Gene Expression from Atherosclerotic Lesions

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Abstract

Macrophage foam cells are integral in the development of atherosclerotic lesions, however gene expression studies are complicated by the cellular heterogeneity of atherosclerotic plaque. This application note describes a protocol for LCM of cells identified immunohistochemically, followed by real-time RT-PCR to selectively analyze RNA from foam cells of apolipoprotein E-deficient mice. The specificity of the procedure and the measurement of gene induction in laser captured cell populations after an *in vivo* perturbation are illustrated. These techniques will facilitate the study of atherosclerosis.

Introduction

Macrophage foam cells are critical in the development of atherosclerosis (1-3). Therefore, a better understanding of the gene expression changes in foam cells during disease progression and regression has become an important goal in order to develop potential therapies and interventions (4-6). However, the study of macrophage foam cell gene expression in arterial lesions is hampered by the cellular heterogeneity of arterial tissue, which, besides macrophages, also contains lymphocytes, smooth muscle cells, endothelial cells, and adventitial fibroblasts. To overcome these technical obstacles, we describe here a method for the use of LCM (7, 8) to selectively procure macrophage foam cells from arterial lesions (identified by the macrophage-specific marker, CD68/ Macrosialin (9, 10). RNA extracted from the laser captured foam cell material was used to quantify, by real-time quantitative RT-PCR, the fold of enrichment and to measure the induction of specific transcripts in response to an inflammatory stimulus. These methods make possible the quantitative analysis of gene expression in macrophage foam cells and add a powerful dimension to the study of atherosclerosis.

Equipment and Reagents

This protocol requires the following reagents:

I. CRYOSECTIONING

- ❑ Isoflurane (Baxter, Cat. #Forane™)
- ❑ Phosphate Buffered Saline (PBS) (Fisher Scientific, Cat. #FLBP399-1)
- ❑ OCT cryoembedding medium (VWR, Cat. #25608-930)
- ❑ ColorFrost Plus™ slides (Fisher Scientific, Cat. #12-550-17)

II. IMMUNOHISTOCHEMISTRY

- ❑ Normal goat serum (Vector Laboratories, Cat. #S-1000)
- ❑ Anti-CD68/Macrosialin antibody (Serotec, Cat. #MCA1957S)
- ❑ SUPERaseIn™ (Ambion, Cat. #2694)
- ❑ Biotinylated rabbit-anti-rat IgG mouse-adsorbed secondary antibody (Vector Laboratories, Cat. #BA-4001)
- ❑ Vectastain™ ABC Alk- Phosphatase Kit (Vector Laboratories, Cat. #AK-5200)
- ❑ Vector Red substrate (Vector Laboratories, Cat. #SK-5100)

III. LASER CAPTURE MICRODISSECTION

- ❑ PixCell II® Laser Capture Microdissection Instrument (Arcturus, Cat. #LCM1104)
- ❑ CapSure® Macro LCM caps (Arcturus, Cat. #LCM0211)

IV. RNA EXTRACTION/ ISOLATION AND QUANTIFICATION

- ❑ PicoPure™ RNA Isolation Kit (Arcturus, Cat. # KIT0204)
- ❑ RNAse-Free DNase Set (Qiagen, Cat. #79254)
- ❑ Ribogreen RNA quantification kit (Molecular Probes, Cat. #R11490)

V. REAL-TIME QUANTITATIVE RT-PCR

- ❑ Gene-specific TaqMan primers and probes (Biosearch Technologies, Cat. # (see Table I)
- ❑ SuperScript™ II reverse transcriptase enzyme (Invitrogen, Cat. #18064-014)
- ❑ 10mM dNTP mix (Invitrogen, Cat. #18427-013)
- ❑ Taq DNA polymerase (Invitrogen, Cat. #10342-020)
- ❑ RnaseOut™ RNase inhibitor (Invitrogen, Cat. #10777-019)
- ❑ Acetylated BSA (1µg/ml) (Promega, Cat. #R9646A)
- ❑ 50X 5-carboxy-X-rhodamine (ROX) internal reference dye (Invitrogen, Cat. #12223-012)

The following laboratory equipment is required to complete the protocol:

- ❑ Disposable gloves
- ❑ Dry ice
- ❑ Cryostat
- ❑ ABI Prism™ 7700 Sequence Detection System (Applied Biosystems)
- ❑ RNase AWAY (Fisher Scientific, Cat. #14-375-35)
- ❑ Nuclease-free pipette tips
- ❑ MicroAmp optical tubes (Applied Biosystems, Cat. #N801-0933)
- ❑ MicroAmp optical caps (Applied Biosystems, Cat. #N801-0935)
- ❑ Nusieve 3:1 agarose (BioWhittaker Molecular Applications)
- ❑ Immunostaining jars

RNase-free Technique

All reagents were maintained under RNase-free sterile conditions.

1. Wear disposable gloves and change them frequently.
2. Pipettmen are wiped with RNase AWAY® prior to use according to manufacturer's instructions.
3. Cryosections are temporarily stored in the cryostat and later into a -80 °C freezer to prevent RNA degradation.
4. Use RNase-free solutions (water prepared by treatment with

0.2% DEPC overnight and autoclaved).

5. Concentration of antibodies and duration of incubations have been optimized so as to preserve the quality of RNA and to yield specific staining having a high signal/noise ratio.
6. Addition of SuperaseIn RNase Inhibitor to the antibody cocktails during immunostaining is recommended as it prevents RNA degradation during the incubation steps. (NOTE: Do not add the SuperaseIn to the ABC Alk-Phos enzymatic reaction as it will completely inhibit the staining).

Special Considerations

1. We have observed that addition of the SuperaseIn RNase inhibitor to the alkaline phophatase enzyme completely inhibits the reaction and does not produce any visible staining.
2. It is important that the alcohols and the xylenes be prepared fresh and not be reused since insufficient dehydration will dramatically affect the ability to perform successful LCM.
3. RNA extraction of the laser captured material yielded 3.5 ng from 30 6 µm thick sections, equivalent to volume of 1.35 x 106 µm3 of CD68+ immunos taining).
4. To generate the standard curves for CD68 and α-actin, total RNA was prepared from thioglycol late-elicited intraperitoneal macrophages and cultured primary aortic smooth muscle cells.
5. The probe, labeled at the 5' and 3' ends with 6-carboxyfluorescein (6-FAM) reporter and tetra methyl-6-carboxyrhodamine (TAMRA) quencher, respectively, is hydrolyzed by the 5' exonuclease activity of Taq DNA polymerase, causing an increase in fluorescent signal that is measured in "real-time" after each cycle of PCR amplification. Standard curves were constructed by plotting log10 RNA starting quantity vs. cycle threshold (Fig. 3A). On the basis of appropriate serially diluted standard RNA, the amount of input standard RNA yielding the same amount of PCR product measured from an unknown sample was calculated.

Method

A. Animals and Tissue Processing

1. Apolipoprotein E-/- mice (Jackson Laboratories), a standard model of human atherosclerosis, are fed a standard chow diet for 20 weeks.
2. Sacrifice mice by exsanguination (under general anesthesia with isoflurane) by intra-vascular perfusion with phosphate buffered saline (PBS). The thorax is opened and a 21-gauge cannula inserted to the left ventricle.
3. Incise the right atrium to allow efflux of blood. Perfuse at physiological pressure (100mmHg) with PBS.
4. Remove heart and aorta en bloc. Transect the heart at the lower poles of the atria and perpendicular to them. This plane is parallel to that of the aortic valve.
5. Place the upper half on the heart (containing the atria) in an embedding mold filled with OCT cryoembedding medium, and allow it to infiltrate into the cavity of the heart.
6. Position the heart flat with the cut surface on the bottom of the embedding mold. Fill the embedding mold with OCT and freeze on dry ice.
7. Store specimens at –80 °C.
8. Section tissue blocks at 6 µm thickness and mount on positively charged Color Frost Plus slides.

B. Immunodetection of CD68+ Macrophages for LCM.

Standard immunohistochemical staining protocols usually require prolonged incubation periods in aqueous media, which results in significant degradation of RNA. To overcome this limitation, a modified rapid immunostaining protocol was developed for macrophage-specific CD68/Macrosialin that does not significantly affect RNA yields (<12% reduction of total RNA in immunostained versus non-immunostained whole tissue sections) (11).

1. Allow frozen sections to air dry for 1 min and then fix in 70% ethanol for 15 sec followed by cold acetone for 5 min.
2. Incubate slides with anti-mouse CD68/macrosialin IgG (1:10 dilution) in 4% goat blocking serum (made in PBS) supplemented with SuperaseIn RNase inhibitor (0.4U/µl) for 1 min at room temperature (RT).
3. Rinse slides in PBS 3 times for 5 min each.
4. Incubate slides with biotinylated rabbit-anti-rat IgG mouse-adsorbed secondary antibody (20µg/ml final) in 4% goat blocking serum supplemented with SuperaseIn RNase inhibitor (0.4U/µl) for 1 min at RT.
5. Rinse slides in PBS 3 times for 5 min each.
6. Incubate slides with Vectastain ABC Alk-Phos reagent (prepared at least 30 minutes in advance according to manufacturer's instructions) for 1 min. at RT (see Special Considerations, Note 1).
7. Rinse slides in 100mM Tris-HCl Buffer pH. 7.5 for 1 min. at RT.
8. Apply Vector Red chromogenic substrate until adequate intensity of staining develops (~5 - 10 min). Immerse slides in RNase-Free water to stop reaction.
9. Sections are counterstained with Harris modified hematoxylin for ~ 5 sec.
10. Sections are subsequently dehydrated in graded ethanols (95% 2X, 1 min each, 100% 3X, 3 min each) and cleared in xylene (3X, 2 min each). Allow slides to air dry under a vacuum hood for 30 min (see Special Considerations, Note 2).

C. LCM and RNA Extraction

Laser capture was performed under direct microscopic visualization on the CD68-positively stained areas by activating a thermoplastic film mounted on optically transparent LCM caps over selected regions. 30 proximal aortic sections from each apolipoprotein E-deficient mouse were microdissected in regions immunostained positive for CD68/Macrosialin and with morphologically-identifiable cells having the characteristic "foamy" appearance. Approximately 300 laser pulses were performed on each section using the PixCell® II LCM Instrument. The following parameters were used: 15µm laser diameter, 40 mW power, 3.0 msec duration.

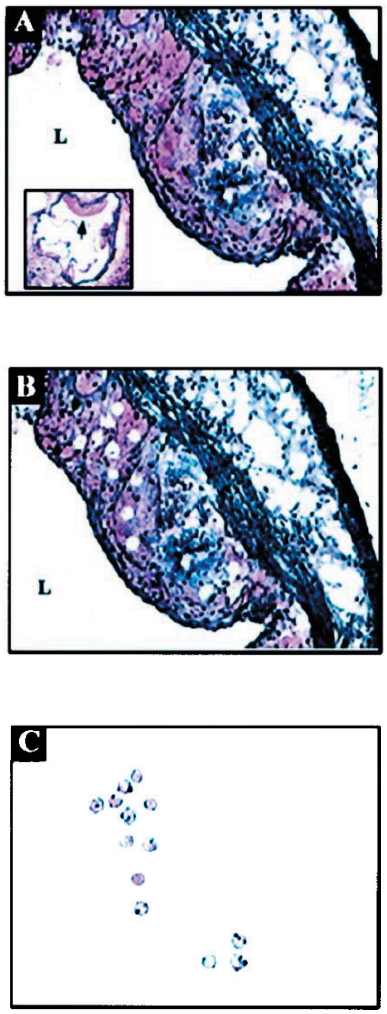

Figure 1. LCM of macrophage foam cells from atherosclerotic lesions of apolipoprotein E-/- mice. Selection of cells for laser capture was guided by immunohistochemical detection of macrophage specific marker CD68/Macrosialin (red staining). A. Proximal aortic lesion is shown prior LCM (200X; inset: 30X). B. Remaining tissue section after LCM. C. The homogeneity of the captured material is confirmed under microscopic visualization prior to processing for RNA extraction.

1. RNA was extracted/isolated using the PicoPure™ RNA Isolation Kit as per manufacturer's instructions.
2. Examine the surface of the LCM cap under the microscope to ensure complete cell extraction.
3. To eliminate potential genomic DNA contamination, RNA samples were treated with the DNase (Qiagen) directly onto the column as per manufacturer's instructions.

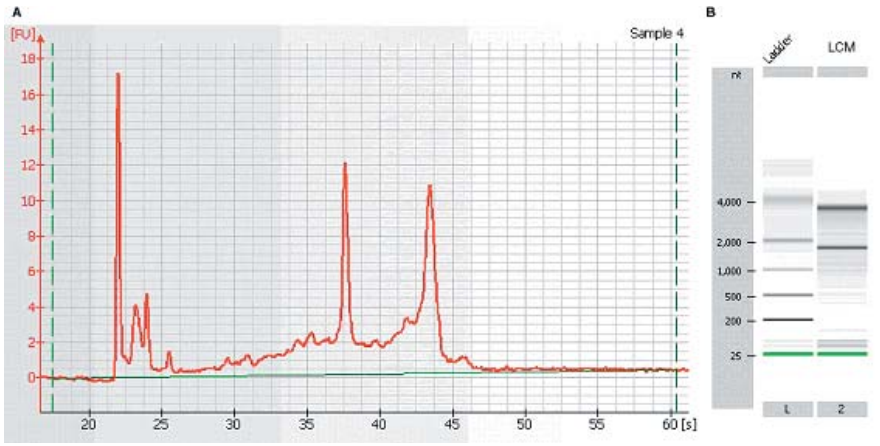

Figure 2. RNA purified from laser captured lesional foam cells assessed for quality. The integrity of RNA isolated from laser captured foam cells was assessed by the Agilent 2100 Bioanalyzer™. A total of 1 ng of total RNA was loaded onto a PicoChip for analysis. The bioanalyzer electropherogram shows two distinct ribosomal peaks corresponding to the 18S and 28S ribosomes. The first peak is that of an internal alignment marker.

4. RNA concentration was measured by a Ribogreen sensitive nucleic-acid dye binding assay according to manufacturer's instructions (see Special Considerations, Note 3).
5. Store LCM RNA sample at –80 ºC. Total RNA may be used immediately or stored at –80 ºC until used.

D. Analysis of macrophage foam cell gene expression by real-time quantitative RT-PCR.

Real-time quantitative RT-PCR is a very sensitive method which allows for measurements of low abundance transcripts and, unlike Northern or RNase protection assays, requires only a very small amount of total RNA (typically 100 pg - 1ng). A comprehensive review of the methodology has been done by Bustin, 2000. RT-PCR and subsequent PCR are both carried out in a single sealed optical tube using gene specific primers and fluorogenic probes.

1. Prepare a master mix containing the following for each reaction: 1X first strand buffer (50mM Tris-HCl pH 8.3, 75mM KCl, 3mM MgCl2), 5mM DTT, 0.3mM dNTP mix, 20U SuperScript II reverse transcriptase enzyme, 2.5U Taq DNA polymerase, 40U RnaseOut RNase inhibitor, 0.625 µg acetylated BSA, and 1X 5-carboxy-X-rhodamine internal reference dye (Invitrogen Life Technologies, Carlsbad, CA) in optical tubes.
2. Add 50nM of the forward primer and reverse primer, and 100nM of the probe to the master mix for each reaction.
3. To 5 µl of samples (100 pg) and appropriate standard RNA (10 ng - 1 pg serial dilutions), add separately 20µl of the master mix (see Special Considerations, Note 4).
4. Set the Sequence Detection System 7700 to the following cycling conditions: RT-PCR stage (95ºC, 10 sec; 45ºC, 50 min; 95ºC, 2min) immediately followed by 40 cycles of PCR amplification (denaturation: 95ºC, 15 sec; annealing/extension: 60ºC, 1 min). The reaction products are separated on a 2% Nusieve 3:1 agarose gel (Fig. 3B) to verify the appropriate size of the amplicons (~63-67 bp; see Special Considerations, Note 5).

Results

To aid in the identification of foam cells for laser capture, aortic sections were immunostained for the CD68/Macrosialin antigen (red color; Fig. 1). RNA extraction of the laser captured material yielded 3.52±0.18 ng of total RNA per animal (mean±SD). The integrity of the RNA was assessed and was found to be of high quality (Fig. 2). Peaks corresponding to the 28S and 18S ribosomal RNAs were clearly visible on the BioAnalyzer™ profile (A) and pseudo-electropherogram (B). There was no detectable genomic DNA contamination, as evidenced by a lack of additional peaks to the right of the 28S peak. Typically, RNase degradation of total RNA samples produces a shift in the RNA size distribution toward smaller fragments and a decrease in fluorescence signal and the 18S and 28S peak can no longer be identified with certainty. Degraded total RNA will lack a smooth baseline and typically contains multiple peaks that are as large as or larger than the ribosomal peaks. For the different gene transcripts measured, the quantitative RT-PCR assay was highly reproducible (median intra-assay coefficients of variation, based on >20 samples run in duplicate, ranged between 2.4% and 7.7%) and highly sensitive (10 pg of starting total RNA resulted in detectable product formation). In Fig. 3A, representative standard curve plots for CD68 (top) and α-actin (bottom) are shown. For all of the genes measured, a linear relationship between the log of the initial RNA concentration (pg) and the threshold cycle held true over a range of five orders of magnitude variation in the starting RNA concentration (for all experiments, the correlation coefficients were between 0.980-0.999). RT-PCR products from the serially-diluted standards and samples were analyzed by gel electrophoresis to confirm the presence of the specific amplicon (Fig. 3B). To show that LCM enriches macrophage-specific transcripts, RNA was extracted either from whole sections (analogous to homogenized tissue) or from LCM-derived CD68 immuno-positive macrophage foam cells. Real-time quantitative RT-PCR for CD68 was performed on equivalent amounts of RNA (100 pg) and the results normalized to the control gene, cyclophilin A. As shown in Fig. 3C, the LCM-derived RNA was significantly enriched in the mRNA

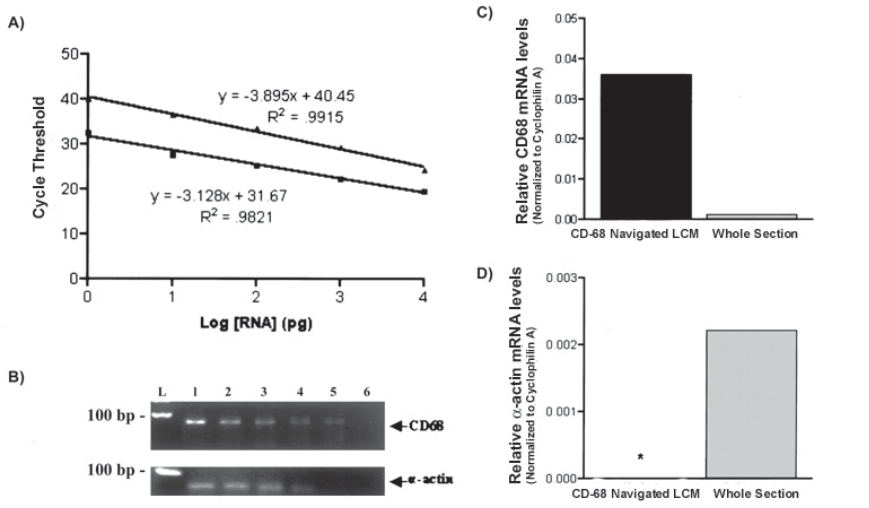

Figure 3. LCM selectively enriches lesional macrophage RNA, as assessed by mRNA transcript levels of cell-specific markers.

A. Representative standard curves from real-time quantitative RT-PCR of CD68 (▲) and α-actin (■). The plot of cycle threshold versus log10 of input starting concentration shows a linear relationship for 5 orders of magnitude of starting RNA. B. Electrophoretic analysis of the quantitative RT-PCR products. The semi-quantitative relationship (i.e., graded intensity of ethidium bromide stained bands) for the serial diluted standards (10 ng – 1pg; lanes 1-5) corresponds to that measured in A. C. The levels of macrophage specific CD68 were markedly increased in the LCM sample compared to the whole section RNA sample. D. There was no detectable amplification for smooth muscle cell-specific a-actin in the LCM foam cell derived RNA. The "*" indicates where the amplification of RNA from non specific cells would have been detected.

for the macrophage specific marker, CD68, compared to whole section RNA (33.6-fold). To determine the potential cellular contamination of the laser captured cells by adjacent medial smooth muscle cells (SMC) either from non-specific tissue adherence to the thermoplastic film or from imprecise laser beam positioning, smooth muscle cell-specific α-actin was measured by real-time quantitative RT-PCR. As shown in Fig. 3D, in contrast to the level of α-actin in RNA from whole sections, in the LCM-derived RNA, α-actin expression was at background levels after 40 cycles of amplification. These results show that lesional macrophage foam cells can be precisely and selectively isolated from atherosclerotic vessels by CD68-guided LCM. To test whether RNA derived by LCM of macrophage foam cells can be utilized to quantitatively assess the transcriptional regulation of target genes implicated in atherosclerosis, laser captured lesional macrophage RNA from proximal aortas of LPS and control stimulated apolipoprotein E-/- mice were analyzed. Mice were administered either an intraperitoneal injection of the bacterial endotoxin lipopolysaccharide (LPS) (100 µg) or vehicle only and, ~4 hours after treatment, the proximal aortas were processed for each mouse as described above. The relative gene transcript levels of the following genes were measured: vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and monocyte chemoattractant-1(MCP-1) (see Table I for gene primer sequences). As shown in Table II, LPS stimulation increased the expression of the Nuclear Factor-κB-mediated inflammatory genes, thereby confirming the ability of LCM and quantitative real-time RT-PCR to measure the regulation of genes after a perturbation or in pathologic (vs. normal) condition.

Table I. Sequences of Real-time quantitative RT-PCR primers and probes of measured gene transcripts.

Gene/Primer	Sequence (5' → 3')	Position	Amplicon Length (bp)
Cyclophilin A (NM_008907)			
Fwd	GGCCGATGACGAGCCC	74-89	64
Probe	TGGGCCGCGTCTCCTTCGA	91-109	
Rev	TGTCTTTGGAACTTTGTCTGCAA	115-148	
CD68/Macrosialin (X68273)			
Fwd	TTGGGAACTACACACGTGGGC	472-490	67
Probe	CGGCTCCCAGCCTTGTGT TCAGC	494-516	
Rev	CGGATTTGAATTTGGGCTTG	519-538	
α-actin (NM_007392)			
Fwd	AACGCCTTCCGCTGCCC	814-829	66
Probe	AGACTCTCTTCCAGCCATCTTTCATTGGGA	832-861	
Rev	CGATGCCCGCTGACTCC	863-879	
VCAM-1 (NM_011693)			
Fwd	CCCCAAGGATCCAGAGATTCA	402-422	63
Probe	TTCAGTGGCCCCCTGGAGGTTG	424-445	
Rev	ACTTGACCGTGACCGGCTT	448-466	
ICAM-1 (X52264)			
Fwd	ATCTCAGGCCGCAAGGG	600-616	66
Probe	TGGCATTGTTCTCTAATGTCTCCGAGGC	617-645	
Rev	CGAAAGTCCGGAGGCTCC	648-665	
MCP-1 (M19681)			
Fwd	TTCCTCCACCACCATGCAG	535-553	64
Probe	CCCTGTCATGCTTCTGGGCCTGC	556-578	
Rev	CCAGCCGGCAACTGTGA	582-598	

*The probes are labeled at the 5' and 3' positions with 6-carboxyfluorescein (FAM) reporter and a 6-carboxy-tetramethyl-rhodamine (TAMRA) quencher, respectively. The positions of the primers and probes are annotated according to the sequences derived from GenBank (accesion numbers given in parenthesis).

Table II. Induction of pro-atherogenic mRNAs by LPS.

mRNA transcript	Control	LPS	Ratio of Means (LPS : Control)
VCAM	2.1 (1.6 - 2.1)	22.1 (11.9 – 32.3)	11.9
ICAM	0.3 (0.2 – 0.4)	9.8 (4.1 – 15.4)	32.5
MCP-1	0.1 (0.126 – 0.128)	3.9 (2.4 – 5.5)	31.0

Real-time quantitative RT-PCR measurements (normalized to the control gene cyclophilin A) of RNA derived from LCM-isolated lesional macrophages from control and LPS-treated apoliprotein E-/- animals are given as a mean (range).

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The data described herein has been published, in part, in Trogan et al., Proc Natl Acad Sci USA, 99: 2234-2239, (2002).

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