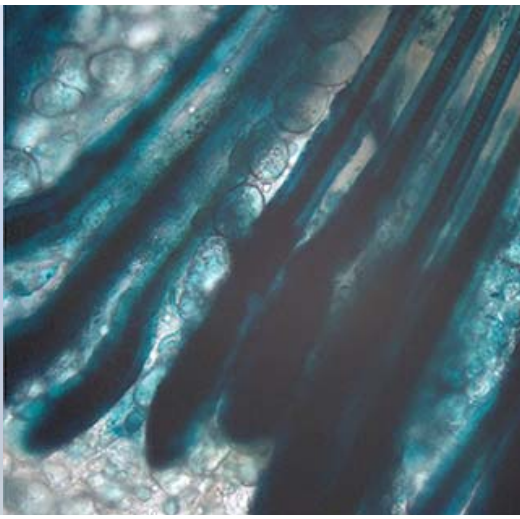
RNA quantification: Sorted Mouse Keratinocyte Stem Cells

Absorbance measurement on the Infinite® M200 microplate reader using the NanoQuant Plate™



Abstract

Quantification of RNA isolated from a minute amount of cells require a highly sensitive measurement method that can use a very small sample volume for the measurement. The isolation of adult stem cells results in very low amounts of cells due to their rare incidence in already formed tissues and organs. Here we describe the preparation and evaluation of RNA samples from a novel population of mouse keratinocyte stem cells marked by Lgr5+ expression. We used tools specifically designed for handling and measuring low RNA quantities including RNA purification and the subsequent quantification with the Infinite® M200 NanoQuant.

Introduction

The mouse hair follicle (HF) has become a very important model in stem cell (SC) biology. The multipotential SC activity is believed to reside within a group of quiescent cells in the so called bulge, marked by CD34 [1].

The gene expression analysis of CD34+ SCs showed genes up-regulated, which are important for the maintenance of the keratinocyte stem cell pool like Tcf3 and Lhx2. Beyond the most up-regulated genes in the CD34+ cells was the orphan G–protein coupled receptor Gpr49/ Lgr5 [2].
We have recently shown that Lgr5 marks a new population of SC, which is located in the lower bulge and the secondary germ of the resting HF (telogen). The Lgr5+ stem cells are partially overlapping with the CD34+ cells in telogen (Fig.1) but distinct during the active phase of the hair follicle (anagen) underlining their proliferative nature [3]. Lgr5+ cells - previously found to mark fast cycling SC in the mouse intestine [4]- encompass the most potent SC in the mouse hair follicle validated by colony forming ability and transplantation assays. Moreover, lineage tracing of Lgr5+ cells show that these cells contribute and are able to maintain all structures of the hair follicle over long periods [3].

Karolinska Institutet

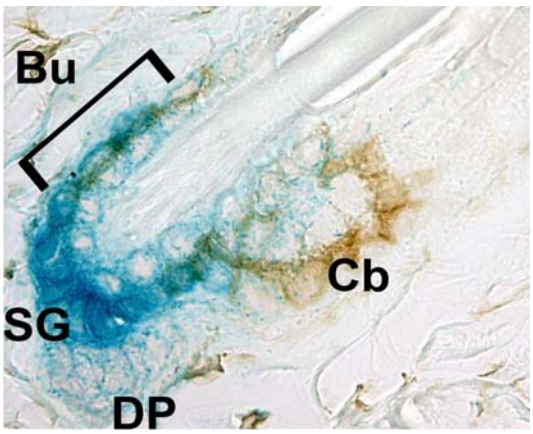


Figure 1. LGR5 expression in the mouse hair follicle is located in the lower bulge and the secondary germ. Immunohistochemical co-staining of β–galactosidase activity (blue) and the expression of CD34 detected using DAB (brown). The samples were obtained from 8 wk old mice (telogen), Lgr5+ cells in the secondary germ area are situated just above dermal papilla and in the lower part of the bulge. CD34 positive cells are situated in the bulge and the club hair. Cb – club hair, DP – dermal papilla, Bu – bulge, SG – secondary germ.

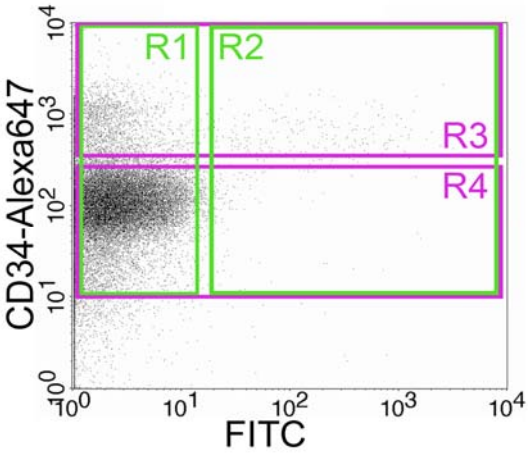


Figure 2. Sorting gates of mouse keratinocytes for CD34 and LGR5$^{+/lacZ}$. The FITC signal intensity reflects the β-galactosidase activity in live cells. R1 and R2 (green) encompass accordingly LGR5- and LGR5+ cells, while R3 and R4 show CD34+ and CD34- cells.

To characterize the gene expression profile of these cells, we isolated the different SC populations using FACS sorting, purified the RNA and performed qPCR. For gene expression profiling using a very low amount of starting material, it is of high importance that the amount of RNA (of the same quality) used for cDNA generation be normalized.

Materials and methods

Instruments

- BD FacsAria™ (BD Biosciences, USA)
- RNAqueous® RNA extraction kit (Applied Biosystems, USA.)
- Infinite® M200 with quad4 monochromator™ detection system (Tecan Austria, Austria)
- NanoQuant Plate™ (Tecan Austria, Austria)
- 2100 Bioanalyzer with 6000 Pico total RNA kit (Agilent Technologies, USA)

Keratinocyte sorting

Mice

For the identification and characterization of the Lgr5-expressing stem cell we used a transgenic mouse model, where the bacterial beta-galactosidase gene lacZ is inserted into the Lgr5 locus. Hence, homozygote Lgr5-LacZ alleles would result in the loss of the endogenous expression of LGR5 [4]. Only hetereozygous mice could be used since complete loss of Lgr5 expression results in perinatal lethality [5].

Cell sorting

Dorsal skin was collected from 7 mice at the age of 8 weeks (telogen) and digested with trypsin as described [6]. For isolation of the lacZ positive (LGR5-expressing) cells the Fluoreporter® LacZ Flow Cytometry Kit (Invitrogen) was used according to the manufacturer's instructions. Briefly, a chemical compound FDG (FITC-digalactopyranoside) was loaded into cells with a hypotonic shock. Note, FDG does not possess fluorescent properties. Beta-galactosidase, when present in the cells hydrolyzes FDG producing free FITC, which can be detected by flow cytometry. The cells were additionally stained with a Anti-CD34-Alexa Fluor® 647 conjugate (BD Biosciences, USA). Subsequently, FITC-positive (LGR5-expressing), FITC-negative, CD34-positive, CD34-negative and cells encompassing all populations (termed "All") were sorted using the BD FACSAria™ (BD Biosciences, USA).

RNA extraction and quality control

RNA extraction was performed using the RNAqueous® RNA extraction kit (Applied Biosystems, USA.), without DNase treatment, according to the manufacturer's instructions. The RNA was eluted in a total volume of 16 µl of RNase free water. The RNA quantity and quality (260 nm/ 280 nm coefficient) was determined using Tecan Infinite® M200 supplied with the NanoQuant Plate™. 2 µl of each RNA sample in a 1:5 dilution was loaded onto the NanoQuant Plate™ and measured. For further validation of the RNA quality, the Agilent 2100 Bioanalyzer was used according to the manufacturer's instructions (Fig. 3).

Results and discussion

In the mouse hair follicle, CD34-positive keratinocyte stem cells reside in the bulge area, whereas lacZ-expressing Lgr5-positive keratinocytes are located in the lower portion of the bulge area and in the secondary germ - located between the bulge and the dermal papilla (DP) (Fig. 1). Since the colony forming and HF reconstitution properties of the Lgr5+ cells surpassed those of the CD34+ cells, the identification of pathways contributing to these properties would greatly enhance our understanding of keratinocyte stem cell regulation.

Two possible approaches would include i) the comparison of the expression of selected genes by real-time RT PCR (qPCR) and ii) the comparison of microarray data. Both approaches require isolation of corresponding pure cell populations and the extraction of intact mRNA. For the qPCR analysis performed with very low amounts of starting material it is of high importance to use the same RNA amount from each sample for cDNA synthesis to obtain reliable results.

For extraction of the Lgr5-expressing keratinocytes, 7 mice were sacrificed and the keratinocytes from the dorsal skin were isolated as described in section “Materials and methods”. Since the Lgr5+ population (0.6 %) is very small, 45 million keratinocytes were initially isolated. The cells were loaded with FDG and stained with anti-CD34 antibody conjugated with Alexa Fluor® 647. The lacZ-expressing keratinocytes appear as FITC-positive cells on the dot plot (R 2, Figure 2). 100,000 cells from each population (R1: Lgr5-, R2: Lgr5+, R3: CD34+ and R4: CD34-) and 200,000 cells of “All” were sorted into growth media, cells were centrifuged (400 g, 7 min) and the RNA was extracted as described in materials and methods. Of note, since primary keratinocytes are extremely sticky, the sorting and centrifugation procedures cause considerable losses in cell numbers. In our situation, the total amount of high quality RNA extracted from 100,000 sorted keratinocytes equals the quantity of 2000 cultured mouse h-TERT keratinocyte cells grown in culture (data not shown). Due to the low RNA amounts, the measurements had to be performed using very low volumes of the RNA sample solution. For the quantification the Infinite® M200 supplied with the 16-channel Tecan NanoQuant Plate™ was used. The results are shown in Table 1. There is a good correlation between starting material and the concentration of the purified RNA; e.g.: the RNA amount extracted from $2x10^5$ sorted cells was nearly two times higher than that of 10^5 cells – including also the variance of the sorting procedure. Non-sorted cells yield proportionally more RNA than sorted cells (110 ng/µl vs. expected 75 ng/ µl accordingly), which might be due to the cell attachment to the collection tube walls during the sorting process. The absorbance ratio 260 nm/ 280 nm was used as purity parameter (pure RNA is 2.0).

RNA replicate 1	Cells $x10^3$	260	280	Conc [ng/µl]	260/280 Ratio
(1) CD34+	100	0.0474	0.0436	3.36	2.12
(2) Lgr5+	100	0.0462	0.0421	3.44	2.41
(3) CD34-	100	0.0487	0.0446	4.00	2.02
(4) Lgr5-	100	0.0467	0.0429	3.60	1.97
(5) All	200	0.0522	0.0458	7.20	2.15
(6) All	200	0.0523	0.0471	6.16	1.89
(7) All	200	0.0525	0.0461	7.68	2.01
(8) All	200	0.0536	0.0469	8.16	2.01
(9) Non-sorted	2000	0.1824	0.1126	109.52	1.99
RNA replicate 2	Cells $x10^3$	260	280	Conc [ng/µl]	Ratio
(1) CD34+	100	0.0482	0.0446	3.60	1.81
(2) Lgr5+	100	0.0474	0.0434	4.00	1.94
(3) CD34-	100	0.0497	0.0453	4.56	1.98
(4) Lgr5-	100	0.0474	0.0433	4.08	1.98
(5) All	200	0.0527	0.0465	7.36	2.01
(6) All	200	0.0534	0.0476	7.04	1.92
(7) All	200	0.0525	0.0462	7.44	2.03
(8) All	200	0.0528	0.0466	7.44	1.99
(9) Non-sorted	2000	0.1843	0.1138	110.56	1.99

Table 1. RNA quantity and quality - isolated from sorted keratinocytes - and measured via using the Infinite® M200 NanoQuant. The different “All” fractions represent sorting quadruplicates; Two technical-replicate measurements are shown.

Since the quality of the RNA is of utmost importance to provide reliable results, the isolated RNA was analyzed with a second independent method using the gel-electrophoresis based 2100 Bioanalyzer and 6000 Pico total RNA kit (Agilent Technologies, USA) (Figure 3). This more complex analysis is usually only available in laboratories/ institutions specializing in gene-expression.

To our surprise, the Bioanalyzer gel-electrophoresis always shows an additional band between the ribosomal 18S and 28S RNA bands when RNA from FACS-sorted cells was analyzed (Figure 3B). RNA from non-sorted cells showed the expected profile. This additional band can give the false negative impression - when looked at the automated analysis results – of poor RNA quality. Interestingly, the existence of this additional peak seen on the gel did not influence the readout of the Infinite® M200 NanoQuant, supported by subsequent testing of the amplification efficiencies, where the same amount of RNA of each sample was used, based on the NanoQuant quantitation. After excluding other possibilities, we believe that the free Alexa Fluor® 647 contaminating the RNA samples most probably caused this peak.

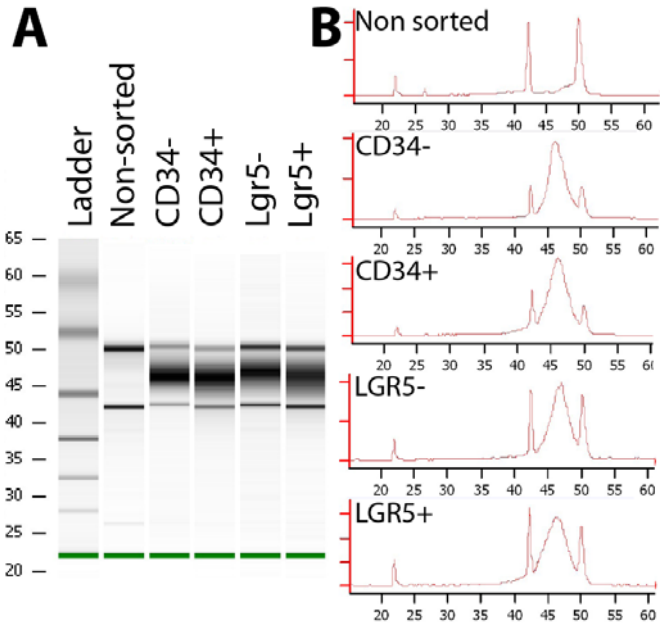


Figure 3. Evaluation of the RNA qualtity on Agilent 2100 Bioanalyzer. (A) Gel electrophoresis of the isolated RNA. 18s and 28 s RNA bands can be seen. An additional band of unknown origin was detected in between 18S and 28S (see text). (B) The corresponding electrophoresis flow charts.

Importantly, the absorption values of this dye are negligible at 260 nm and 280 nm wavelengths, thus the contamination is not expected to interfere with the measurements on the Infinite® M200 NanoQuant instrument. The amount and quality of the isolated RNA in line with the RNA quantitation was sufficient for performing analysis of more than 30 selected genes by qPCR (data not shown).

Conclusion

The value and reliability of gene expression profiles from rare cells (e.g.: adult stem cells) is largely dependent on the RNA integrity. Here we used the Infinite® M200 instrument and the NanoQuant Plate™ for RNA quantitation and quality verification. The data acquired on the Infinite® M200 NanoQuant instruments exhibited the required sensitivity and reliable RNA purity readouts when measured very low RNA concentrations. The NanoQuant Plate™ is the tool of choice for all routine quantitation and quality measurements based on absorption due to i) its ability to process multiple samples and ii) its user-friendly software interface.

Acknowledgements

Tecan would like to thank the Karolinska Institute, particularly Rune Toftgård and members of his research group, Maria Kasper and Viljar Jaks, for performing the experiments, providing their data and writing this application note. Also thanks to their co-laborators Nick Barker and Hans Clevers who provided the Lgr5 mice.

References

1. Trempus, C.S., et al., *Enrichment for living murine keratinocytes from the hair follicle bulge with the cell surface marker CD34.* J Invest Dermatol, 2003. **120**(4): p. 501-11.
2. Trempus, C.S., et al., *Comprehensive Microarray Transcriptome Profiling of CD34-Enriched Mouse Keratinocyte Stem Cells.* J Invest Dermatol, 2007.
3. Jaks, V., et al., *Lgr5 marks cycling, yet long-lived, hair follicle stem cells.* Nature Genetics, 2008.
4. Barker, N., et al., *Identification of stem cells in small intestine and colon by marker gene Lgr5.* Nature, 2007. **449**(7165): p. 1003-7.
5. Morita, H., et al., *Neonatal lethality of LGR5 null mice is associated with ankyloglossia and gastrointestinal distension.* Mol Cell Biol, 2004. **24**(22): p. 9736-43.
6. Wu, W.Y. and R.J. Morris, *Method for the harvest and assay of in vitro clonogenic keratinocytes stem cells from mice.* Methods Mol Biol, 2005. **289**: p. 79-86.

Austria T +43 62 46 89 33 **Belgium** T +32 15 42 13 19 **China** T +32 15 42 13 19 **Denmark** +45 70 23 44 50 **France** +33 4 72 76 04 80
Germany +49 79 51 94 170 **Italy** +39 02 215 21 28 **Japan** +81 44 556 73 11 **Netherlands** +31 18 34 48 174 **Portugal** +351 21 000 82 16
Singapore +65 644 41 886 **Spain** +34 93 490 01 74 **Sweden** +46 31 75 44 000 **Switzerland** +41 44 922 89 22 **UK** +44 118 9300 300
USA +1 919 361 5200 **ROW** +43 62 46 89 33

www.tecan.com

395 673 V1.0, 10-2008