APPLICATION NOTE

Optimization of RNA Isolation Methods for RNASeq Analysis Using the Harpera™ Microbiopsy Device

Jay P. Tiesman[1], Kimberly Kozak[1], John Snowball[1], Florian Lapierre[2]
1) Procter & Gamble, Mason, OH, USA
2) Trajan Scientific and Medical, Melbourne, Australia

Introduction

RNASeq is a powerful tool being used to understand skin biology at the molecular level in dermatology applications. The insights gained from RNASeq analysis of skin can be used to help characterize the molecular heterogeneity of inflammatory skin diseases such as Atopic Dermatitis and Psoriasis or help tailor precision therapies to individual patients by understanding the specific responses to treatments. RNAseq can also uncover the underlying mechanisms of skin diseases by analyzing gene expression patterns for the discovery of new biomarkers and therapeutic targets.

However, one key challenge for using this technique on human subjects is the requirement to obtain a skin specimen of sufficient quality and quantity for the downstream steps of RNA isolation, library preparation, and sequencing. The collection of skin specimens is usually accomplished using biopsy procedures (Zuber, T.J., Am Fam Physician 2002;65:1155-8), that are known to be invasive, painful, and require extended wound care for the patient. It is also time consuming (15-30 minutes for a single punch biopsy procedure) and expensive, requiring qualified medical personnel and multiple surgical instruments, making the procedure unsuitable for conducting longitudinal monitoring of the skin condition. Furthermore, depending on the size and the location of the biopsy, it is challenging to obtain clinical ethical acceptance, especially where there is a cosmetic concern and/or potential scarring post-procedure.

Dermatologists are therefore seeking minimally invasive skin sampling procedures that enable the collection of quality skin specimens that would be particularly useful for targeted biomarker/molecular analysis. Optimally, the procedure would be minimally invasive and would allow for dermatologists to collect skin specimens more often, enabling new possibilities that have been unexplored using conventional skin sampling methods.

The Harpera™ Microbiopsy Punch provides an attractive, minimally invasive alternative to conventional punch biopsies. The device is designed to be simple and rapid to operate, nearly painless, and leaves no visible scar. The microbiopsy specimen is significantly smaller than the one collected via a traditional punch method, but it allows for collecting multiple skin specimens – using several single use Harpera devices – from a single individual whether at one site and/or over time. This enables dermatologists to collect skin specimens from cosmetic and sensitive areas with ethical and patient's acceptance (Figure 1).

However, because of the low skin input (a microbiopsy), it is important to optimize the analytical methods before performing RNASeq analysis. The procedure stated in this report provides a manual method to isolate RNA samples from pooled Harpera microbiopsies that has been optimized for standard RNASeq applications.

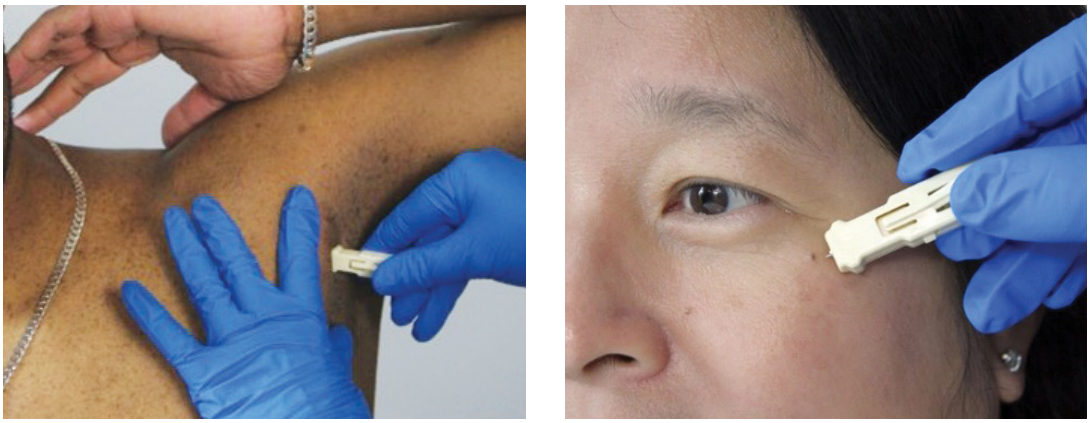


Figure 1. The microbiopsy procedure with the Harpera™ device can be performed on sensitive and cosmetic skin areas such as (a) under the arm pit and (b) the face.

Materials and methods

RNA extraction and purification:
Process flow map for skin sample collection and RNA Extraction

1. Sample Collection

- Skin specimen collected using Neoteryx Harpera™ Microbiopsy™ devices (Trajan Scientific and Medical, Melbourne, P/N#: B123-1005).
- 2 or 5 microbiopsies pooled per site.
- 3 individual arms sampled per pool.
- Collection procedure follows Neoteryx Harpera Instruction for Use (MN-1440).

2. Initial Sample Processing

- Microbiopsy collectors removed from plastic housing.
- Microbiopsy collectors placed side down in 2 mL microcentrifuge tubes containing 350 µL of RLT lysis buffer (QIAgen RNeasy Micro Kit, P/N: 74004).
- Samples stored on ice.
- Tubes vortexed for 30 sec and prepared for either shipment or immediate extraction.

3. Sample Shipment or Immediate Extraction

- **For Shipment:**
  - Flash frozen and stored at -80°C or shipped on dry ice.
- **For Immediate Extraction:**
  - Processed using QIAgen RNeasy Micro Kit (cat. no. 74004) via TissueLyser Protocol.
  - No β-mercaptoethanol (β-ME) or 2 M dithiothreitol (DTT) added.

4. RNA Extraction Procedure

- Tubes disrupted with TissueLyser/TissueRuptor at 30 Hz for 3 min.
- Centrifugation for 3 min at >13,000 x g.
- Supernatant transferred to a 1.5 mL microcentrifuge tube.
- 350 µL of 70% ethanol added and mixed.
- Sample transferred to RNeasy MinElute spin column in 2 mL collection tube.
- Centrifugation for 15 sec at ≥ 8000 x g; discard flowthrough.
- 350 µL Buffer RW1 added; centrifuge for 15 sec at ≥ 8000 x g; discard flowthrough.
- DNAse I treatment:
  - Add 80 µL mixture of DNAse I (10 µL) and Buffer RDD (70 µL).
  - Incubate at room temperature (20-30 °C) for 15 min.
- 350 µL Buffer RW1 added; centrifuge for 15 sec at ≥ 8000 x g; discard flowthrough.
- Column placed in fresh 2 mL collection tube.
- 500 µL Buffer RPE added; centrifuge for 15 sec at ≥ 8000 x g; discard flowthrough.
- 500 µL of 80% ethanol added; centrifuge for 2 min at ≥ 8000 x g; discard flowthrough.
- Column dried by centrifugation for 5 min at full speed with lid open.
- Column placed in 1.5 mL collection tube.
- 14 µL RNAse-free water added to center of membrane; centrifuge for 1 min at full speed.

5. RNA Quality and Yield Assessment

- RNA concentration evaluated using Qubit 4 Fluorometer and Qubit RNA HS Assay Kit (ThermoFisher Scientific, P/N: Q32855).
- RNA quality checked using Agilent Bioanalyzer and RNA 6000 Pico Kit.

6. Preparation for RNA Sequencing

- Purified RNA moved to RNASeq analysis (Illumina® Stranded mRNA Prep).

RNASeq Analysis:

RNA-Seq was performed using purified RNA on the Illumina® Nextseq2000 platform. In short, approximately 25 ng of total RNA from each microbiopsy sample was polyA selected, fragmented, and converted to cDNA (Illumina Stranded mRNA Prep, P/N 20040534). The cDNA fragments were adenylated, and anchors were ligated to the ends of the fragments to provide a binding site for unique dual index sequences (IDT® for Illumina RNA UD Indexes, Illumina P/N 20040553). The resulting products were purified and amplified before being pooled for sequencing on the Illumina NextSeq2000 (NextSeq 2000 P3 Reagents, P/N 20040560). After sequencing, FASTQ files were trimmed by TrimGalore. Trimmed files were aligned to RefSeq GRCh38.p14 using STAR aligner via RSEM. RSEM was then used to generate FPKM and count files. Functional enrichment analyses was performed on top expressed non-ribosomal, non ATP/NDU genes using ToppFun. Markers used for specific skin cell types are well established but were validated using control, forearm, skin, single cell data (Tabib et al., Nature Communications 2021; 12.1:4384.)

Results and Discussion

Evaluation of RNA Quantity/Quality:

RNA quantity and quality (as indicated by RNA Integrity Numbers or RIN's) are listed in Table 1 and were low overall at about 1.9 ng/µL (RIN: 3.7) and 3.14 ng/µL (RIN: 6.5) for a pool of 2 or 5 microbiopsy specimens, respectively. Such results can be explained by the low sample input that reached the limit of quantification of both the Qubit and Agilent 2100 instruments.

However, pooled microbiopsies boosted the yield to obtain sufficient input for RNASeq experiments. Indeed, the Illumina Stranded mRNA prep recommend 25 ng to 1 µg of total RNA input at a concentration of 2 ng/µL. Although there was some expected variability in yield and quality, pools of both 2 and 5 microbiopsies provided sufficient material for sequencing.

		Qubit		Agilent 2100	
Sample	**# Pooled Biopsies**	**RNA (ng/uL)**	**Yield (ng)**	**RNA (ng/uL)**	**RIN**
1	5	3.14	43.96	3.0	8.6
2	5	3.32	46.48	3.4	5.7
3	5	2.98	41.72	3.0	5.3
4	2	1.63	22.82	2.0	3.1
5	2	1.82	25.48	2.3	3.1
6	2	1.90	26.60	1.8	5.1

Table 1. RNA quantity and quality (RNA Integrity Numbers (RIN's)) measured via Qubit and Agilent 2100 on pooled of 2 or 5 microbiopsy specimen.

Evaluation of RNASeq Results:

All 6 samples were sequenced successfully and passed standard NextSeq2000 quality control metrics. The total number of reads and mapped reads for each sample are outlined in Table 2. There appears to be no direct correlation between the number of microbiopsies in a pool and total sequenced reads, which range from 10 million to 15 million reads per sample. There does appear to be a slightly better mapping percentage to both the reference genome and exons in the larger pools but the differences are minor. Although the number of reads per sample is lower than the 20-30 million reads recommended for high-quality RNASeq analysis, the range is sufficient to provide reliable data for standard gene expression profiling applications.

Sample	**# Pooled Biopsies**	**Total Sequence Reads**	**% Reads Mapped to Genome**	**% Reads Mapped to Exons**
1	5	13'592'779	90.11	87.86
2	5	15'784'027	89.73	87.08
3	5	9'969'162	90.71	88.67
4	2	14'244'759	89.80	86.41
5	2	13'838'781	89.53	86.91
6	2	10'061'007	89.00	86.53

Table 2. Total sequenced read and % reads for each of the 6 samples extracted from pooled microbiopsy specimens

Although this experiment was not designed to determine differential gene expression, an analysis of the genes highly expressed (FPKM>100) in these samples indicated an enrichment of genes associated with canonical epidermal biological processes (Table 3). Along with Immune, melanocyte and keratinocytes specific markers, markers for known dermal skin cell types including fibroblast and endothelial cells were detected in micro biopsy samples indicating this technique has the ability to capture cells from both the epidermis and dermis (Table 4).

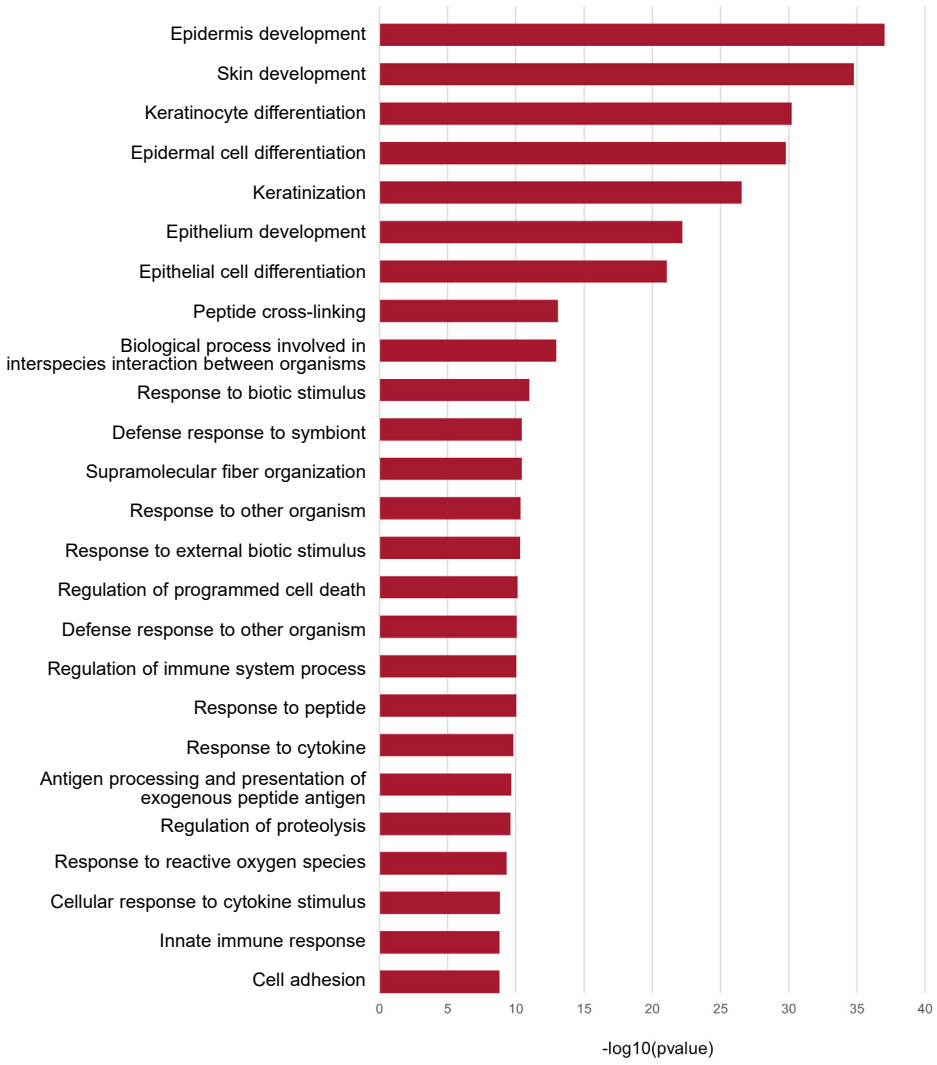


Table 3. Biological processes associated with Top Expressed Genes from microbiopsy samples

Gene	Average FPKM	Cell Type Marker
LCE2B	3261.295	Granular
LCE1C	2620.346667	Granular
SPRR2E	2016.945	Granular
LCE2C	1511.08	Granular
LCE1A	1319.27	Granular
LCE1F	1045.031667	Granular
SPRR2G	852.3983333	Granular
SPRR1B	217.79	Granular
KRT10	10119.345	Spinous
KRT1	5257.57	Spinous
KRT2	4308.97	Spinous
KRT14	3149.538333	Spinous

Gene	Average FPKM	Cell Type Marker
FLG	736.8683333	Spinous
KRT5	2653.188333	Basal
KRT15	271.795	Basal
VIM	306.3183333	Fibroblast
COL1A1	196.8133333	Fibroblast
COL6A2	92.54166667	Fibroblast
COL6A1	75.34833333	Fibroblast
TYRP1	191.0183333	Melanocyte
PMEL	185.8566667	Melanocyte
PTPRC	60.46333333	Immune
PECAM1	19.92166667	Endothelial

Table 4. List of specific immune, melanocyte and keratinocytes markers analyzed from microbiopsy samples.

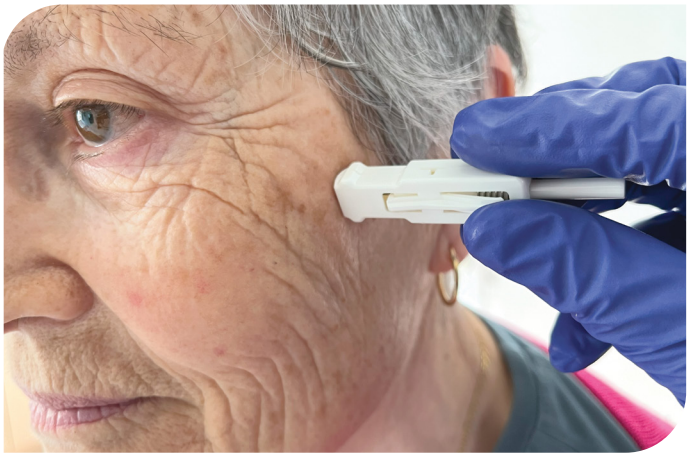
Conclusion

This report provides user indication that microbiopsy specimen collected with the Harpera™ Microbiopsy Punch can be utilized for RNAseq applications. While microbiopsy specimen are of low volume, the pooling of 2 and 5 microbiopsy specimens boosted the yield to obtain sufficient input for RNASeq experiments, which ranged from 10 million to 15 million reads per sample and up to 90 % reads mapped to Genome and 87 % reads mapped to Exons.

To the best of our knowledge, this is the first report on the use of microbiopsy skin sampling procedure for RNAseq applications and the use of such procedure could support the longitudinal monitoring of skin conditions more efficiently and with minimal disruption to patient compared to current skin sampling standard of care.

Some additional work still remains to further explore the optimization of the analytical workflow from the specimen collection to the analysis, including the optimal storage and shipment of such specimens. Moreover, the protocol described in this report is manual, thus automation workflow would need to be further evaluated in order to support fully the longitudinal monitoring of dermatology patients with skin conditions.

In conclusion, the Harpera Microbiopsy Punch provides a minimally invasive, highly efficient alternative to traditional biopsy methods, enabling dermatologists to collect high-quality skin specimens with minimal patient discomfort and no visible scarring. Its ability to collect smaller samples while maintaining quality makes it ideal for repeated, longitudinal sampling, particularly in sensitive or cosmetic areas. These features, coupled with its simple, rapid operation, significantly enhance patient comfort and convenience. By facilitating frequent, non-invasive skin sampling, the Harpera Microbiopsy Punch opens up new possibilities for advanced molecular analysis and biomarker discovery, supporting more precise and personalized dermatological care.



Visit the Harpera product page

Visit us at www.neoteryx.com or contact neo.support@trajanscimed.com for assitance and further information.

Harpera™ Microbiopsy Punch is a manual surgical device intended to enable the collection of a specimen from the cutaneous skin surface by a healthcare professional. FDA-registered for marketing in the US, Harpera is made available as an investigational use only (IUO) product for use in performance studies outside of the US.