# Q&As

Webinar: Total RNA sequencing of liquid biopsies

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## 1 What is the role of cell-free RNA in biofluids?

It's not entirely clear yet. RNA has a documented role in intracellular communication. We cannot rule out that some or the majority of RNAs reflect by-products of processes like dying cells. Whatever its function is, it can be exploited as a biomarker as long you can show sensitivity and specificity of the biomarker.

## 2 Would you recommend to study platelet-free or platelet-rich blood plasma?

I briefly discussed the tumor educating platelet hypothesis and we were initially considering studying platelet-rich plasma. However, our pilot experiments indicate that RNA from tumors is not necessarily higher, if not lower in platelets. We reconsidered, and we favor platelet-free plasma.

## 3 What is the RNA concentration in the liquid biopsy eluate?

I don't know since we never measure it. Typical measurement methods (spectrophotometry and fluorimetry) are just not sensitive enough for robust measurements. We use 200µL of urine or plasma and simply add spikes at the RNA extraction level and in the eluate, and we use these spikes to monitor our process, and to correct for variables in RNA input amounts.

## 4 What means per unit of biofluid volume? ml?

Yes, per ml (or µl), whatever you want to use.

## 5 Working with urine as source biofluid can be very challenging as even collecting the same quantities, it can be really concentrated or diluted. How to normalize the data? Some suggestions?

Normalization comes at the end. We recommend working on the standardization of the collection method first. Take morning urine or midstream for example, and work on standardizing that collection method (make sure that concentration of certain metabolites or salts is very similar for instance). Once you standardized the collection method, do a quick spin to remove cells, take supernatant and add spikes during extraction and library preparation. Different suggestions for normalization methods: spikes are used to normalize on urine volume (if you used spikes during RNA extraction) or normalize on RNA volume if you used spikes present in the eluate, or normalize using endogenous RNAs. We have seen different scenarios where different normalization methods are better. How then to assess if normalization works? For example, if data makes more sense (more differentially expressed gene, or differential pathways by confronting with the literature), large fold changes, or

more significant p values, or combinations of these three. There's no good or bad method as long as you clearly state the method you used.

## 6 What is the purpose of using ERCC and why the concentration of ERCC and sequin running opposed to each other?

ERCC spikes were developed by the External RNA Controls Consortium (ERCC) and are made commercially available by ThermoFisher. The opposing order between ERCC and sequin is not extremely important, we just wanted to have approximately equal spike concentrations in all samples.

## 7 How long we can store EDTA tubes at -80 before serum isolation?

EDTA is an anti-coagulant to prepare plasma. Serum results from coagulation. Anyway, we have not tested the freezing of blood followed by plasma or serum preparation. I would not recommend it, as blood cells will likely lyse and leak into the extracellular space.

## 8 I use Trizol coupled with Monarch RNA kit (NEB) to isolate extracellular vesicle RNA from pleural fluids. After I isolate RNA I precipitate it with Etoh precipitation, I get good qPCR results but my 260/280 and 260/230 are abysmal. How can I solve this?

Don't worry! It's not a problem. We don't look at these ratios. These ratios are only meaningful if measured in pH neutral solutions. In my experience, these ratios have never been predictive for quality of RT-qPCR or RNA-seq data.

## 9 What % of the circulating mRNA is cell-free and bound in vesicles? How do their stabilities compare?

Regarding the stability, for now, we don't know. We plan to perform SLAM-seq (metabolic labeling). By doing so, we will be able to do a proper assessment of stability. The majority of mRNA is outside vesicles. It is different for miRNAs, where we show that miRNAs are more abundant in vesicles than longer RNAs.

## 10 How much volume of CCM starting material is generally enough to isolate EVs for RNA-seq?

Please see our paper in Scientific Reports, 2019. If you have more questions, please reach out to EV expert - Prof. An Hendrix, Ghent University.

## 11 What is the "best"/"easiest" input type of material to use?

For a liquid biopsy, serum and plasma are easy to obtain. Both work well for RNA profiling. When it comes to plasma, I recommend platelet-free plasma.

## 12 How does hemolysis affect the results of the lncRNA analysis? How do you assess the hemolysis?

During hemolysis, red blood cells (full of RNA) lyse and RNA content contaminates the extracellular RNA. When studying lncRNAs, fewer reads will go to these lncRNAs (because consumed by red blood cell RNA). You can assess by OD measurements at 414 nm.

## 13 How did you extract the RNA from FFPE samples for sequencing?

It was Qiagen for FFPE and we also have good experience with Promega (there may be some other vendors). The extraction of RNA from FFPE is currently not the biggest challenge and sequencing not anymore as well.

## 14 Is there a need for doing an RNAse Digest to exclude cfRNA and just end up with EV miRNA?

Yes, this is the recommendation. To make sure that RNA resulting from EV purification is RNA contained inside the vesicles, it is strongly recommended treating vesicles with RNase and

sometimes also with proteases. By doing so, you can have a very good understanding of whether the RNA is inside the vesicles or bound to vesicles.

## 15 How many plasma samples should be used to get reliable data if searching for specific circulating lncRNA with RNA-seq in patients/control type of study?

More! A strong recommendation is to include as many as possible. A good start is 24 patients per group (treated vs untreated) for group-based analysis. If you have matched samples from the same patient or donor, you can get away with fewer patients or donors (12 per group to start with). In that case, the intrinsic variability or interindividual variability is compensated by looking at differences over time in the same patient or donor. Learn from these pilot studies and then do a proper power analysis to determine the numbers you need. LncRNAs are typically low abundant. With the low abundance and noisy data, you will need more data points and you will need to increase sample input volume. For instance, instead of working with 2-4 ml of plasma, use 4-5 ml of sample input. This will lead to a much better signal, better data and hence better statistics.

## 16 I am going to do a research on non-small lung cancer. Can I perform NGS for 12 biopsies, for example, then validate the NGS of miRNA abundance by using plasma? Or, should I use plasma for NGS and also for validation? For healthy and NSCLC patients, I will use RT-qPCR to validate miRNAs expressions.

There is not so much in the literature on matched tissue and fluid profiling. We have done similar studies for esophageal cancer. We have fluids and matched diseased tissues and we are analyzing that. You can, of course, combine RT-qPCR and NGS. We prefer to use longer RNAs using this total RNA-seq approach than miRNAs because the number of biomarkers is typically 1-2 orders of magnitude larger; miRNAs being in the range of 400-600 and longer RNAs in the range of 6,000-10,000 genes per sample. We have preliminary evidence that mutant RNA from cancer can be detected in plasma. However, I cannot comment further on sensitivity, but it is possible to detect mutant RNA.

## 17 Is there already a publication made from the methods and/or results from Case 2 about the extracellular vesicles?

This is published in the Scientific Reports 2019, in which we presented the Pico v2 method.

## 18 Which tube is recommended for urine sample collection and which RNA extraction kit is recommended?

Any container is good for urine. The Colli-Pee device is quite convenient. The best RNA extraction kit depends on the volume you have and RNA biotype you are interested in. As RNA conc. is low in urine, I would recommend Qiagen's ccfDNA/RNA that can process up to 4 ml.

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