STUDY OF PRE-ANALYTICAL VARIABLES IN PLASMA AND BREAST BIOPSIES TO BE USED FOR PROTEOMIC AND GENOMIC STUDIES

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ABSTRACT

Pre-analytical variables introduced during clinical sample collection and processing can significantly impact the molecular integrity of specimens and bias the results from biomarker studies. As part of our bio-banking effort we have performed pilot studies to better understand the issue of pre-analytical variability in blood and biopsy specimens and its effects on DNA, RNA and protein analyses. I n the first study we investigated the effect of collecting blood in tubes containing protease inhibitors (P100 tubes) or platelet activation inhibitors (CTAD tubes). We assessed the effects of different processing protocols and of delaying processing by keeping samples at room temperature for up to 6 hours. We measured the levels of 27 cytokines using a Bio-Plex assay and the levels of 55 mid- and high abundant proteins using MRM/MS technology. In addition, we present results from our study on breast core biopsies looking at methods of collection and processing for use in genomic applications. As part of our tissue banking initiative we collect core biopsies from primary breast tumors and metastatic lesions. Three core biopsies are collected, two in RNAlater and one snap frozen. B iopsies in RNAlater are stored for 72h at 4°C and then washed with 1xPBS and embedded in OCT or snap frozen for later OCT embedding. We compared to biopsies processed similarly but without the 1x PBS wash step. Cryostat sectioning of a single slice from OCT embedded biopsies was done to analyze percent tumor cells. DNA and RNA were extracted from whole biopsies containing at least 70% tumor and we evaluated DNA and RNA yield as well as RNA and DNA integrity. In addition, we assessed tissue morphology using H&E staining. Results from our pilot studies have guided us in the establishment of standard operating procedures for the collection of plasma and breast biopsies for biomarker studies.

Introduction

• Biomarker driven clinical trials integrating the collection of tissue and blood during patient treatment are essential for the development of personalized medicine.

• The collection of biospecimens using standard operating procedures is essential to control for preanalytical variability and ensure the quality of biospecimens collected. However, the Implementation of SOPs remains a challenge in the clinical setting, particularly in the case of multicentral trials.

• There are numerous factors to consider when implementing clinical biomarker studies:

- Is Freezing possible at different sites? If not, what are the alternatives?
- The plasma proteome can be affected by how blood is collected, processed and stored.
  - Protein degradation or protein release from platelets can bias the results from biomarker studies.

•Processing of the biospecimen will depend on the molecular analysis/biomarker to be analysed.
- Use of formalin, RNAlater® or frozen sample

•Need to perform a QC of collected samples to make sure they qualify for downstream molecular a nalysis :
- •% tumor cells
- •RNA and DNA integrity
- •Protein degradation

Objectives

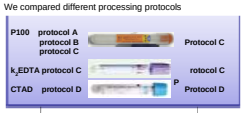
• To analyse the effect of plasma collection and processing on low abundant cytokines and mid and high abundant plasma proteins.

•To develop protocols for the collection and processing of breast biopsy specimens to ensure high quality biospecimens for downstream genomic applications.

Methods of blood collection and processing

• We collected blood from 10 healthy volunteers in:
- •P100 tubes containing k-EDTA and cocktail of protease inhibitors
- •CTAD tubes which inhibit platelet activation
- •K-EDTA tubes

We compared different processing protocols



• Samples were processed within 15 minutes of collection (time 0).

•The levels of cytokines were measured with the Human Cytokine 27-plex assay (Bio-Rad)

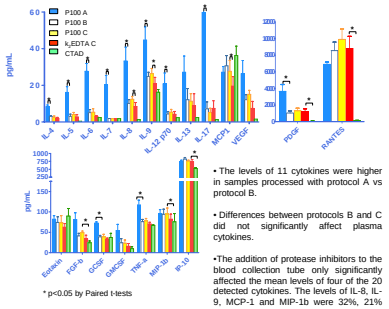
• Samples were processed at different time points after collection (time 0, 2 hours and 6 hours)

•The levels of 55 mid and high abundant proteins were measured with LC-MRM/MS

Protocol	RCF (x g)	Time (minutes)	Temperature
A	2000	5	8ºC
B	2500	20	RT
C	1300 2500	10 15	RT RT
D	1300	10	RT

Plasma Analysis

Cytokine Measurement



* p<0.05 by Paired t-tests

• The levels of 11 cytokines were higher in samples processed with protocol A vs protocol B.

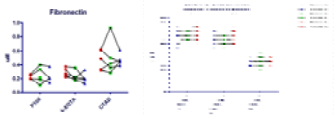
• Differences between protocols B and C did not significantly affect plasma cytokines.

•The addition of protease inhibitors to the blood collection tube only significantly affected the mean levels of four of the 20 detected cytokines. The levels of IL-8, IL-9, MCP-1 and MIP-1b were 32%, 21% 29% and 13% higher in P100 tubes compared to k_2EDTA respectively.

•Only 11 cytokines could be detected in CTAD samples. Lower levels of cytokines were measured with PDGF and RANTES being the most affected.

Multiple Reaction Monitoring

LC-MRM/MS analysis of plasma digests was performed as previously reported (Kuzyk et al 2009).



2 hours and 6 hours after collection respectively.

Conclusions from plasma analysis

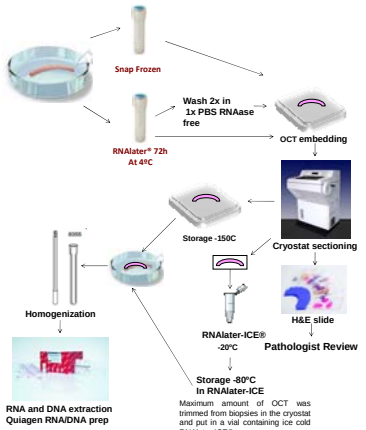
•The addition of protease inhibitors to blood collection tubes do not confer any advantage over k-EDTA tubes for proteomics studies.

•Temperature and speed during centrifugation have a significant impact on low abundant cytokines.

•Levels for most cytokines are low or undetectable in samples collected in CTAD tubes which inhibit platelet activation.

•Platelet activation also affects the levels of mid and high abundant plasma proteins.

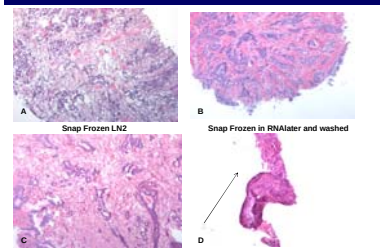
Methods of tissue collection and processing



Maximum amount of OCT was trimmed from biopsies in the cryostat and put in a vial containing ice cold RNAlater-ICE®.

Tissue Analysis

H&E staining for histological analysis



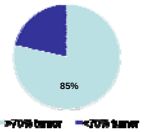
•Treating tissue with RNAlater does not affect tissue integrity, morphology is compatible with histological interpretation and assessment of percent tumor cells.

•It was not possible to obtain a cryostat section for H&E staining from specimens treated with RNAlater that were not washed (D). Arrow points to section of missing tissue.

•The presence of RNAlater impedes proper embedding, cutting and transfer to the slide.

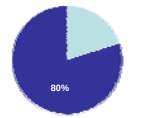
Tumor content in biopsies from primary tumor and metastatic specimens

Sample	% Tumor Cells
100-A	70%
100-C	70%
101-A	70%
101-C	90%
95-A	90%
95-B	95%
86-A	90%
86-B	0%
87-A	90%
87-B	80%
88-A	0%
88-B	0%
94-A	90%
94-B	90%



•Percent tumor cells in breast biopsies from 7 different patients. Two biopsies were collected per patient and put in RNAlater® (A or C) or snap frozen (B).
•There is good concordance in % tumor content between matched bio psies. O nly in one patient both biopsies did not contain any tumor.
•85% of collected breast biopsies met the quality control criteria of >70% tumor cells

Sample	Metastasis	% Tumor Cell
BM-04	Skin	0%
BM-05	Skin	50%
BM-06	Skin	0%
BM-07	Skin	?
BM-08	Liver	90%

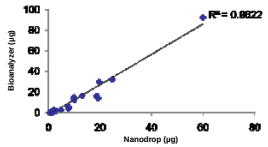


•Percent tumor cells of biopsies from metastatic sites. Only the liver biopsy had high tumor content.

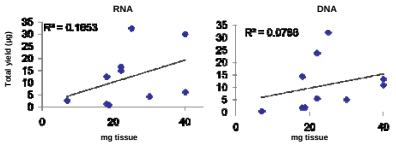
•None of the punch skin biopsies passed the quality control criteria.

•This prompted us to modify our method of performing skin punch biopsies.

Nucleic Acid Isolation



The level of RNA was measured in 27 biopsy and mastectomy samples with a Bioanalyzer or Nanodrop. There is a good correlation between the Levels measured with both technologies, however, the Bioanalyzer was more sensitive. We used the Bioanalyzer reading for subsequent analyses.



There is no direct correlation between RNA or DNA y ield with biopsy weight (mg).
DNA and RNA were simultaneously extracted (Qiagen DNA-RNA kit) from 10 breast biopsies containing at least 70% tumor.

Average RNA and DNA yield

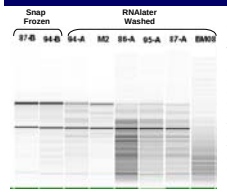
Sample type	Processing pr or o OCT embedd ng	Processing after cryostat sect oning	Average RNA yield (µg)	Average µg RNA mg t ssue	Average DNA yield (µg)	Average µg DNA mg t ssue
Biopsy	Snap frozen	-150C	22.7	0.716	17.35	0.216.8
Biopsy	RNAlater® wash	-150	11.53	0.552	9.87	0.442
Biopsy	RNAlater® no wash	-150	3.69	0.111	7.53	0.215
Biopsy Like	Snap frozen	RNAlater- Ice	1.275	0.0368	0.92	0.026
Biopsy Like	RNAlater® wash	RNAlater- Ice	1.36	0.054	1.88	0.062
Biopsy like	RNAlater® no wash	RNAlater- Ice	3.477	0.206	3.94	0.151

RNA and DNA yield obtained from biopsies (>70% tumor) or biopsy like specimens obtained from mastectomies (>50% tumor cells).

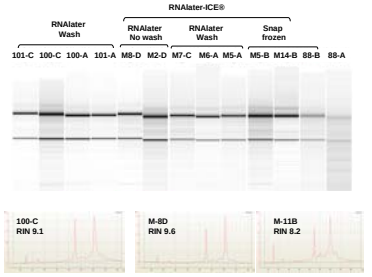
•High yields of R NA and DNA were obtained from snap frozen and RNAlater treated biopsies .

•RNAlater-ICE® was used to facilitate the extraction of the tissue sample from the OCT, OCT pealed off easily from the biospecimen and the pieces could be weighed and homogenized, however t his significantly decreased R NA and DNA yield.

RNA and DNA quality



Gel view of Bioanalyzer results for RNA integrity. Biopsy specimens were snap frozen or stored in RNAlater for 72 h. All samples were embedded in OCT, RNAlater samples were washed before embedding. After cryostat sectioning of the slide to be used for H&E staining, the biopsy was taken out of the OCT block by pouring RNAlater on top and pulling out the biopsy with tweezers. In this first set of samples only snap frozen biopsies had good quality RNA (RIN> 7) which resulted in modifications to the wash procedure.



Bioanalyzer results for RNA integrity.
• A second set of samples were processed and the washes were done with freshly prepared ice cold RNAse free 1X PBS and manipulation during the wash was done on dry ice.
•For frozen OCT blocks, the specimen was recovered by pouring RNAlater on top of the block and pulling out the biopsy with tweezers.

•All different methods yielded high quality RNA: RIN>7 and Nanodrop absorbance 260/280 >2.0

•For all samples DNA quality assessed by Nanodrop was high: absorbance 260/280 >1.8

•Sample 88A from the previous batch shows RNA degradation as seen before.

Conclusions from tissue analysis

• Histological control of percent tumor cells in biopsy specimens is necessary to:
- ensure optimal representation of tumor in the specimen (>70%)
- to identify the need for modifying methods of biopsy collection if necessary

• Cryostat sectioning of RNAlater® treated OCT embedded tissue is only feasible if RNAlater® is washed out prior to OCT embedding

• Breast tissue morphology for histological analysis is well preserved in RNAlater® treated tissue that has been washed and in snap frozen tissue

• Simultaneous extraction of DNA and RNA from snap frozen tissue and tissue treated with RNAlater® (washed) yield nucleic acids of optimal concentration and quality for downstream genomic applications.

•The use of RNAlater-ICE® facilitates the recuperation of OCT embedded tissues, yields good quality RNA however the yield of RNA and DNA are significantly decreased.

•Careful washing procedure is necessary to guarantee integrity of RNA since tissue is no longer protected after RNAlater® has been washed out.

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