

## Before You Analyze a Human Specimen, Think Quality, Variability, and Bias

Mark David Lim and Anthony Dickherber

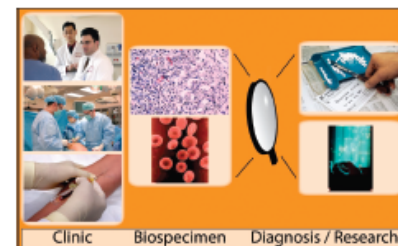
AAAS Science and Technology Policy Fellowship Program and National Cancer Institute, National Institutes of Health

Carolyn C. Compton

National Cancer Institute, National Institutes of Health

Personalized medicine requires capabilities to detect and measure health-associated biomarkers with increasingly specific and sensitive methods, putting analytical chemists at the front lines of translational research. Analytical scientists must be upstream in the experimental design process because the analysis of a biospecimen (tissue, blood, etc.) presents technical and experimental design complexities. (To listen to a podcast about this feature, please go to the *Analytical Chemistry* multimedia page at [pubs.acs.org/page/ancham/audio/index.html](http://pubs.acs.org/page/ancham/audio/index.html).)

To eliminate the barriers between biomedical discovery and clinical application, translational research must bring together experts from different disciplines to collectively share knowledge and skill sets with the goal of improving patient care. This new paradigm for biomedical research is essential for making "personalized medicine" a reality and creates an environment for clinicians and scientists to match unmet needs with innovative approaches. Analytical scientists are essential members of these interdisciplinary teams because the vision of personalized medicine is to improve the standard of medical care by including an analysis of the patient's molecular profile as part of the decision-making process. Unlike the current practice that depends on a clinician's ability to diagnose using symptoms and measurement of oftentimes broadly interpretable biomarkers (such as cholesterol), this next generation of biomarkers aims to use more precise, higher resolution assays to target an individual's biology. Ideally, this profile would contain customized information that aids in the early detection of disease, and once detected, another assay would monitor the disease's natural course using prognostic markers. Predictive markers embedded within a device would guide the prescription of a personalized therapeutic regimen, determine proper dosing, and directly monitor efficacy of treat-



ment in real time. Examples of combined therapeutic-diagnostic interventions that are currently on the market include Genentech's Herceptin and Novartis's Gleevec, which diagnose and treat patients with Her2/neu positive breast cancer and Philadelphia chromosome positive chronic myeloid leukemia, respectively.

As the source of molecules used to detect disease, the "raw material" used for clinical diagnosis is a human tissue or biofluid, generically known as a biospecimen. These biological materials are an observation window into a patient's health and are essential for the discovery and validation of biomarkers that serve as the foundation for most next-generation clinical technologies, including clinical and pathologic diagnostic tests, therapeutic drugs, and imaging agents.

The research discipline of the biospecimen sciences focuses on rigorously identifying the impacts of pre-analytical variability to a biospecimen's molecular composition and developing systems for annotated data to better inform the downstream researcher. As an integral component of translational research, this science aligns with the quality demands brought about by rapid advances in analytical methodologies and tools, the increased lability and

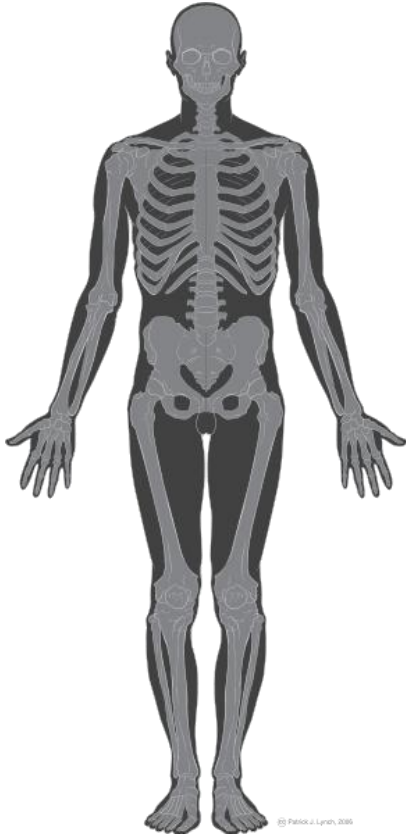
# Before You Analyze a Human Specimen, Think... Quality, Variability, and Bias

Mark David Lim, Ph.D.



Montreal In Vivo 2011

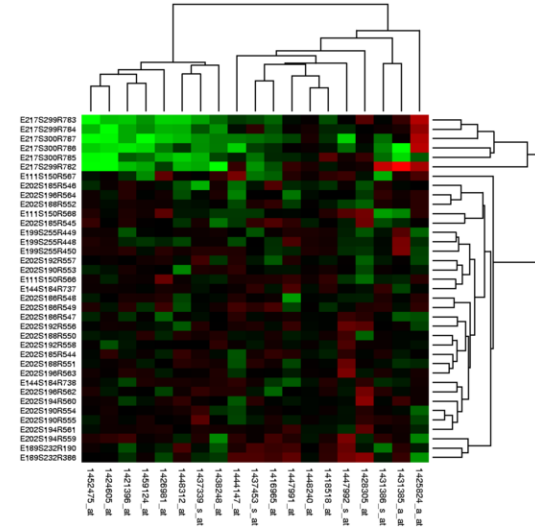
# Personalized Medicine



**Molecular-based profile**



**“biomarkers”**



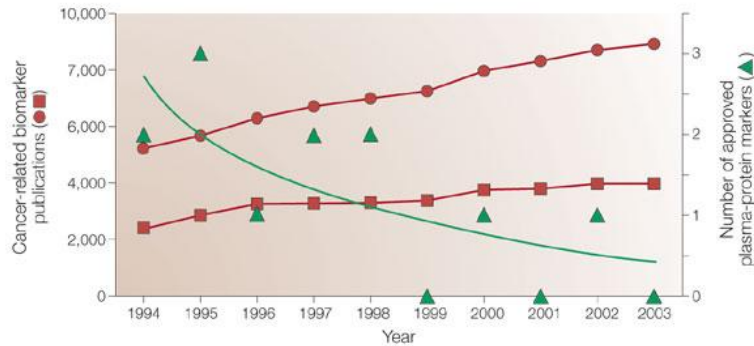
- predict a patient's response to therapy
- early detection of disease
- predict the course of disease
- monitor efficacy of treatment

*The right drug for the right person for the right duration.*

*Diagnostics that can predict, diagnose or track disease; or, can be used to track treatment outcome.*



# Biomarkers: lost in translation?



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Nature Reviews | Cancer

June 2011, JAMA

**REVIEW**

## Comparison of Effect Sizes Associated With Biomarkers Reported in Highly Cited Individual Articles and in Subsequent Meta-analyses

John P. A. Ioannidis, MD, DSc  
Christie A. Panagoulas, MD

**M**ARKERS IN BIOMARKER studies are usually proposed to predict the prognosis of disease risk, prognosis, or response to treatment. The practice of statistically significant associations to increase expectations for improvement tends to appear as "biomarkers" in the medical literature. However, many biomarkers are not validated in a rigorous manner. The translation of biomarkers from bench to bedside for the effect size and potential for clinical use is a complex process. It is particularly challenging in the case of biomarkers that are highly cited, as they often have a large effect size and are often cited in highly cited meta-analyses. This review compares the effect sizes of biomarkers reported in highly cited individual articles and in subsequent meta-analyses.

**OBJECTIVE:** To compare whether the magnitude of the effect sizes of biomarkers reported in highly cited studies is accurate or overestimated.

**DESIGN:** Systematic review. We included biomarker studies that had a relative risk associated with the biomarker in the abstract, eligible articles were those that had received more than 50 citations in the ISI Web of Science and that had been published in one of the highly cited biomedical journals. We also searched PubMed for subsequent meta-analyses on the same biomarkers from 1990 to 2009.

**SETTING:** The highly cited studies, abstracts were focused on the disease outcome, biomarker under study, and had reported relative risk in the abstract. From each meta-analysis, we extracted the overall relative risk and the relative risk in the largest study. Data extraction was performed independently by 2 investigators.

**RESULTS:** We included 17 highly cited biomarkers. For 20 of the 25 (80%) highly cited studies had a stronger effect estimate than the largest study. For the largest study was also the highly cited study, and only 10% (2 of 20) highly cited studies, the corresponding meta-analysis found a smaller effect estimate. Only 13 of the meta-analyses were statistically significantly superior based on the largest meta-analysis of these only 7 had a relative risk point estimate greater than 1.3.

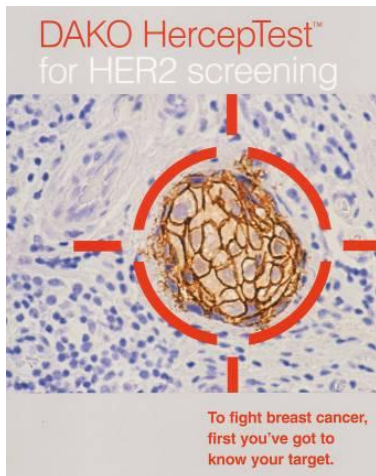
**CONCLUSION:** Highly cited biomarker studies often report large effect estimates for biomarkers associated with disease outcomes. However, subsequent meta-analyses evaluating the same associations...

**KEY WORDS:** Biomarkers, effect sizes, meta-analysis, relative risk, highly cited studies.

**INTRODUCTION:** We compared biomarkers that had a relative risk (RR) in the abstract of the top 10% of biomarker studies published in the ISI Web of Science and that had received more than 50 citations in the ISI Web of Science until December 2010.

**CONCLUSION:** Highly cited biomarker studies often report large effect estimates for biomarkers associated with disease outcomes. However, subsequent meta-analyses evaluating the same associations...

## Herceptin® and HercepTest®



Amplification of HER2/neu (ErbB2) protein (20-30% of early-stage breast cancers)

*Reduces risk of relapse by 50% in adjuvant setting*

Level of overexpression is the determined by an **immunohistochemical** assay

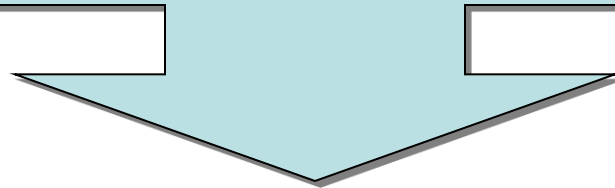
14-16 % False positive  
\$70,000 treatment

18-23 % False negative



# The *biospecimen* - The *personalized* part of personalized medicine

***Window into the health of an individual***



**Identify targets for drug development, treatment and prevention**

**Identify biologic variations that determine drug efficacy and drug toxicity**

**Define markers for susceptibility, screening and reoccurrence**

**Validate new therapeutics and diagnostics**

# Biofluids are a valuable source of biomarkers

**Table 3** Human biological fluids: a source for biomarker discovery.

Human biological fluid	Cancer type
Plasma	Broad spectrum of diseases
Serum	Broad spectrum of diseases
Cerebrospinal fluid	Brain
Nipple aspirate fluid	Breast
Breast cyst fluid	Breast
Ductal lavage	Breast
Cervicovaginal fluid	Cervical and endometrial
Stool	Colorectal
Pleural effusion	Lung
Bronchoalveolar lavage	Lung
Saliva	Oral
Ascites fluid	Ovarian
Pancreatic juice	Pancreatic
Seminal plasma	Prostate and testicular
Urine	Urological

Kulasingam V and Diamandis EP (2008) Strategies for discovering novel cancer biomarkers through utilization of emerging technologies  
*Nat Clin Pract Oncol* doi:10.1038/ncponc1187

# A biospecimen roadmap

The world of the analytical scientist



**Bias**

Results may be reproducible, but not truly representative of the broader healthy / diseased populations because of non-comparable patient selection criteria (as one example of a potential source).

**Pre-analytical Variability**

Results are dependent on collection, processing, storage conditions.

## Bias in patient selection (Case study: breast biopsy)

Representing "healthy"	Representing "diseased"
	Sex
	Age
	Medications
	Specimen Storage/Processing Differences

***Not detectable by larger sample sets or statistical massaging  
Reproducibility does not represent the lack of bias***

## Pre-analytical Variability

*Biospecimen is still reacting to stresses during its collection, processing, and storage*

*How can we tell biologic fact from biologic artifact?*

Pitfalls for translational research using human specimens:

- **Varying methods** of collection, processing, and storage can alter the physical/biologic state of the specimen
- **Varying associated specimen data** elements alter what the scientist knows about the character/nature of the specimen
- **Variable clinical information** alters what the scientist knows about the patient (biologic context of the specimen)
- **Variable restrictions** (patient consent; other ethical, legal, and policy issues) alter what the scientist may do with the specimen and/or data

# Tissue Biospecimens

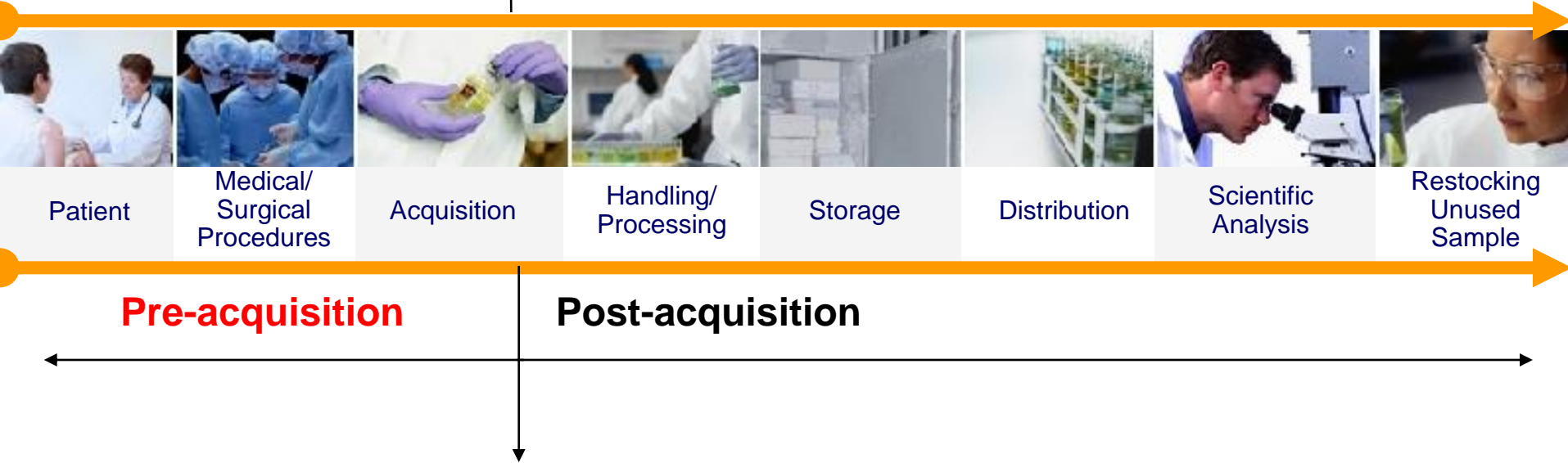
## Variables (examples):

- Antibiotics
- Other drugs
- Type of anesthesia
- Duration of anesthesia
- Arterial clamp time

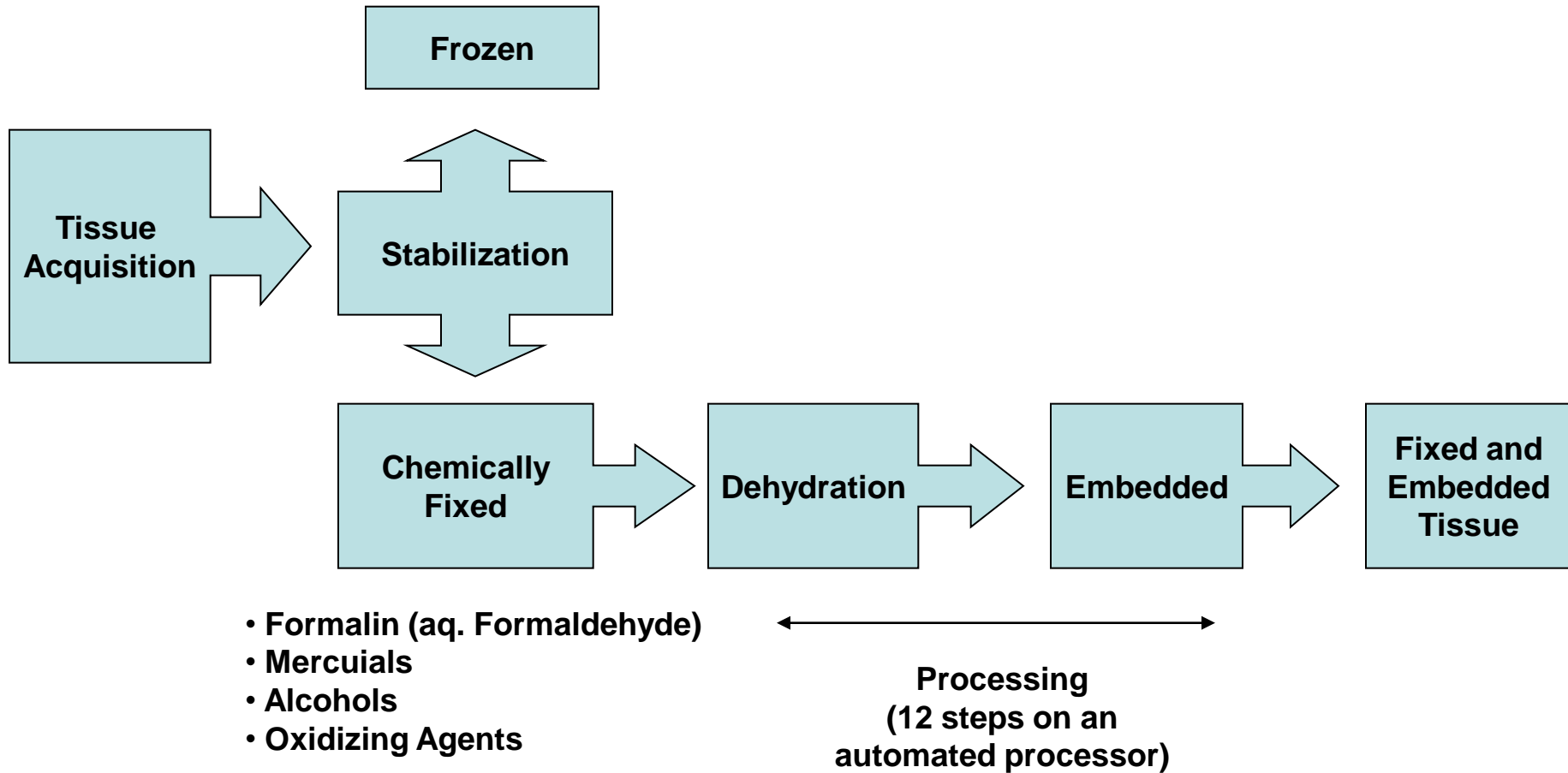
Time 0

## Variables (examples):

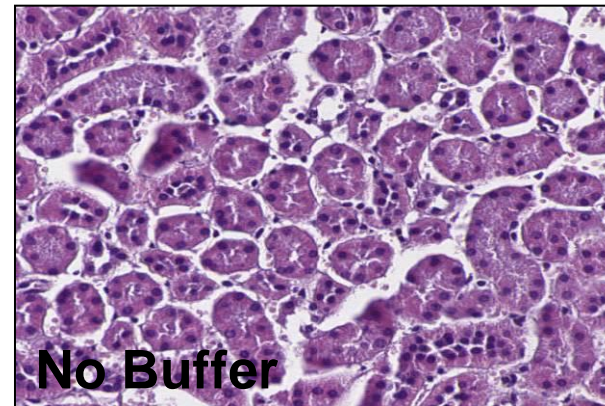
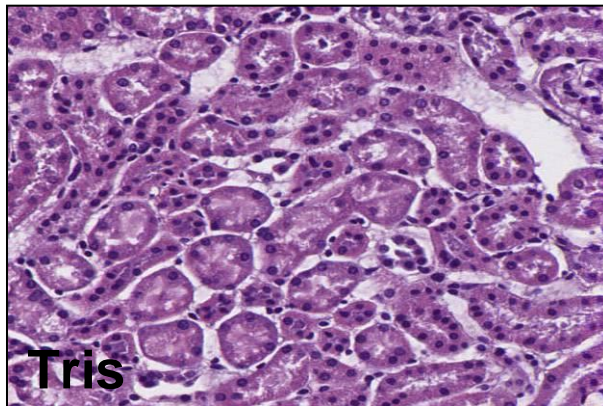
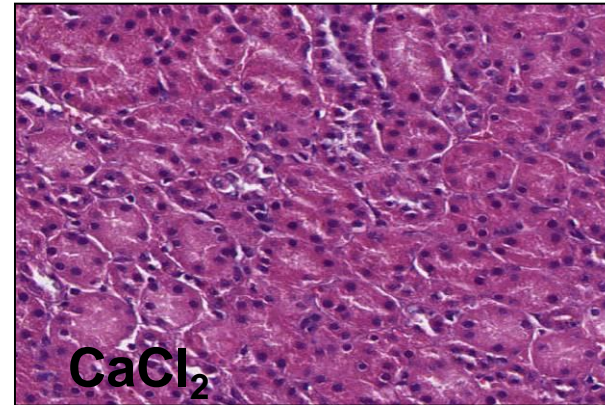
- Time at room temperature
- Temperature of room
- Type of fixative
- Time in fixative
- Rate of freezing
- Size of aliquots



# Pre-analytical variability: Tissue Preservation

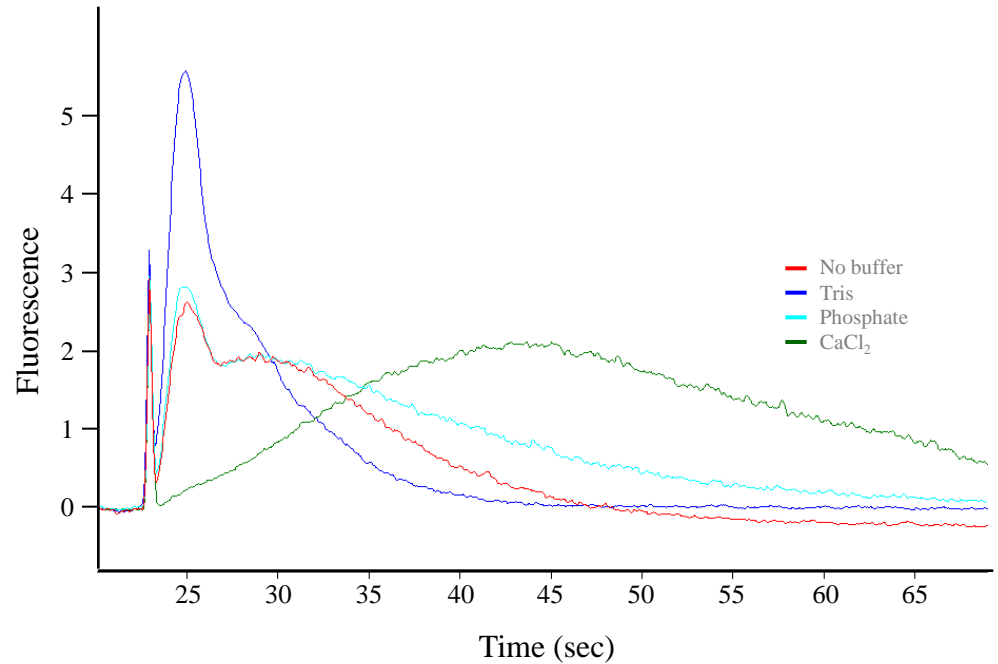
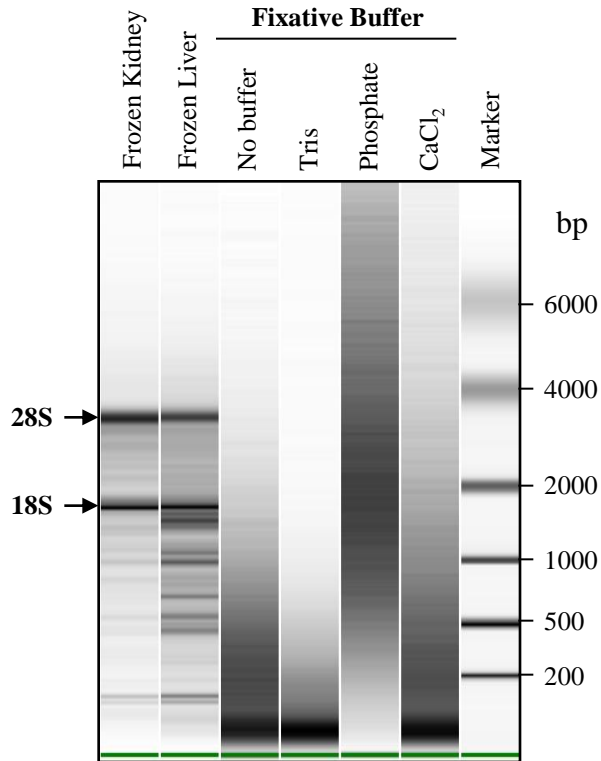


# Effects Of Formalin Buffer On Histology (courtesy of S. Hewitt MD PhD, NCI)



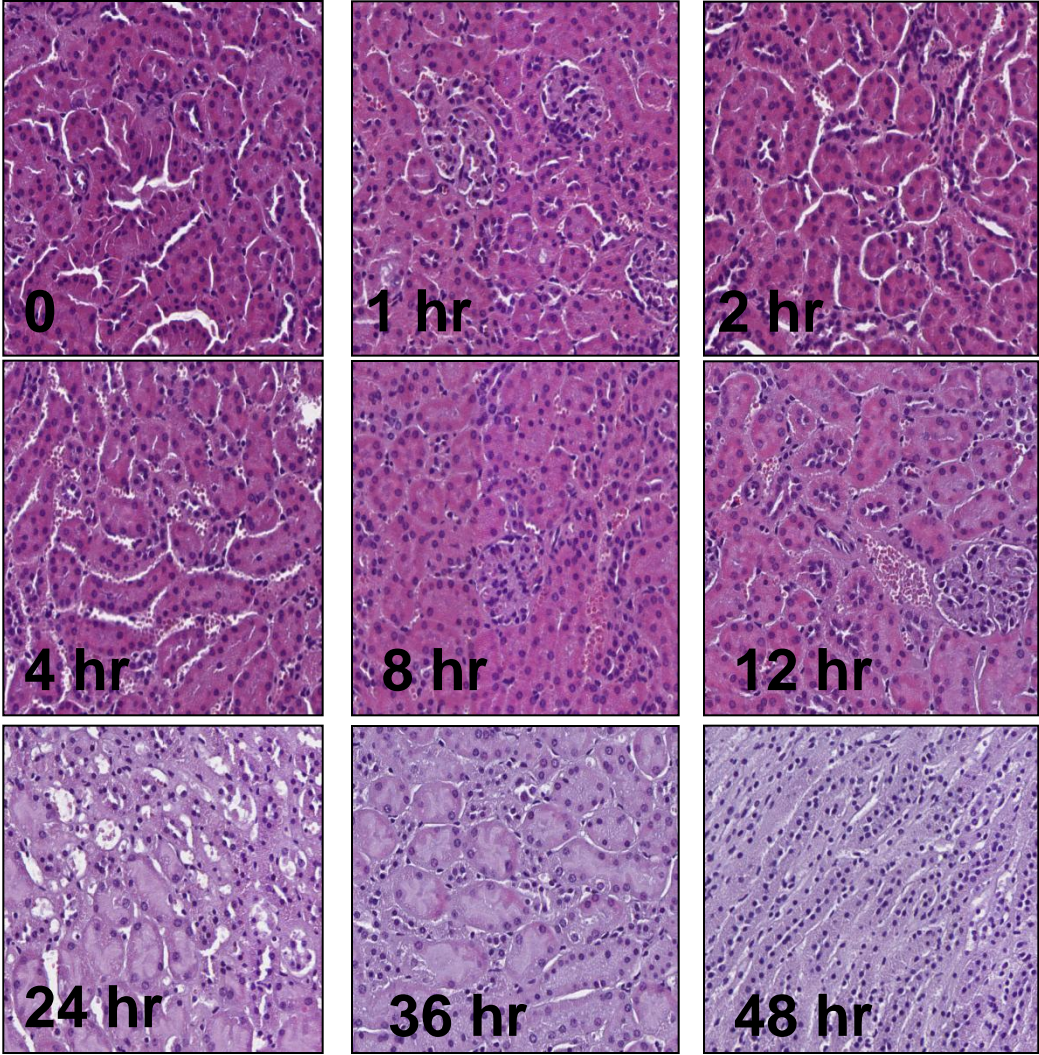
← Shrinkage

# Effect Of Formalin Buffers On RNA Quality



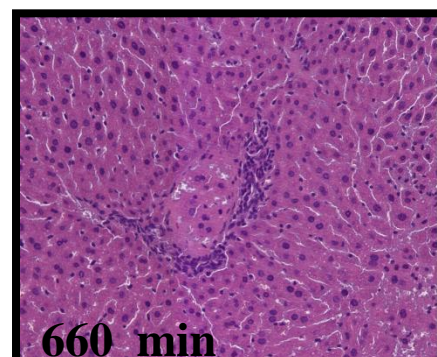
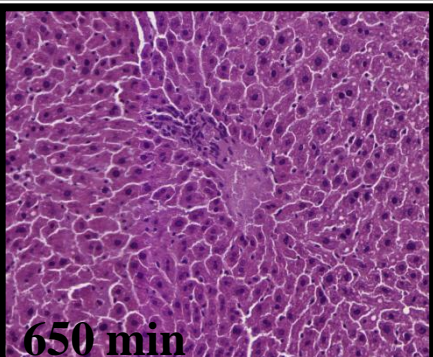
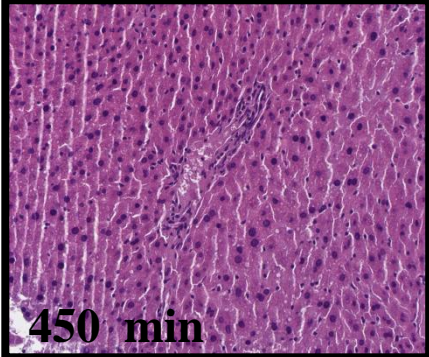
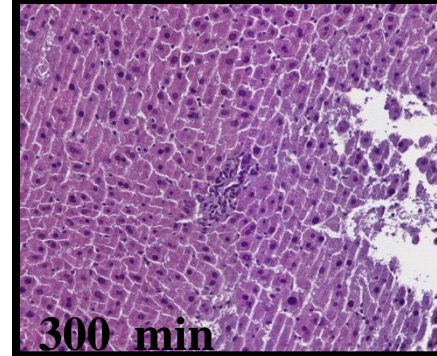
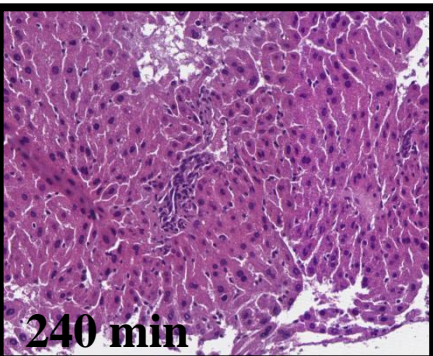
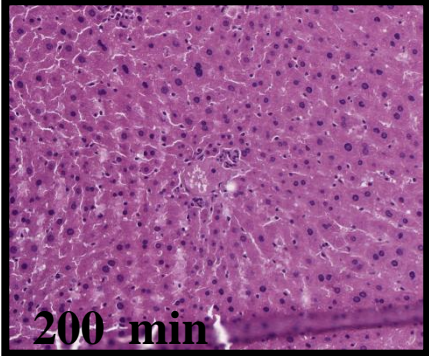
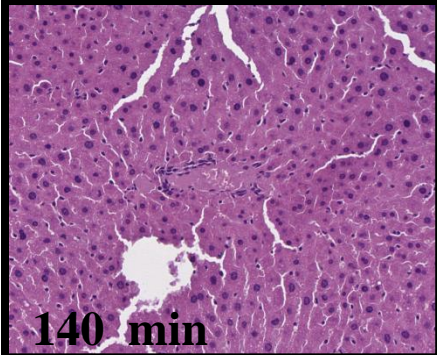
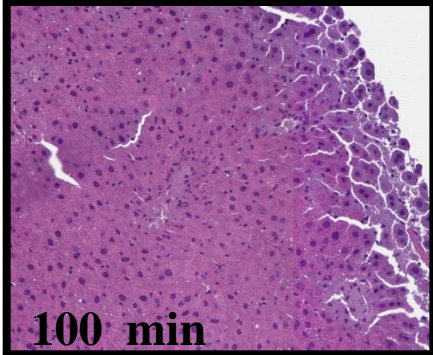
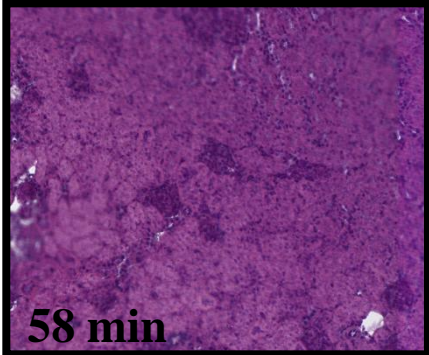
**Conclusion: Phosphate - CaCl<sub>2</sub> > No buffer > Tris**

# Effects Of Fixation Time On Histology



← Smeared

# Effect Of Processing Time On Histology



# Blood is a complex biospecimen



- Travels through all tissues in the body
  - 60,000 miles of vessels
  - Important for tumor growth and metastasis
- Readily collected in the clinic

## Numerous Roles:

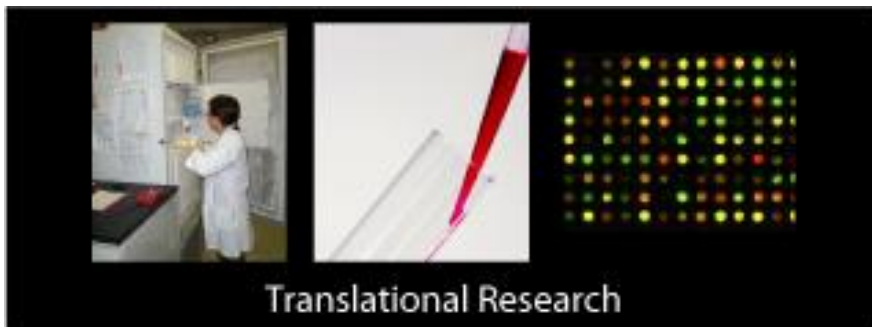
- delivery of nutrients
- removal of metabolites
- delivery of oxygen
- removal of carbon dioxide
- immune response
- response to injury
- etc...

## Complex Composition:

### *Biologically active!*

- red blood cells
- white blood cells
- platelets
- almost every protein
- genomic transcripts
- etc...

# Blood Collection and Processing Variables



# Blood Collection and Processing Variables



**Patient Health, Hydration, Diet**

**Venipuncture vs Finger Stick**

**Patient Position**

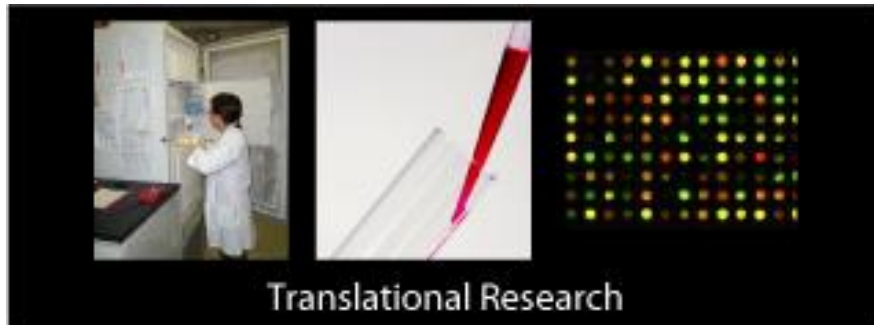
**Tourniquet time duration**

**Gauge of needle**

**Order of draw**

**Type of tube, additives**

# Blood Collection and Processing Variables



**Serum vs plasma vs whole blood**

**Processing Procedure**

**Processing Temperature**

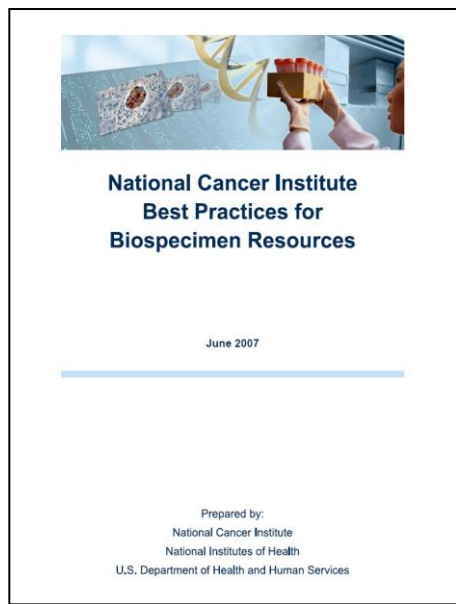
**Centrifuge Speed**

**Centrifuge Temperature**

**Storage Temperature**

**Freeze-thaw**

# NCI Best Practices for Biospecimen Resources



*“Living document”  
updated  
to stay current with  
state-of-the-science  
and policies*

## NCI Best Practices - Six operating principles:

- Evidence-based, data-driven **technical and operational standards** to ensure quality and enable reproducible molecular analysis
- High-quality **biospecimen annotation** with pathological and clinical data
- Biospecimen **access** through a timely, centralized peer review process
- **Ethical and privacy compliance** through a chain of trust with research participants
- State-of-the-art **informatics systems** to track biospecimens, associated data, and research participant informed consents
- **Communication and outreach efforts**, particularly with investigators, to ensure greatest impact

<http://biospecimens.cancer.gov>

# So what does this mean for the biomarker technology developer?

- **The analytical scientist needs to collaborate with other communities**
  - Pathologists
  - Clinical Research Methods / Experimental Design
  - Biostatisticians
  - Epidemiologists
- **Annotation is important**
  - Source of biospecimen including patient data (as allowable)
    - Sex, medication, age, etc. – depending on how the biomarker is used
  - Biospecimen handling, processing and storage conditions
    - Freeze-thaw, stabilization chemistry, storage temperature, etc.
- **Ensure that sample preparation doesn't introduce technology-specific bias**
  - Validation via side-by-side comparisons of different technologies
  - Etc...

# Anal. Chem. 2011, 83, 8–13

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REVIEW

# Questions?

Mark D. Lim, Ph.D.

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## JAMA, 2011, 2200-2211

### Comparison of Effect Sizes Associated With Biomarkers Reported in Highly Cited Individual Articles and in Subsequent Meta-analyses

John P. A. Ioannidis, MD, DSc  
Christis A. Panagiotou, MD

**M**ANY HIGHLY CITED BIOMARKER studies have been proposed as potential determinants of disease risk, prognosis, or response to treatment. The plethora of statistically significant associations<sup>1,2</sup> increases expectations for improvements in risk appraisal,<sup>3,4</sup> however, many markers are not evaluated only in 1 or a few studies.<sup>5</sup> Assessing evidence in a more comprehensive, less biased clinical practice<sup>6</sup>

This translational situation requires better studies. Are the effect sizes reported in the literature accurate or overestimated? It is interesting to address this question in particular for biomarker studies that are highly cited. Many of these risk factors are not evaluated in meta-analyses<sup>7</sup> that allow cross-checking of the evidence. However, some meta-analyses may suffer from their own set of reporting, especially among small data sets<sup>8,9</sup>; often large studies may provide more unbiased evidence.

Here, we examined biomarkers that had been associated in at least 1 highly cited study and/or which at least 1 meta-analysis had been performed for. That same amount of time, we aimed to compare the effect size of these associations in the recent highly cited studies to what was observed in the largest studies and the corresponding meta-analyses.

For additional content see p. 2229.

**Context** Many biomarkers are proposed in highly cited studies as determinants of disease risk, prognosis, or response to treatment. Do they consistently translate to clinical practice?

**Objective** To examine whether the magnitude of the effect sizes of biomarkers reported in highly cited studies is accurate or overestimated.

**Data Sources** We searched ISI Web of Science and MEDLINE until December 2010.

**Study Selection** We included biomarker studies that had a relative risk presented in their abstract. Eligible articles were those that had included more than 400 citations in the ISI Web of Science and that had been published in any of 29 highly cited biomedical journals. We also searched PubMed for subsequent meta-analyses on the same associations (same biomarker and same outcome).

**Data Extraction** In the highly cited studies, data extraction was focused on the disease outcome, biomarker under study, and first reported relative risk in the abstract. From each meta-analysis, we extracted the overall relative risk and the relative risk in the largest study. Data extraction was performed independently by 2 investigators.

**Results** We examined 25 highly cited associations. For 20 of the 25 (80%), the highly cited studies had a more precise estimate than the largest study. For 1 the largest study overlapped the highly cited study and only tests were the effect size estimate to go in the largest study. For 20 of the 25 (80%) highly cited studies, the corresponding meta-analysis found a smaller effect estimate. Only 13 of the associations were normally statistically significant based on the largest studies, and of these only 7 had a relative risk point estimate greater than 1.37.

**Conclusion** Highly cited biomarker studies of low report larger effect estimates for good clinical associations than are reported in subsequent meta-analyses evaluating the same associations.

DOI: 10.1093/aje/kwq120

**METHODS** We considered biomarkers that had a relative risk (RR) estimate presented numerically in the abstract of an article that had received more than 400 citations in ISI Web of Science until December 2010. The threshold of 400 citations was decided a priori, to target approximately the top 1% of biomarker studies published in influential journals. Of these, we focused further on biomarkers with published meta-analyses on the same associations.

**Author disclosures of potential conflicts of interest and author contributions are found in the text. Address correspondence to Dr. Ioannidis, Department of Medicine, and Department of Health, Behavior and Society, Harvard School of Public Health, 665 Huntington Ave, Boston, MA 02115; or Department of Biostatistics, Harvard School of Public Health, Room 5308, 201 Longwood Ave, Boston, MA 02115; [panagiotou@hsph.harvard.edu](http://panagiotou@hsph.harvard.edu).**