Pre-Analytical Variables in Validation of Methylated Biomarkers in Blood Plasma

Biospecimen Research Network (BRN) Symposium:

Advancing Cancer Research Through Biospecimen Science

March 13-14, 2008

www.epigenomics.com

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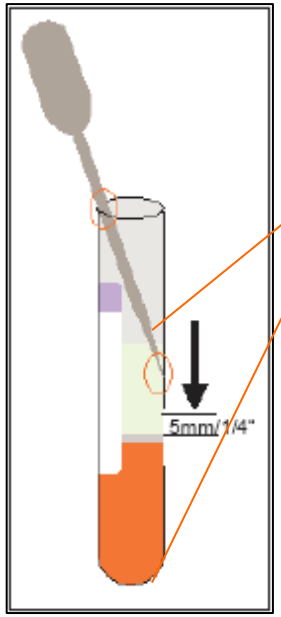
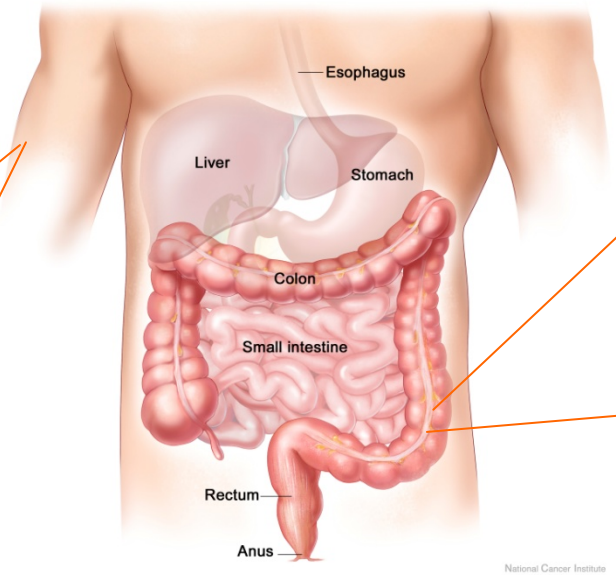
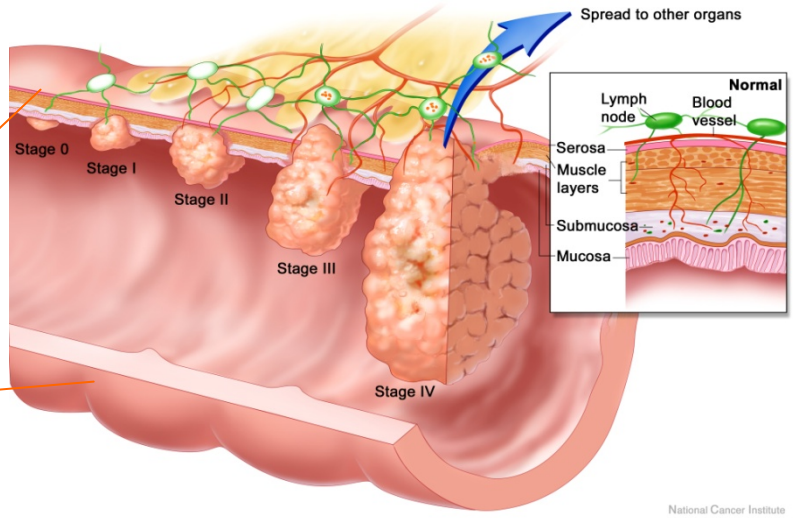
Our goal: A simple blood test to assess risk for colorectal cancer and direct patients to colonoscopy

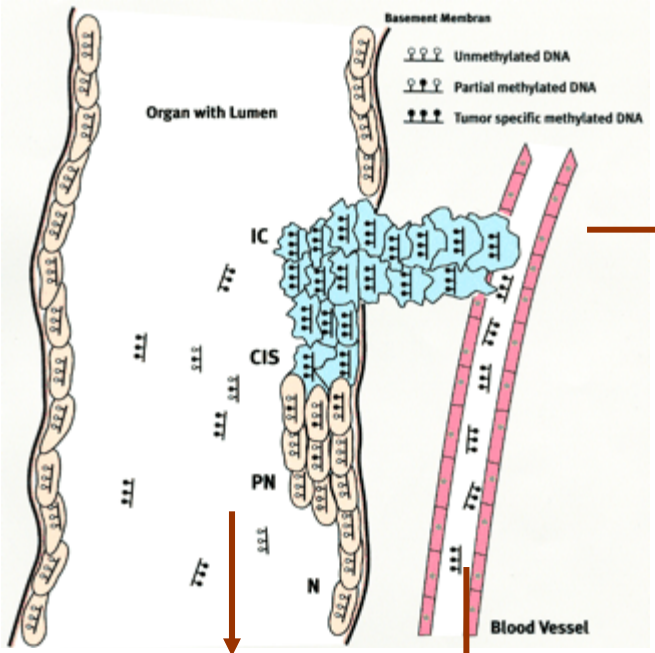






Images from NCI website

Tumor DNA Detectable in Blood



DETECTABLE IN:

-Tissue

DETECTABLE IN:

-Sputum
-Stool
-Urine
-Ejaculate
-Ductal Lavage

DETECTABLE IN:

-Serum
-Plasma

Widschwendter M, Jones PA ,
Clin Cancer Res. 2002 Jan;8(1):131-7.

Tumor DNA in Blood

- Serum/plasma DNA well known in autoimmune diseases
- First report in cancer– 1971
- Leon et.al. 1977
  - Normal mean 13 ng/ml
  - Correlated with cancer type and metastatic state
- Stroun, Anker et.al 1987
  - Extracted DNA – determined fragment size, structure

- CNAPS – biannual symposium focused on body fluid nucleic acids

- To date 1000's of publications measuring tumor DNA in plasma and serum

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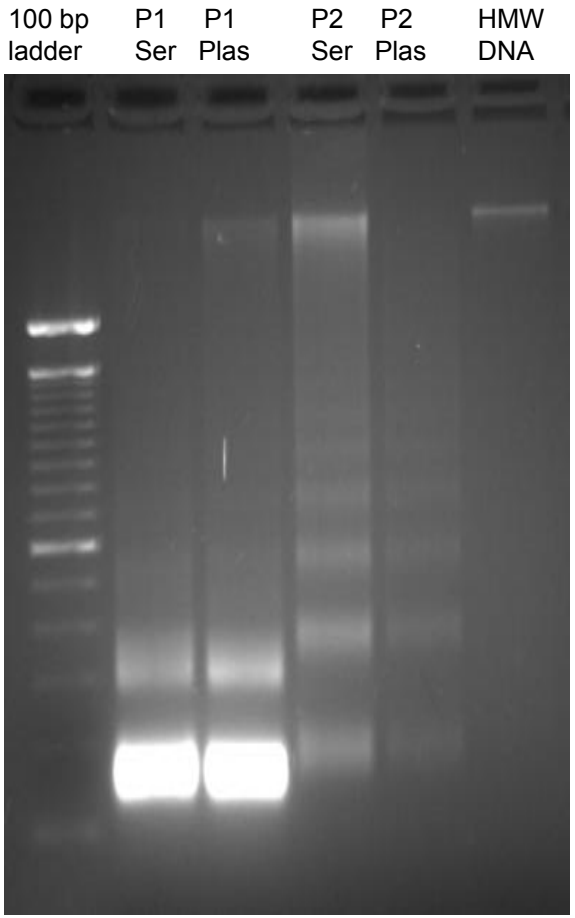
Cell Free DNA in Plasma & Serum

- Tumor DNA present as extracellular DNA in both serum and plasma
- Total serum DNA concentration higher than plasma concentration - in our experience 2-3 fold
- Increase a consequence of serum preparation which results in a greater degree of cell lysis
- Both fractions are likely to work for markers that are highly specific



- We focused on plasma to give the most favorable ratio of target : background

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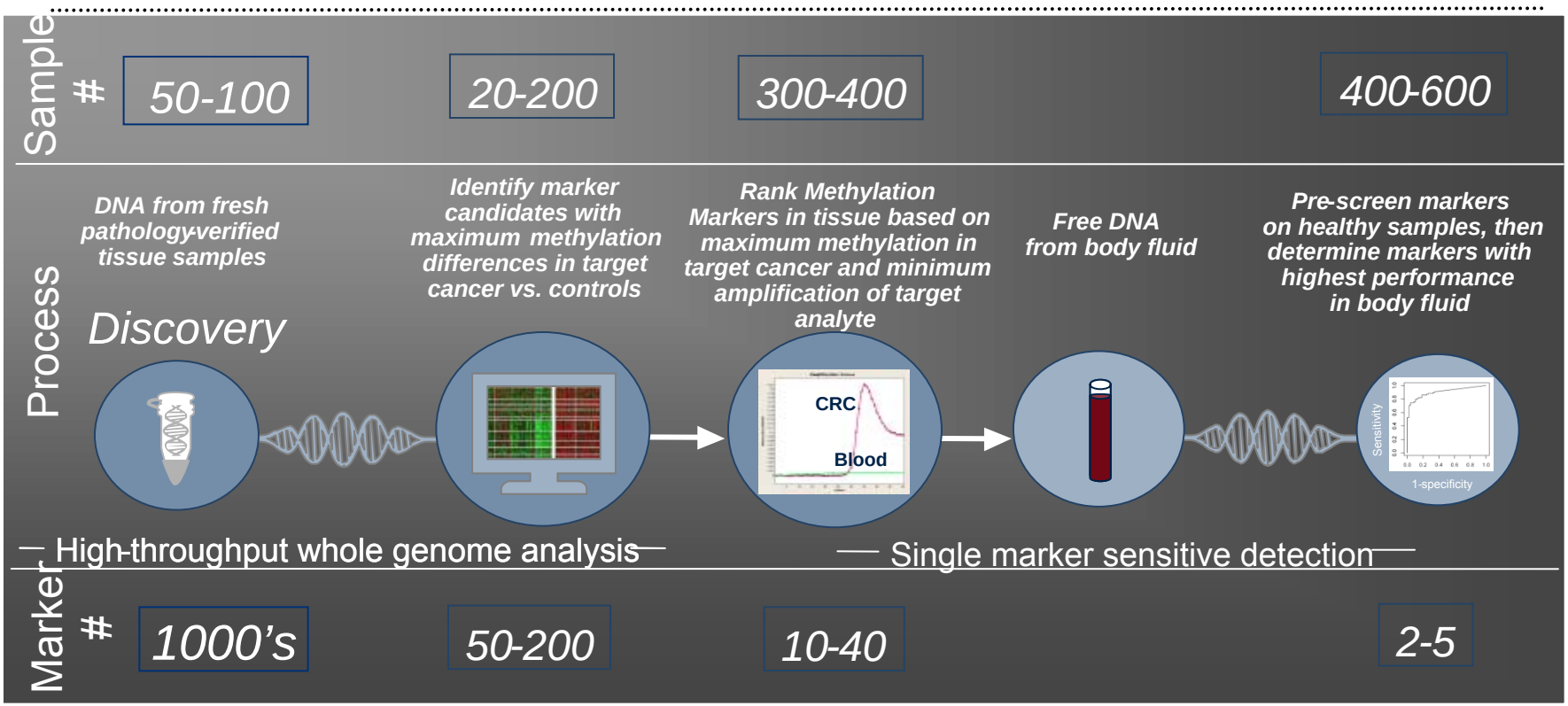
Methylation Biomarker Discovery & Validation Process



- Markers Selected for
  - Maximum methylation differences in target cancer vs. controls
  - Applicability to highly sensitive real-time assays
  - Best performance in body fluid

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SEPT9 – a colon cancer biomarker

- Septin 9 gene promoter is highly methylated in colorectal cancer tissue and hypomethylated in normal colon epithelial tissue.
- Based on a panel of 200 colon cancer tissues, normal colon epithelia, other cancers and other normal tissues, methylated SEPT9 represents a candidate CRC biomarker
- By HM real time PCR, SEPT9 showed high specificity on Peripheral Blood Leukocytes, whole blood DNA and normal plasma
- Hypermethylated SEPT9 was measured in the plasma of CRC patients

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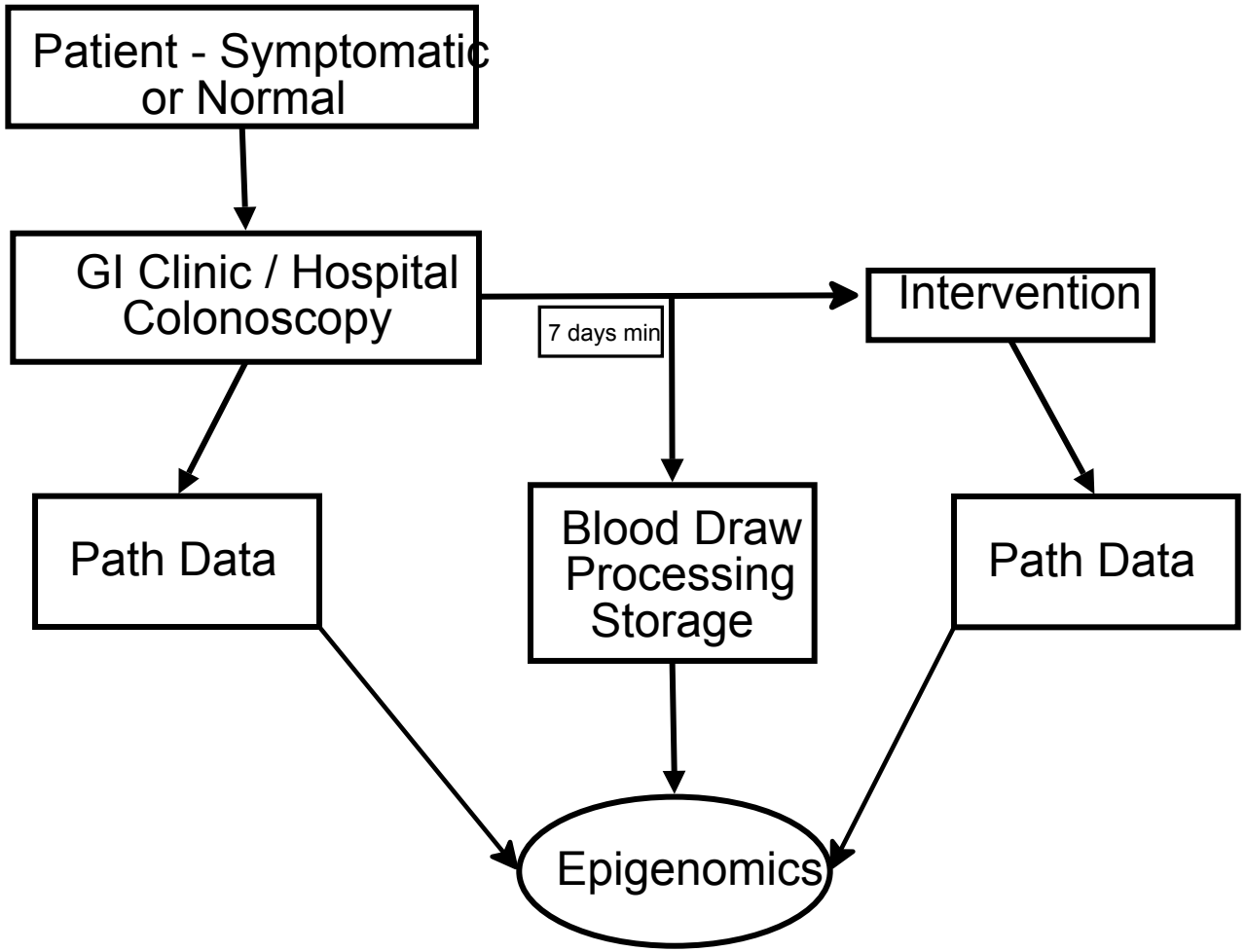
Case Control: Validate SEPT9

Objective:

- Validate Performance of plasma SEPT9 and other methylation markers for detection of colorectal cancer patients

- Cases: patients positive for colorectal cancer as determined by colonoscopy

- Controls: colonscopy verified patients in the same age range with normal findings



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Validation of Marker in Case Control Studies - Requirements

- Limited to one specimen type
  - Collection logistics – IRB determines acceptable sample volume
  - Experience indicated performance improvement with increasing sample volume per test
  - A number of candidate markers were selected for validation

PLASMA

  - Reduce background from lysed cellular fraction in serum
  - Plasma processing protocols well described
  - Precedent molecular diagnostic tests used plasma

- Collection Volume
  - Sufficient for multiple markers / multiple replicates

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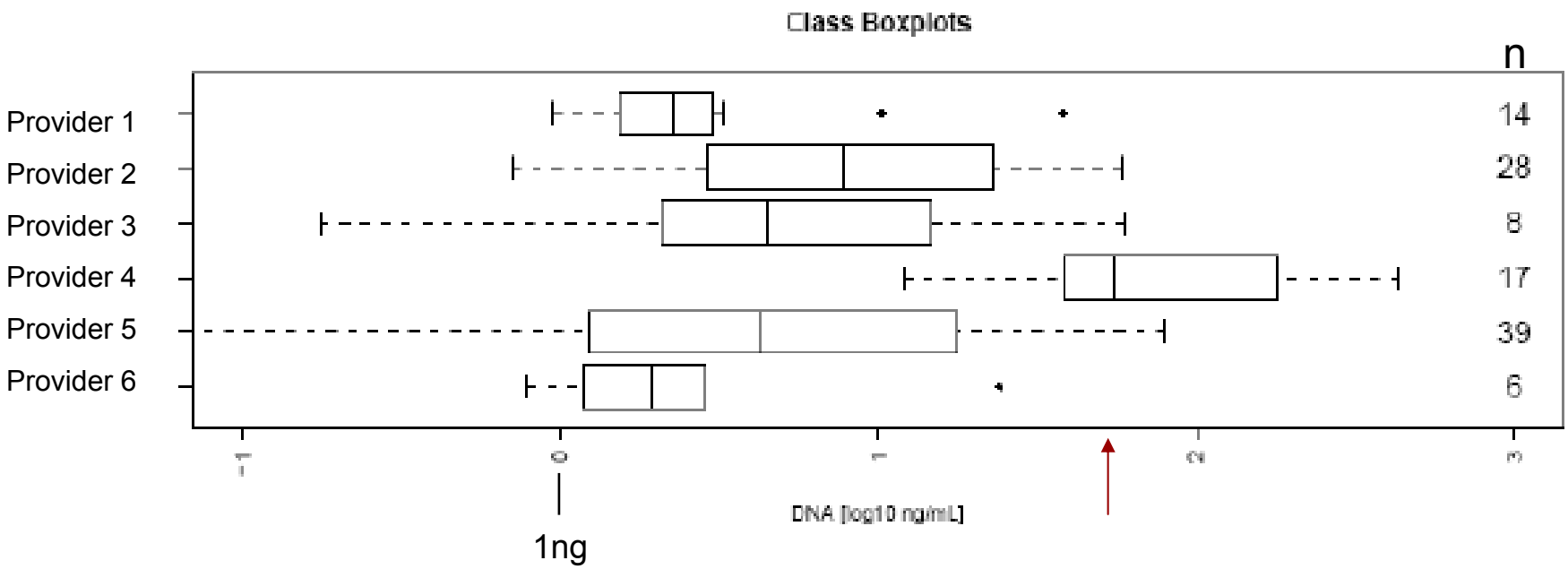
Total DNA recovery: Non CRC Control Patients



Total DNA measured by real time PCR

- Differences in provider protocols result in DNA recovery differences

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Initial Sample Collection Experience

2004	Tube	Centrifu gation	Timing	Other
Collection SOP	**BD Vacutainer 10 mL glass – K3-EDTA**	**2x**	**Prior to colonoscopy or 7 days after – prior to surgery**	
Provider A	Sodium Heparin Tube	1x	Before colonoscopy	
Provider B	Sarstedt Plastic K3-EDTA	2x	After colonoscopy Before surgery	Arterial
Provider C	As Specified	1x	Before colonoscopy	Controls outside age range

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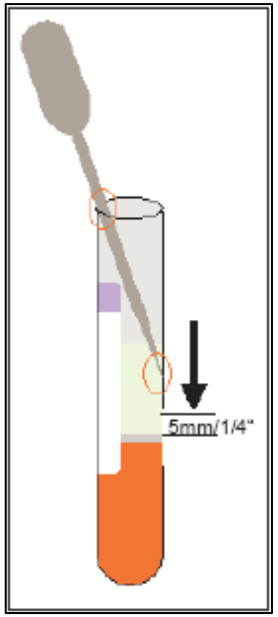
Case Control - Sample Collection

Collection:

- Optimize cell free DNA
- Limit lysis of nucleated cell fraction in blood
- Maximize recovered volume – analyte present at low concentration
- Prevent DNase activity – EDTA tube



Provide Collection Kit

Provide Processing Equipment

Provide Collection SOP

Provide On Site Training

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Sample Collection

- Provide Kit
  - SOP
  - Needles
  - Vacutainers
  - Tubes
- Provide Training
- Require Compliance
- Audit Performance

1. EDTA Vacutainer tubes plastic (1-5 pcs.)
2. Needles (2 pcs.)
3. Vacutainer/Needle holder (1 pc)
4. 15mL Centrifuge tubes (1-2 pcs.)
5. Bulb pipettes (1-3 pcs.)
6. 50mL Pooling tube (1 pc)
7. 5mL cryovials for plasma (1-5 pcs.)
8. 2mL cryovial for Buffy Coat (1 pc)
9. Small bag for filled cryovials from each patient (1 pc)
10. Case report form/process checklist

Together with the collection kit the following forms will be provided:

1. Case report form
2. Process checklist

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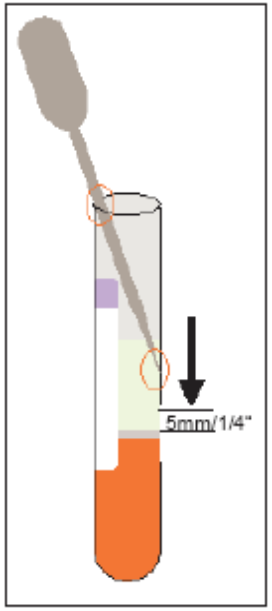
Plasma Preparation Protocol

1) Comply with manufacturers protocol - time (4 hrs) – temp.
2) Request careful avoidance of buffy coat
3) Require a second centrifugation to improve clearance of cells
4) Store onsite for batch shipment



Overview of the Plasma Separation Process

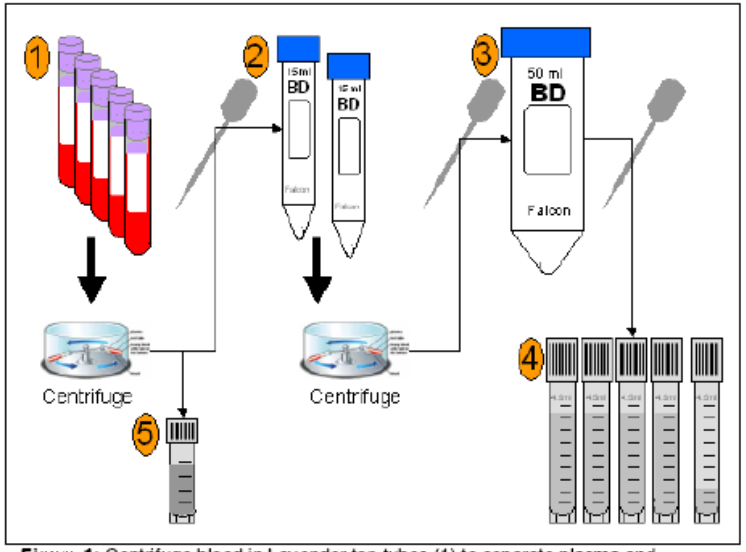


Figure 1: Centrifuge blood in Lavender top tubes (1) to separate plasma and transfer it to the 15ml Falcon tubes (2) for a second spin. The second pipette is used to transfer the purified plasma from both centrifuge tubes to the pooling 50ml falcon tube (3) before aliquotting it in the 4.5ml cryovials (4). The mini-cryovial (5) is used to collect buffy coat from one of the Lavender top tubes (1) after the first spin AND AFTER transferring the plasma.

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Plasma Collection: Tube Type

	PPT	Standard
Price	1 USD / tube	0.1 USD / tube
Required number tubes (5mL) plasma	2	1
Established in routine	rarely	broadly
Time until centrifugation	max. 2h	4 up to 6h
Plasma separation	Pouring	Careful Pipeting
FDA Approved	Yes – 5mL	Yes
Specified for Molecular Testing	Yes	Yes
EDRN Protocol		Yes
PPT tubes included in case control collection to compare performance		

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Sample Collection Data

- Case Report Form includes sample collection data
  - Patient Age / Gender
  - Clinic Location Blood Draw
  - Date / Time of Blood Draw
  - Centrifugation parameters
  - Date / Time in Freezer

Process Information (both tube types)			
Shipment abnormalities	☐ None ☐ Hemolysis	☐ Broken tube ☐ Other, please specify:	
First Centrifugation	☐ 12 min, 1200g, RT	Brake	☐ on ☐ off ☐ NA
PURPLE top tubes only Note: Residual volume in tubes after pipetting should be >= 0.5ml			
Second centrifugation	☐ 12 min, 1200g, RT	Brake	☐ on ☐ off ☐ NA
Buffy coat collected	☐ 2ml (small) cryovial		

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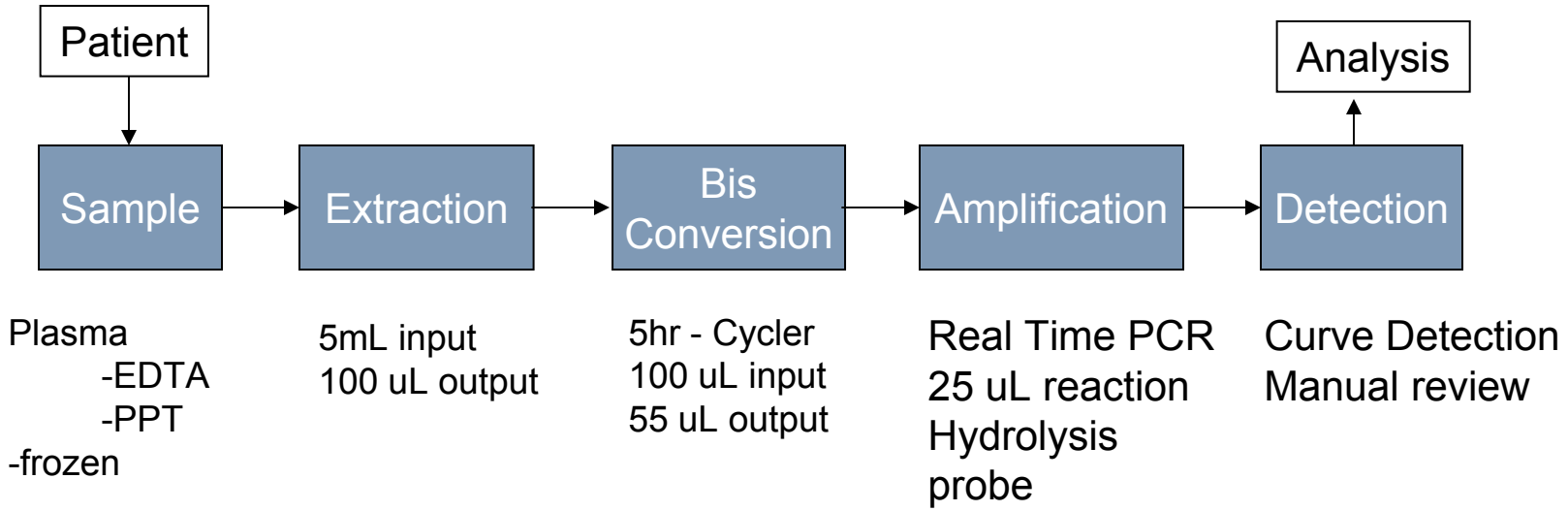
Sample processing protocol



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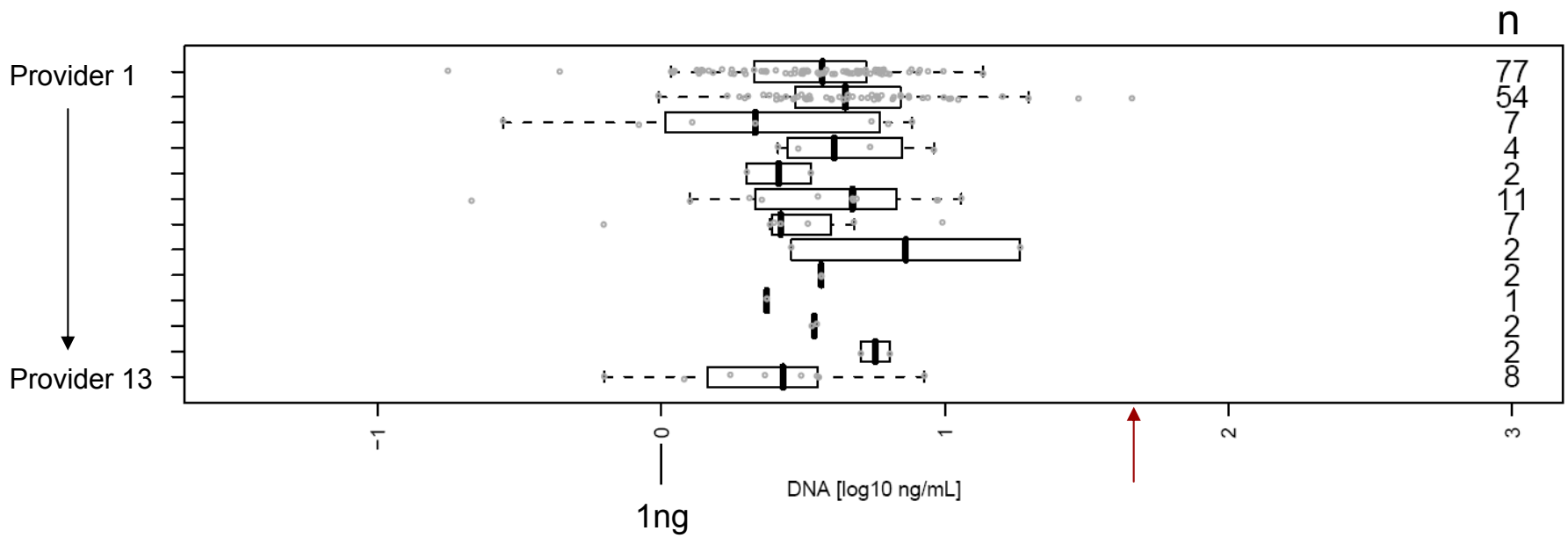
Total DNA recovery: Non CRC Controls



Total DNA measured by real time PCR

- Reduction in variability

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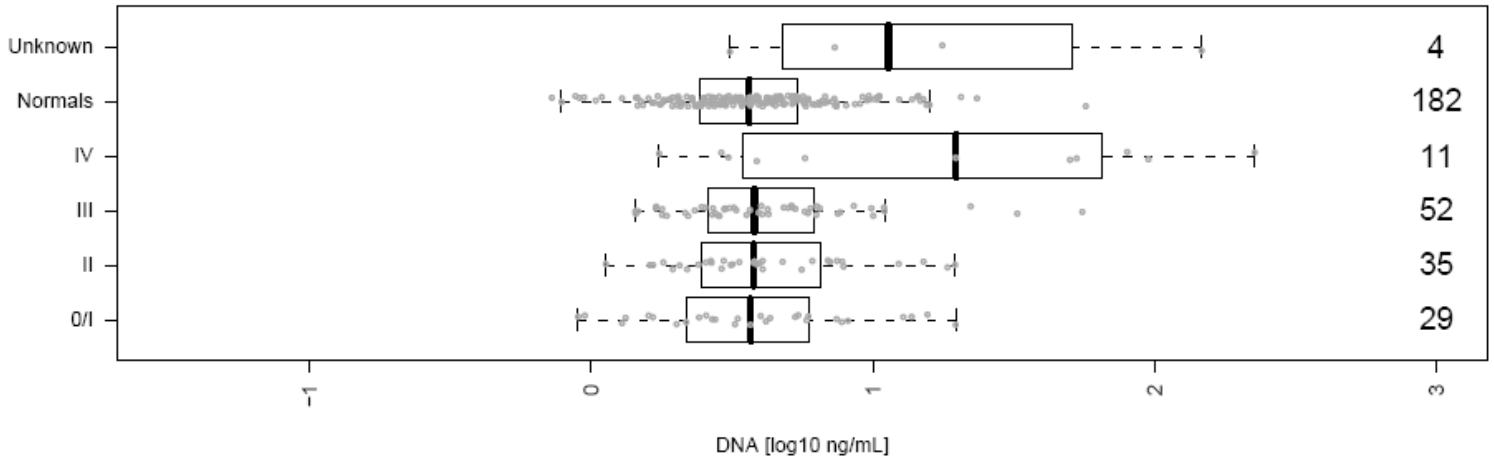
Total DNA recovery: CRC cases

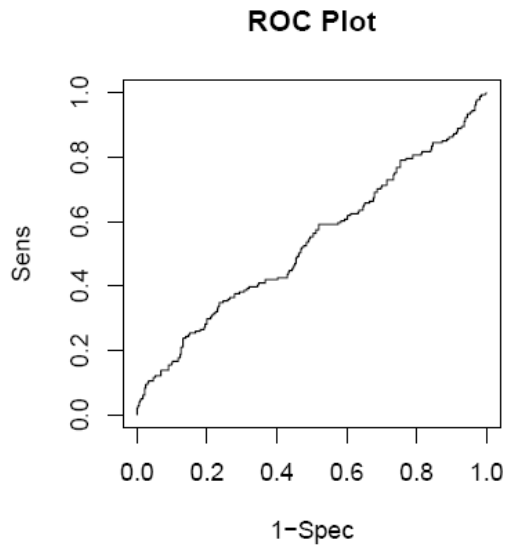


Total Genomic DNA:

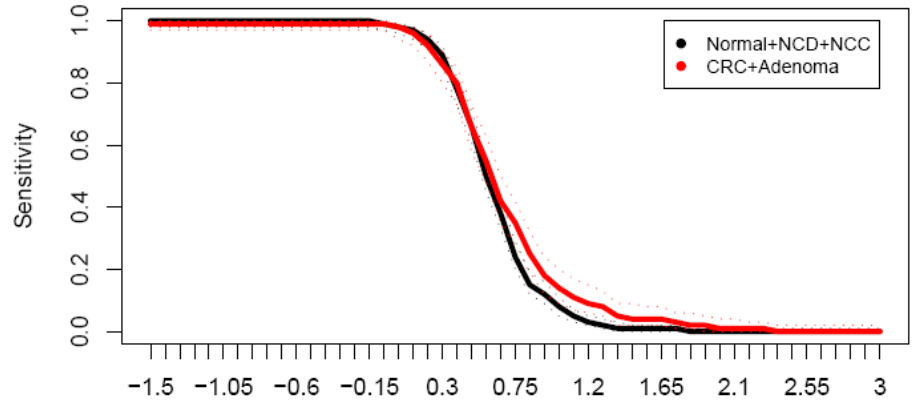
Reduced Variability

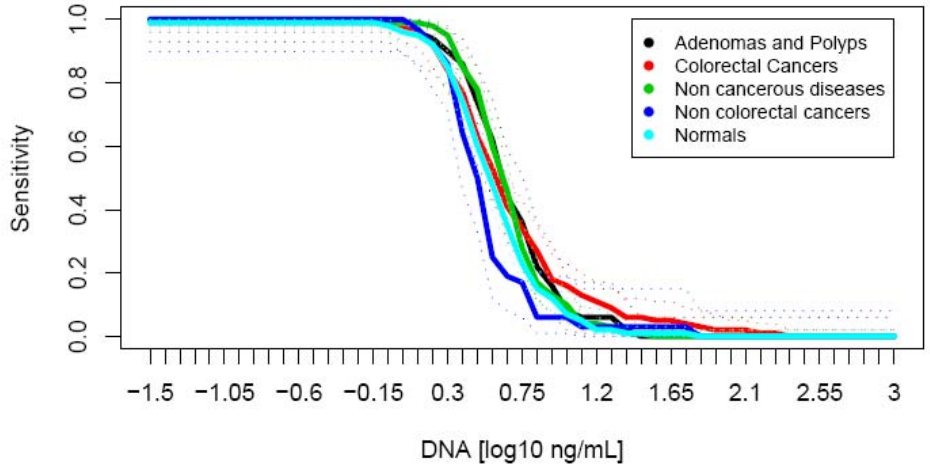
Elevated levels in stage IV

Total Genomic DNA is not a general marker for Colo-rectal Cancer



AUC	0.53 [0.49,0.58]
Sens / Spec	0.12 / 0.95
Sens / Spec cut off	1.1
Wilcoxon *P*	0.2083
CRC+Adenoma (pos)	181
Normal+NCD+NCC (neg)	365





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Additional Quality Control Questions

- Is the plasma in my sample tube from the patient listed on my case report form?
  - Use Gender PCR on the extracted plasma DNA and compare with case report.
    - We identified a small number of samples with mismatched genders
    - Follow up – Audit the CRF information – most resulted from clerical errors
- When collecting multiple aliquots – are all of the aliquots from the same patient?
  - Use Gender or Genotyping PCR as above
    - Not detected as a problem

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Additional Quality Control Questions

- When collecting serial samples – are the samples from the same patient?
  - Collect Buffy Coat samples – Use Gender / Genotyping PCR
    - In early collections found a common genotype in multiple samples from one provider
    - Follow up – site audit revealed inconsistent data tracking – corrective action taken

- These solutions must included in the protocol, in the informed consent and require IRB approval

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Ongoing research

- Sample Storage
  - Systematic Data not available yet
    - Complicated by the introduction of a new sample processing workflow at the end of 2007
  - Anecdotal Data
    - Aliquots of samples tested in 2006 used in workflow development studies in 2007/08:
      - Total DNA and Bisulfite DNA recovery similar
      - Marker positive samples measured – marker positive in 2007/8
      - Overall test performance (sensitivity and specificity) similar for aliquots tested in 2006 and 2007
  - Systematic studies ongoing:
    - Known marker positive plasma samples diluted in marker negative control plasma. Dilution series aliquots stored and will be tested at intervals

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Summary

- Plasma is a viable specimen for measuring DNA methylation biomarkers
- Currently we use the 10 mL BD lavender Vacutainer blood collection tube in our standard collection
- Plasma collection following the manufacturers guidelines, with attentive pipeting and a second centrifugation step reduces variability in total DNA recovery
- Case control collections require additional effort beyond the clinical routine – eg. Larger volume, Multiple Tube types, Modified Storage
  - Additional QC steps should be applied
    - Site Qualification and Auditing
    - Gender / Genotype analysis confirmation of CRF
    - Total DNA measurement in addition to marker to check sample collection
    - Matched sample collections (cases & controls) from each site

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Summary cont'd

- Based on >3000 specimens:
  - median genomic DNA content is ~ 5 ng / mL plasma
  - total genomic DNA recovery in early stage cancers is similar to noncancer controls
  - Total genomic DNA increases in late stage (IV) cancers

- In Case Control studies, the presence of hypermethylated SEPT9 in plasma is predictive for the presence of colorectal cancer

- Next Steps: Prospective Study

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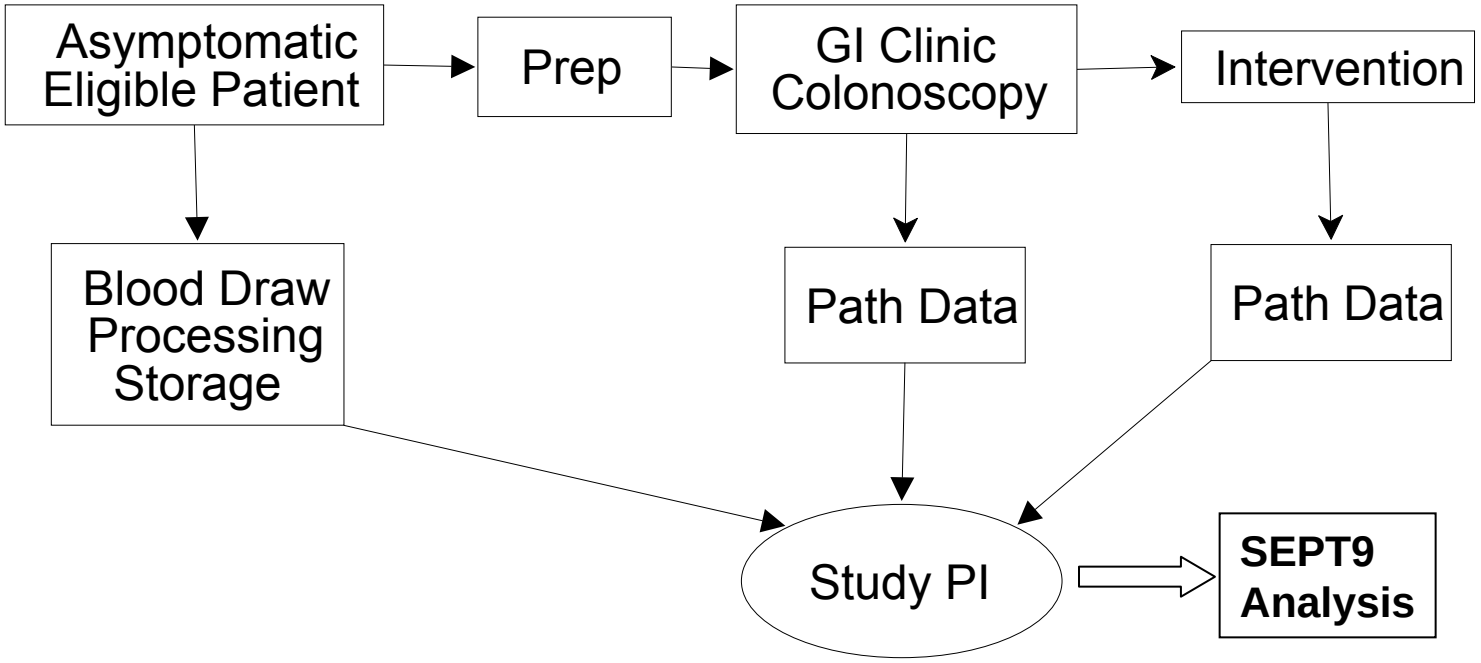
Prospective Study

Performance of SEPT9 in a screening setting



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