



Pre-Analytic Issues Relating to Molecular Testing

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Setting Up a Molecular Laboratory



- Spatial separation of pre- and post-amplification work areas
 - ◆ Area 1 – Reagent preparation
 - ◆ Area 2 – Specimen preparation
 - ◆ Area 3 – Post-PCR processing

Source: CLSI MM5-A, Vol 23 (17) Nucleic Acid Amplification Assays for Molecular Hematopathology; Approved Guideline



Alternative to Spatial Separation



- Alternative: enclosed countertop box
- Dedicated pre-amplification equipment (pipets, racks etc.)
- Dedicated pre-amplification reagent refrigerator or freezer



Setting Up a Molecular Laboratory



- Unidirectional work flow
 - ◆ Avoidance of returning to the pre-amplification area after working in the post-amplification area
 - ◆ Minimizes the risk of amplicon carry-over on clothing, hair and skin

Source: CLSI MM5-A, Vol 23 (17) Nucleic Acid Amplification Assays for Molecular Hematopathology; Approved Guideline





Alternative to Unidirectional Workflow



- Hairnet
- Dedicated safety glasses
- Disposable labcoat/gown
- Gloves
- Shoe covers

Note: Even when unidirectional workflows are feasible, not everyone washes their hair every night or their clothes after one time wear!

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The Small, but Important Details...



■ *Equipment*

- ◆ One time use, cotton plug tips (pre-amplification area)
- ◆ Dedicated pre-amplification pipets
- ◆ DNA extraction best in BSC
 - ★ after use, wipe area with bleach and turn on UV light
- ◆ UV crosslinker
- ◆ Note: do not use frost-free freezers for enzyme storage





More of the Small, but Important Details...



■ *Processes*

- ◆ Dedicated PPE for pre-amplification processes
- ◆ Use of nuclease free or autoclaved water
- ◆ Wipe surfaces with bleach after use

■ *DNA integrity*

- ◆ Aliquot oligonucleotides – multiple freeze thaws will cause degradation
- ◆ Always include a negative control to check for contamination





Selecting a DNA Extraction Method for DBS

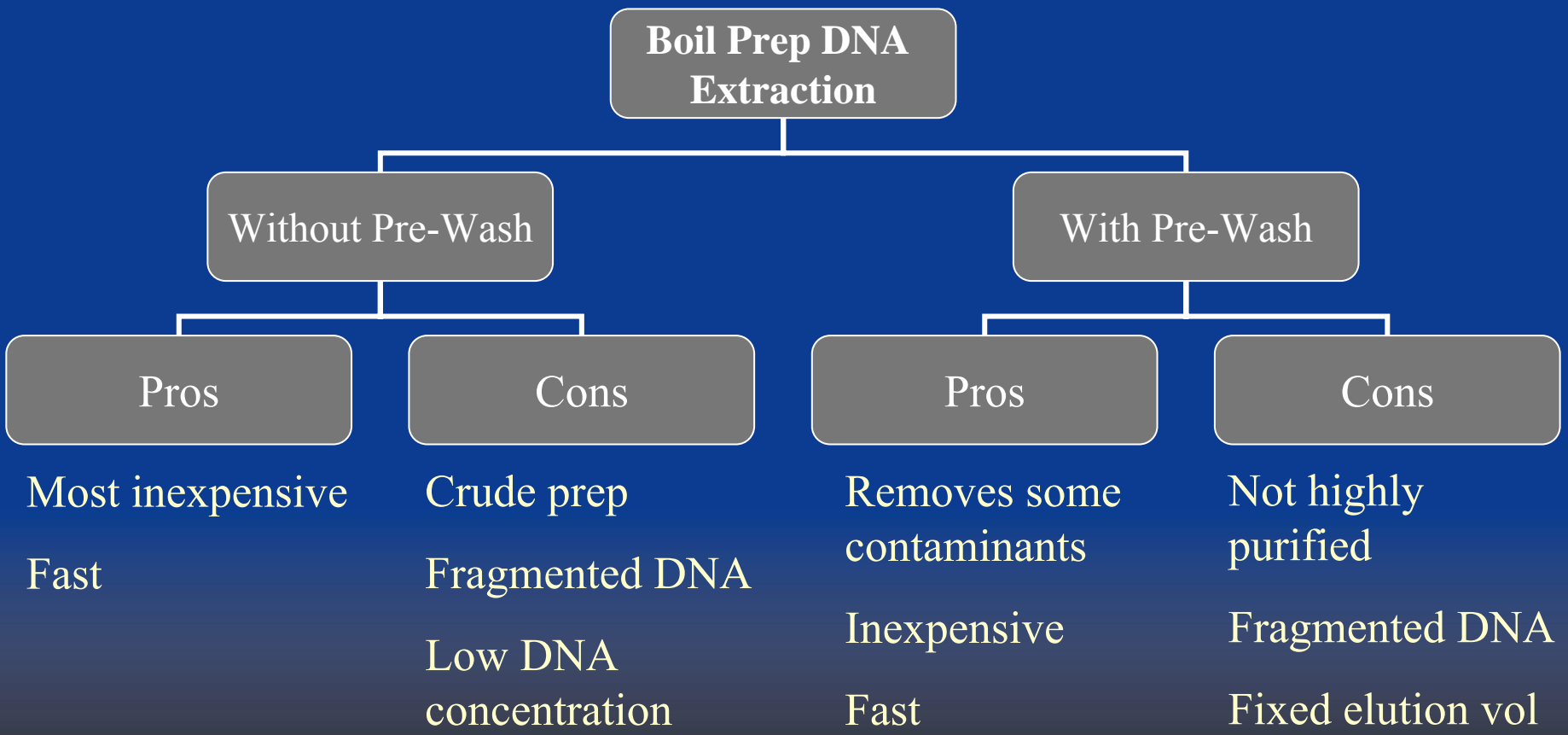


- Boiling Procedure
 - ◆ With or without an initial wash
- Column Based Purification
- Magnetic Bead Based Purification



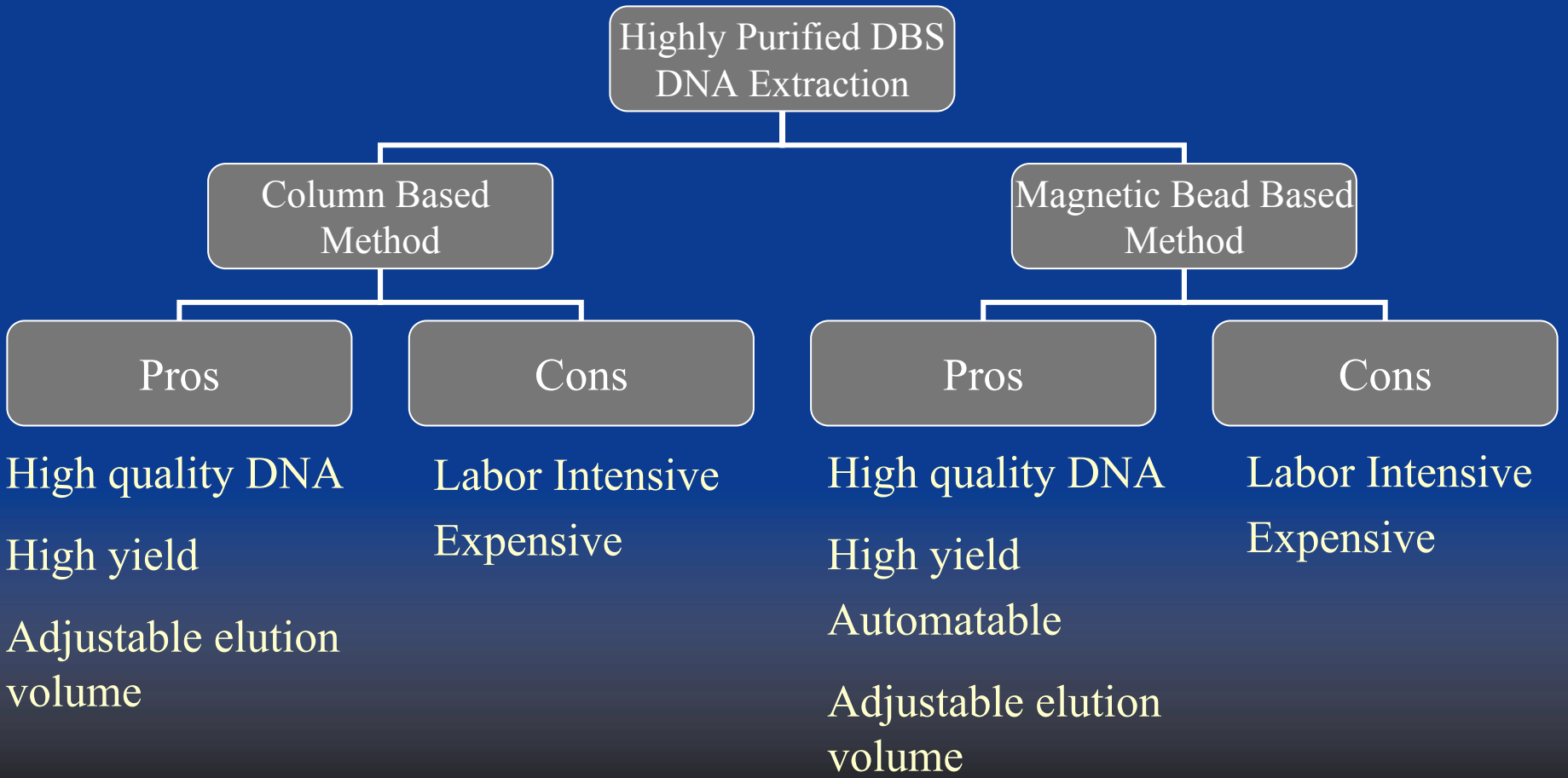


Selecting a DNA Extraction Method for DBS





Selecting a DNA Extraction Method for DBS





Potential PCR Inhibitors



- Inherent in blood or may be introduced during collection (anticoagulants)
 - ◆ Hemoglobin
 - ◆ Immunoglobulin G
 - ◆ Lactoferrin
 - ◆ Heparin
- Impact should be determined for each assay
 - ◆ Assay robustness
 - ◆ Assay sensitivity





Quantitating DNA

When and How?

- Typically unnecessary for routine PCR based assays
- Important for validating new assay limits and sensitivity
 - ◆ Too little DNA may lead to allele drop-out (not always obvious)
 - ◆ Some assays require a minimum DNA quantity





Quantitating DNA

When and How?



- ***Absorbance***
 - ◆ Measure not specific to DNA
 - ◆ DBS DNA contains contaminants resulting in inaccurate measures
 - ◆ Not recommended for DBS DNA
- ***Pico-green***
 - ◆ Measure specific to double stranded DNA
 - ◆ Recommended for DBS DNA
- ***Quantitative PCR***
 - ◆ Measure specific to amplifiable DNA
 - ◆ PCR inhibitors may underestimate DNA concentration
 - ◆ Different genomic targets may give different concentrations
 - ◆ Recommended for DBS DNA





Expected DNA Yield from a 3.2 mm DBS punch



Sample Type	N	minimum DNA conc (ng/ul) ^a	maximum DNA conc (ng/ul) ^a	average DNA conc (ng/ul) ^a	std dev	average DNA total yield (ng) ^b
Adult DBS	42	1.1	3.8	2.3	0.7	104
CF-affected Adult DBS (hematocrit-adjusted)	10	1.6	7.0	4.5	1.7	202
Newborn DBS (stored 10 yrs)	20	2.2	12.0	5.2	2.2	234
Newborn DBS (stored 1 yr)	6	3.3	8.3	5.5	1.9	247

^a DNA extracted from 3.2mm punch (3.4μL volume of blood) taken from a 75μL DBS

^b Average DNA yield was calculated by multiplying the average DNA concentration by 45μL (volume resuspended)





Specimen to Specimen Contact – Does it Cause Problems?



Photo compliments of Brad Therrell

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Specimen to Specimen Contact – Does it Cause Problems?

- Specimen to specimen contact results in minute amounts of contamination
 - ◆ Is this contamination detectable?
 - ◆ Would a DNA extraction with a pre-wash result in less contamination?
 - ◆ Would this contamination interfere with current NBS CF molecular tests?





Experimental Design to Test for Contamination

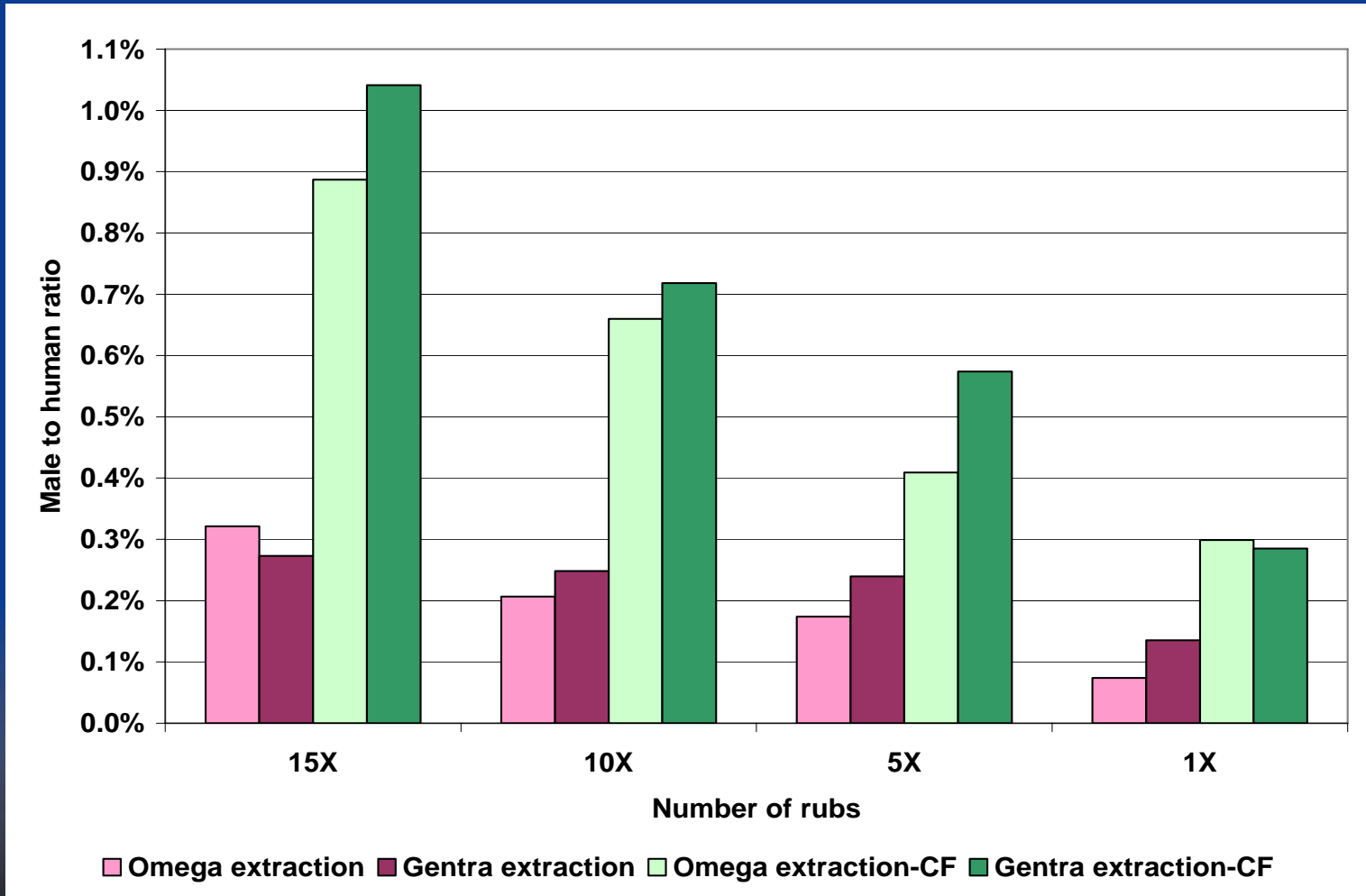


- Rub male DBS against female DBS 1x, 5x, 10x and 15x
 - ◆ Adult healthy males against adult females
 - ◆ Adult CF-affected males against adult females
- Measure human and male specific DNA
 - ◆ calculate male DNA using quantitative PCR
 - ◆ DNA extraction methods – Gentra and Omega
 - ★ boil prep with pre-wash vs. magnetic bead



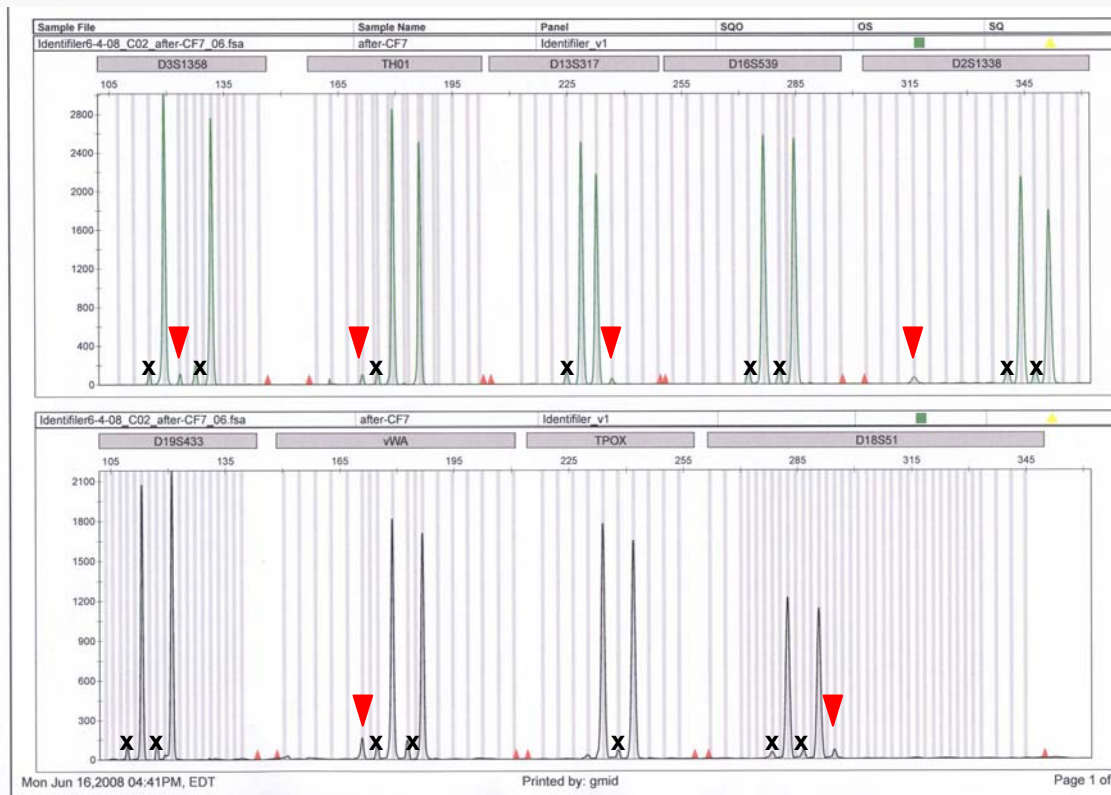


Specimen to Specimen Contamination Data



Forensic Results from Contaminated DBS

Forensic Short Tandem Repeat (STR) analysis detects 4 bp repeat sequences found in the human genome – acts like a DNA fingerprint



Male contaminant = ~3% of total DNA yield (threshold = 5%)



CF Mutation Analysis

- Contaminated samples tested by
 - Hybridization assay for 36 mutations (Innogenetics)
 - DNA sequencing of Exon 10 (F508del)
- **No CF mutations seen in either assay**

Assessment of DNA contamination from dried blood spots and determination of DNA yield and function using archival newborn dried blood spots. Clinica Chemica Acta 2009 402:107–113.

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Summary

- Spatial separation of work spaces
- Unidirectional workflow through pre- and post-amplification processes
- Pros and cons of different DNA extraction methods
- When and how to quantitate DNA
- Specimen to specimen contact and contamination
 - ◆ Contamination is present at very low levels
 - ◆ Pre-wash does not impact contamination
 - ◆ Contaminant is not seen in two CF assays

