DBS DNA Extraction, Validation & Quantitation

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Molecular Quality Improvement Program

NBS Molecular Training Class
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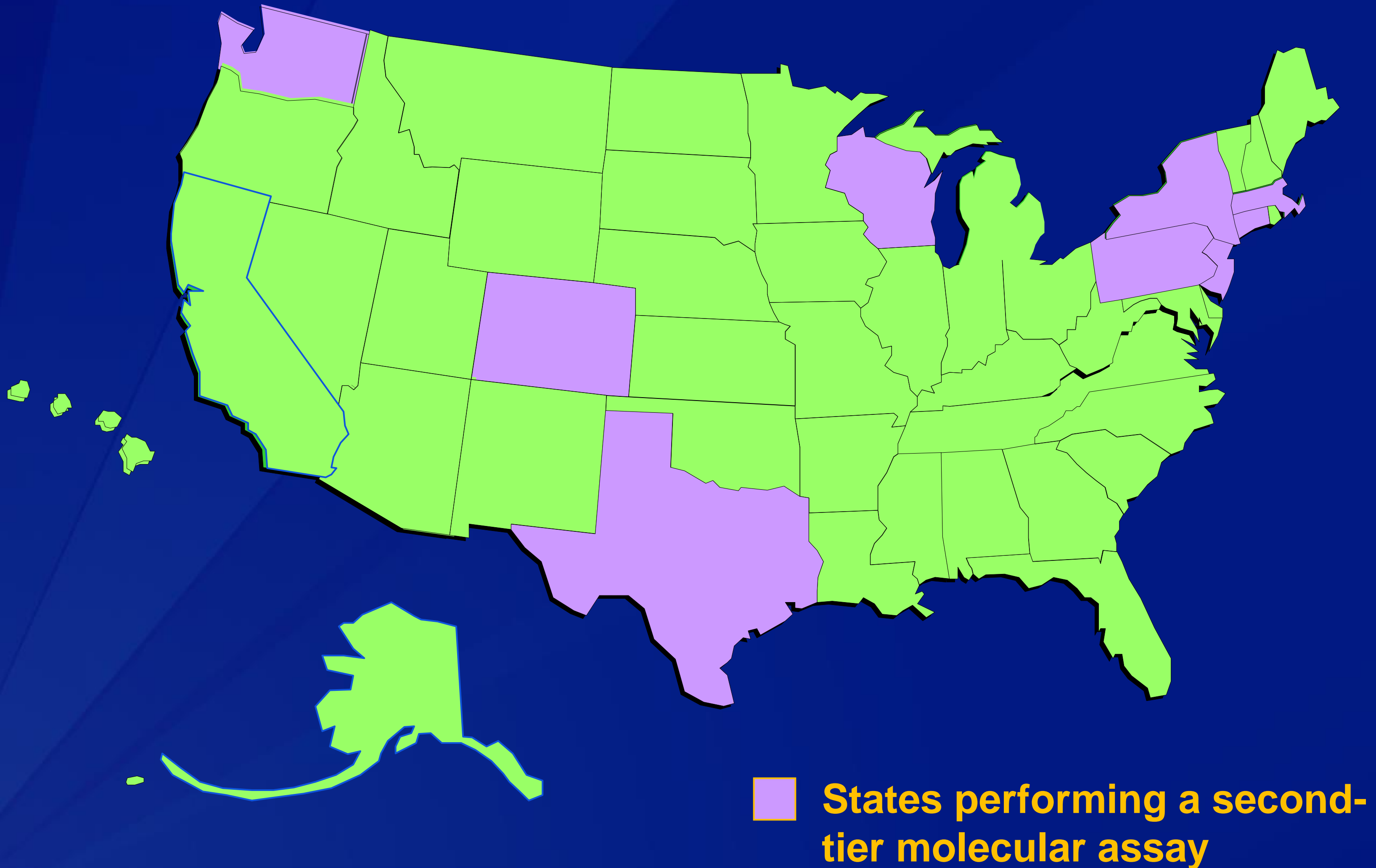
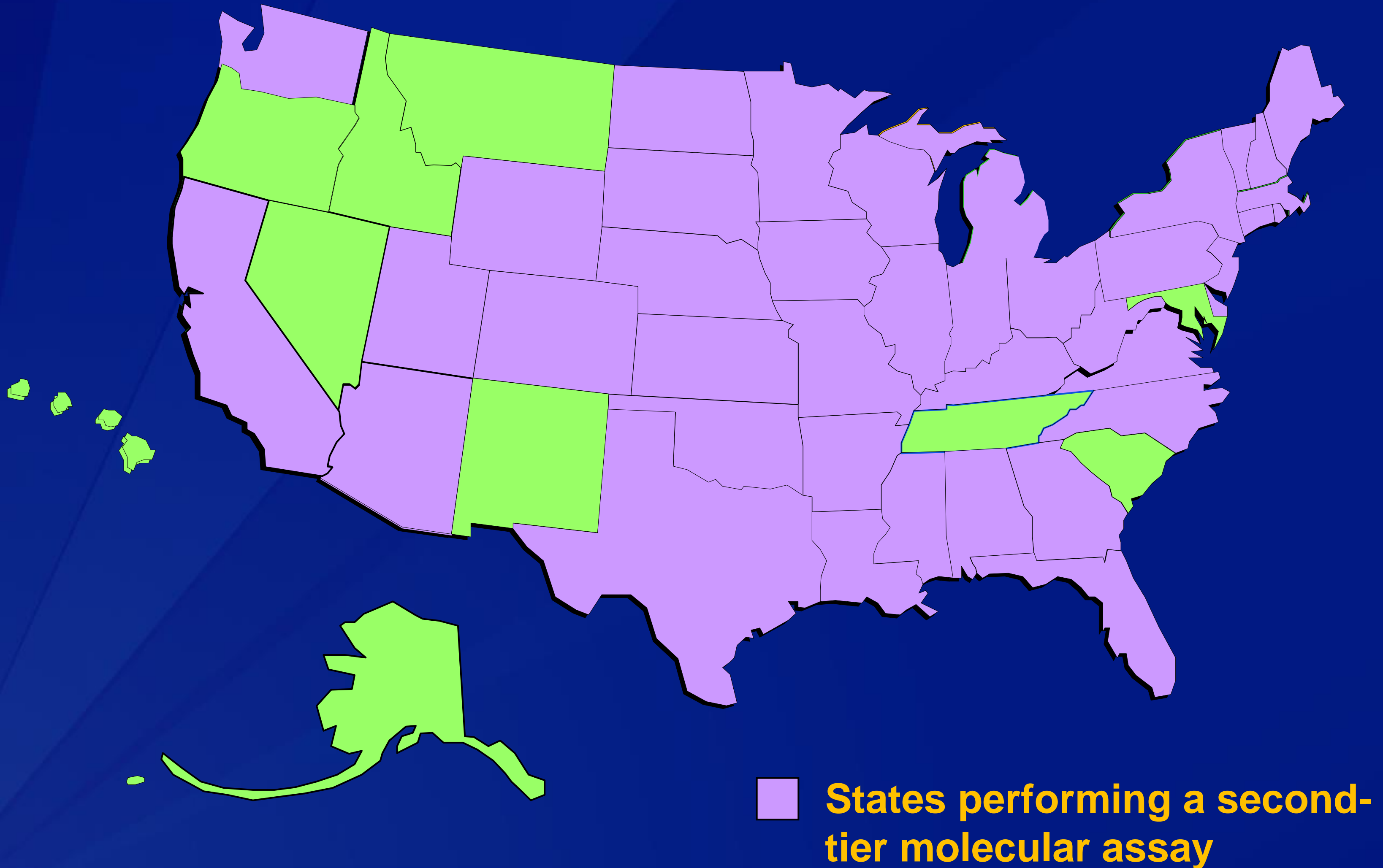
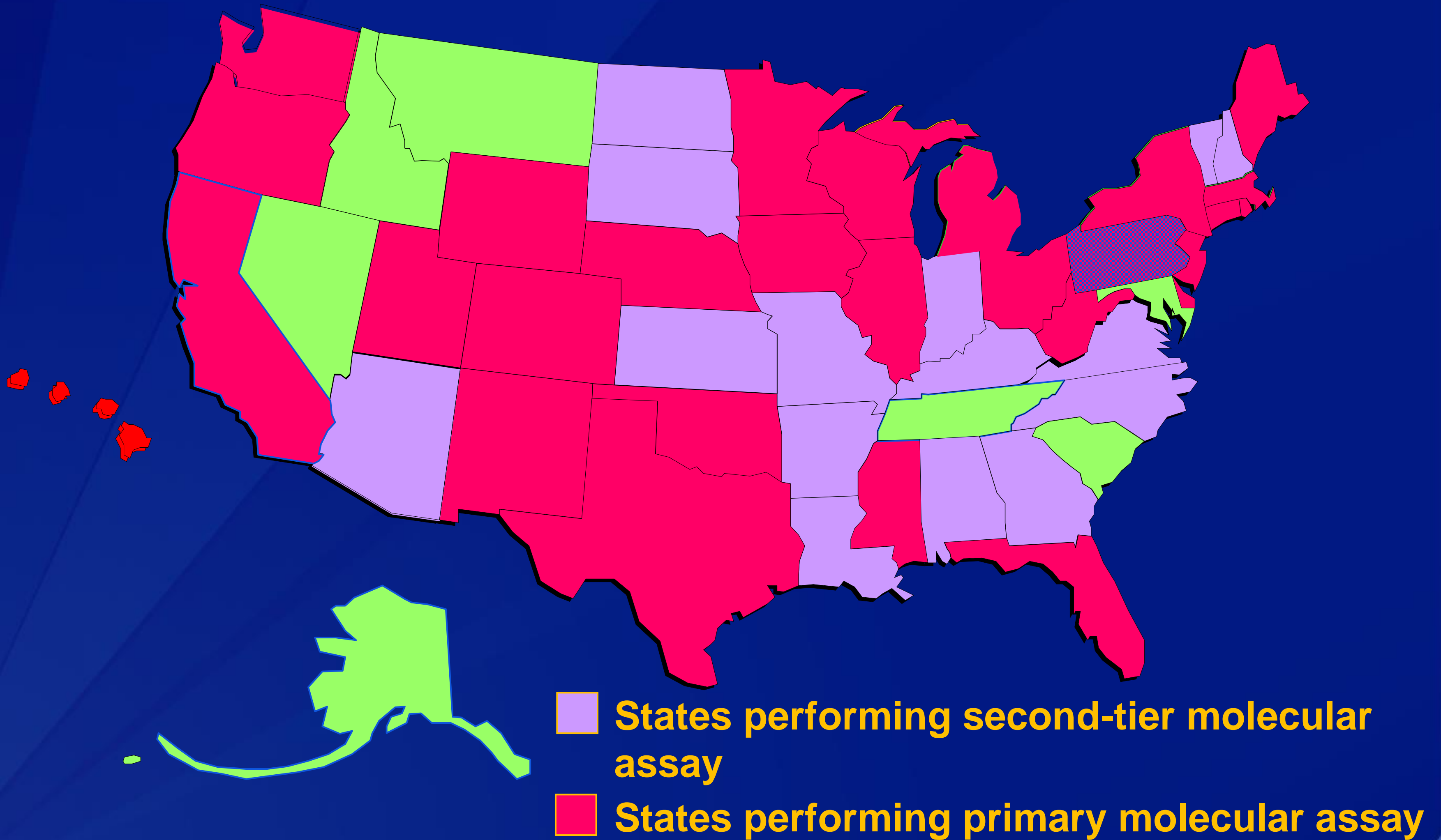
State of Molecular Screening in 2004
(second-tier only)



State of Molecular Screening February 2015 (second-tier only)



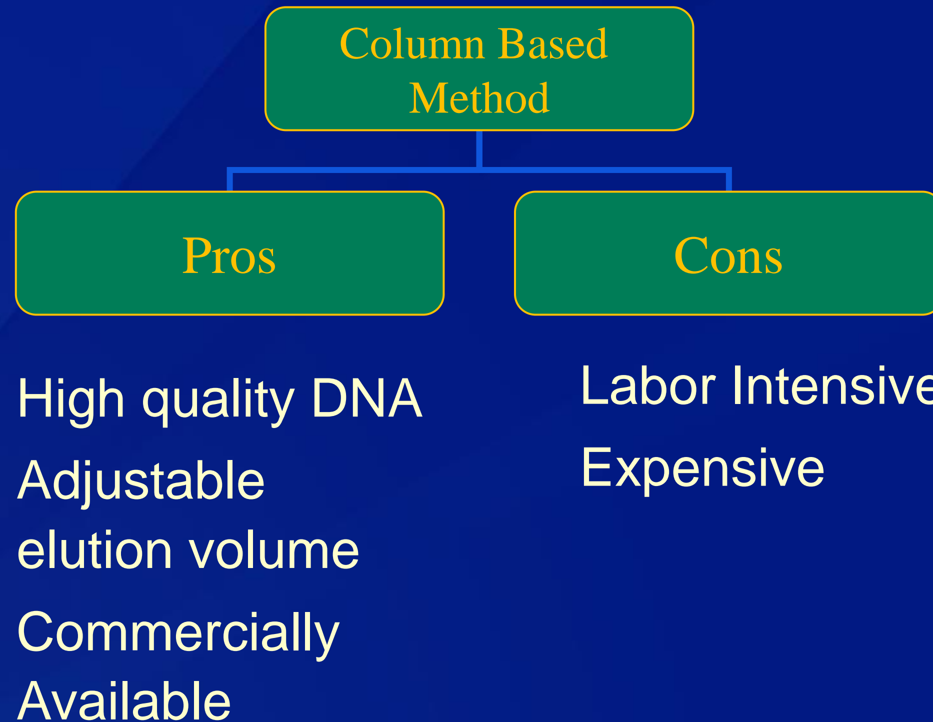
State of Molecular Screening February 2015
(primary and second-tier)



DBS DNA Extraction Methods

- **Column Extraction**
  - Highly purified DNA extraction
- **Boil Prep Generations Method (Qiagen)**
  - Solutions 1 & 2
  - Multiple wash steps, followed by boil
- **Boil Prep Method**
  - No wash, followed by prolonged boil
- **Methanol Boil Prep Method**
  - Fixation of proteins, followed by prolonged boil

Highly Purified DBS DNA Extraction Method



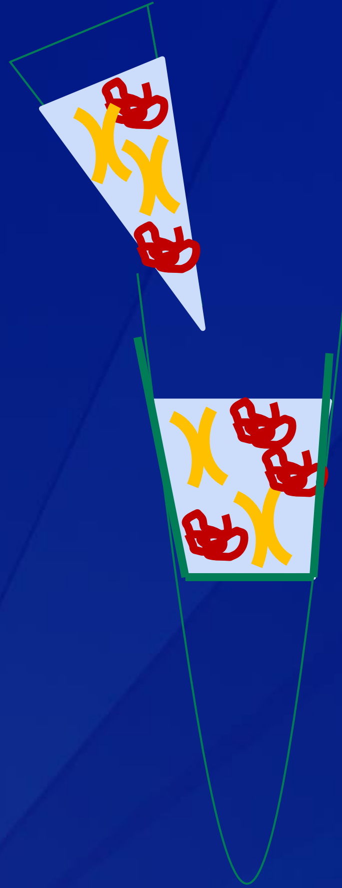
Column-based DNA Extraction

Step 1:
Add buffer to DBS and heat to remove lysed blood into solution

Add binding buffer so the DNA will bind to the column matrix

Remove solution in preparation to apply to the column

Column-based DNA Extraction cont.



Step 1:
Add buffer to DBS and heat to remove lysed blood into solution

Add binding buffer so the DNA will bind to the column matrix

Remove solution in preparation to apply to the column

Step 2:
Add solution to column

Column-based DNA Extraction cont.

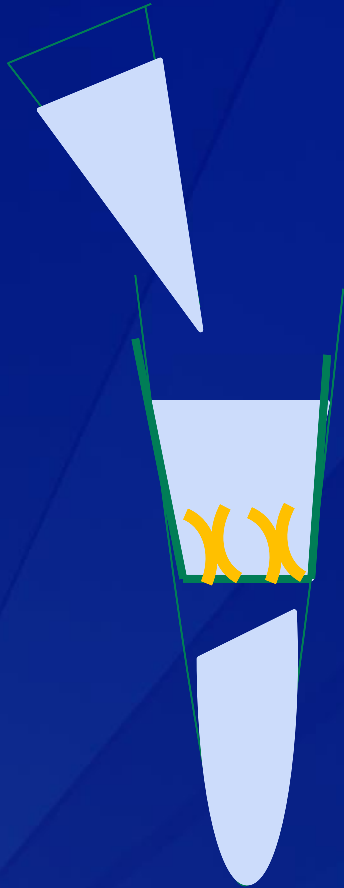
Step 1:
Add buffer to DBS and heat to remove lysed blood into solution

Add binding buffer so the DNA will bind to the column matrix

Remove solution to apply to column

Step 2:
Add solution to column
Centrifuge column to push proteins through the matrix – DNA does not pass through

Column-based DNA Extraction cont.



Step 1:
Add buffer to DBS and heat to remove lysed blood into solution

Add binding buffer so the DNA will bind to the column matrix

Remove solution in preparation to apply to column

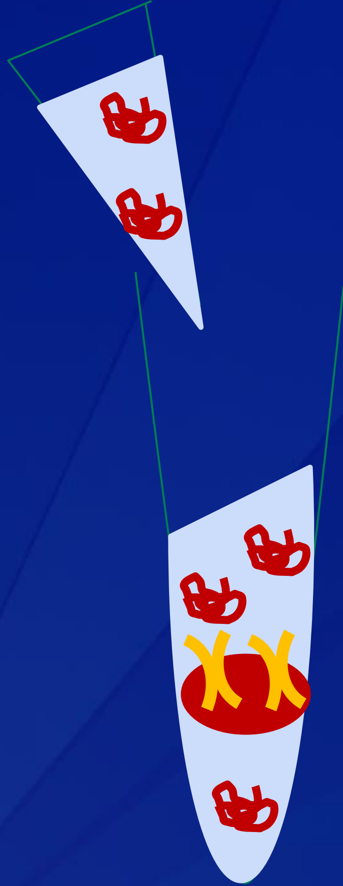
Step 2:
Add solution to column
Centrifuge column to push proteins through the matrix - DNA does not pass through

Step 3:
Add DNA elution reagent and centrifuge to elute DNA into tube

Less Pure DBS DNA Extraction Methods

- Boil Prep DNA Extractions
  - Without Pre-Wash
    - Pros
      - Most inexpensive
      - Fast
    - Cons
      - Crude prep
      - Fragmented DNA
      - Low DNA concentration
      - Homebrew
  - With Pre-Wash
    - Pros
      - Removes some contaminants
      - Inexpensive
      - Fast
      - Commercially available
    - Cons
      - Not highly purified
      - Fragmented DNA

Boil Prep Generation DNA Extraction

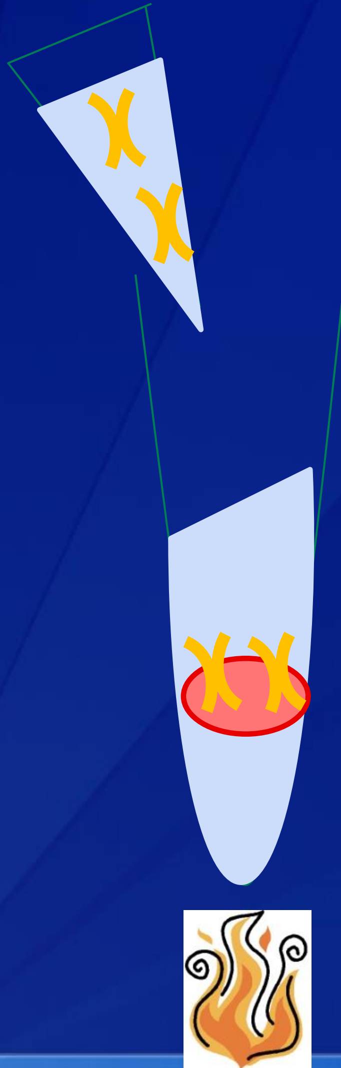


Step 1:
Add solution 1 to DBS to wash the punch

Remove supernate to wash away contaminants including heme & other proteins

Repeat process

Boil Prep Generation DNA Extraction cont.



Step 1:
Add solution 1 to DBS to wash the punch

Remove supernate to wash away contaminants including heme & other proteins

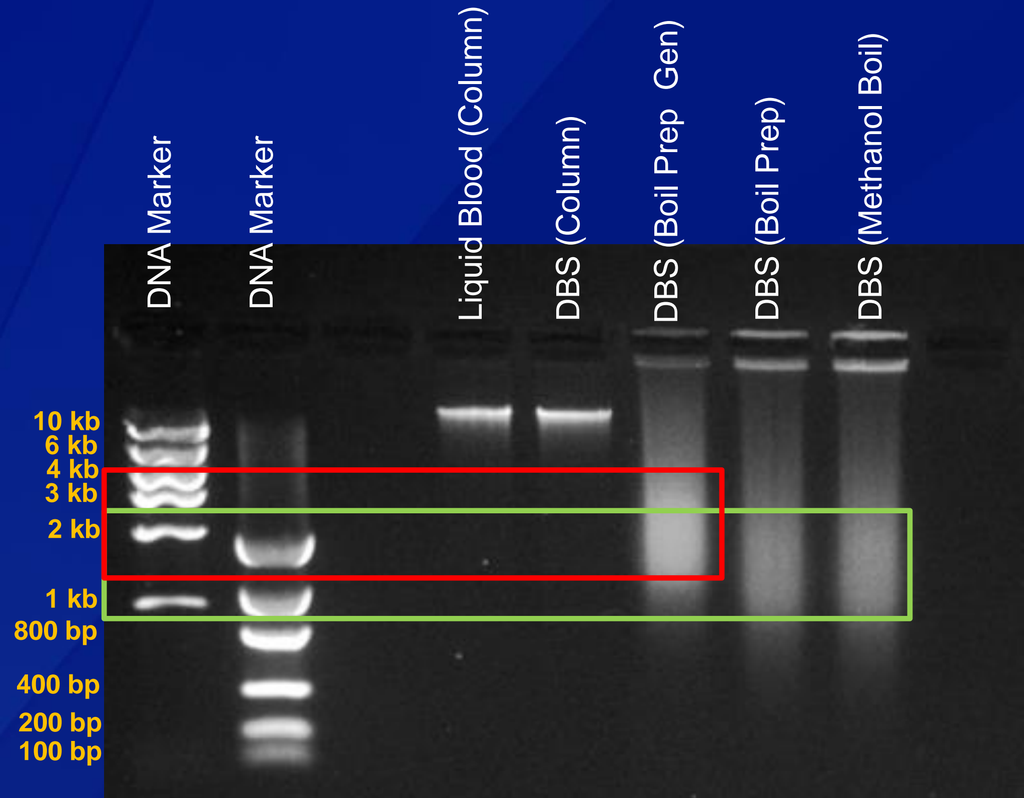
Repeat process with Soln 1 and a second time with solution 2

Step 2:
Add DNA elution solution (Soln 2) and heat to remove DNA from the DBS

How DNA Becomes Fragmented

- Exposure to prolonged high temperatures
- Mechanical shearing – pipetting, mixing, etc.
- DNAse enzyme activity

Genomic DNA Fragmentation



- Most NBS assays are small target sizes (< 1kb)
- Fragmented DNA often results in better amplification
- Can amplify 6 kb fragment from Boil Prep (Generation)

DBS DNA Quantitation: 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

Commonly Used DNA Quantification Methods

- **Absorbance**
  - Measures aromatic compounds
- **Pico-green**
  - Measures double stranded DNA
- **Quantitative PCR**
  - Measures target in amplifiable DNA

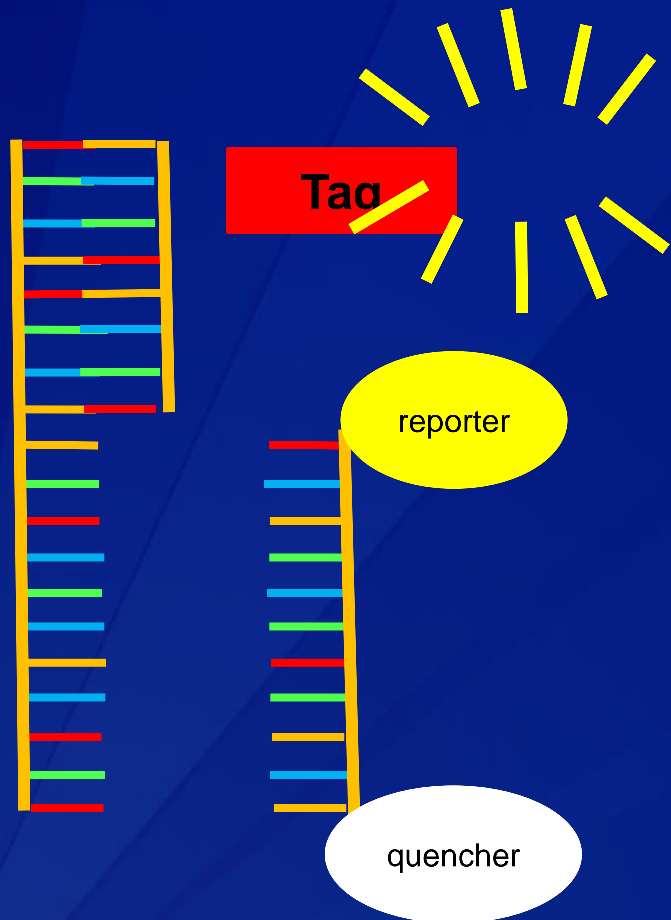
DNA Quantitation: Absorbance

- Spectrophotometer reads the amount of light that passes through a DNA sample at A260
  - (Ex: Nanodrop, SMAX)
- Does not distinguish between dsDNA, ssDNA, RNA or aromatic organic compounds
- Measure is sensitive to protein contamination (A280)
  - A260/280 ratio should be 1.8 if sample has little to no protein contamination

DNA Quantitation: Picogreen

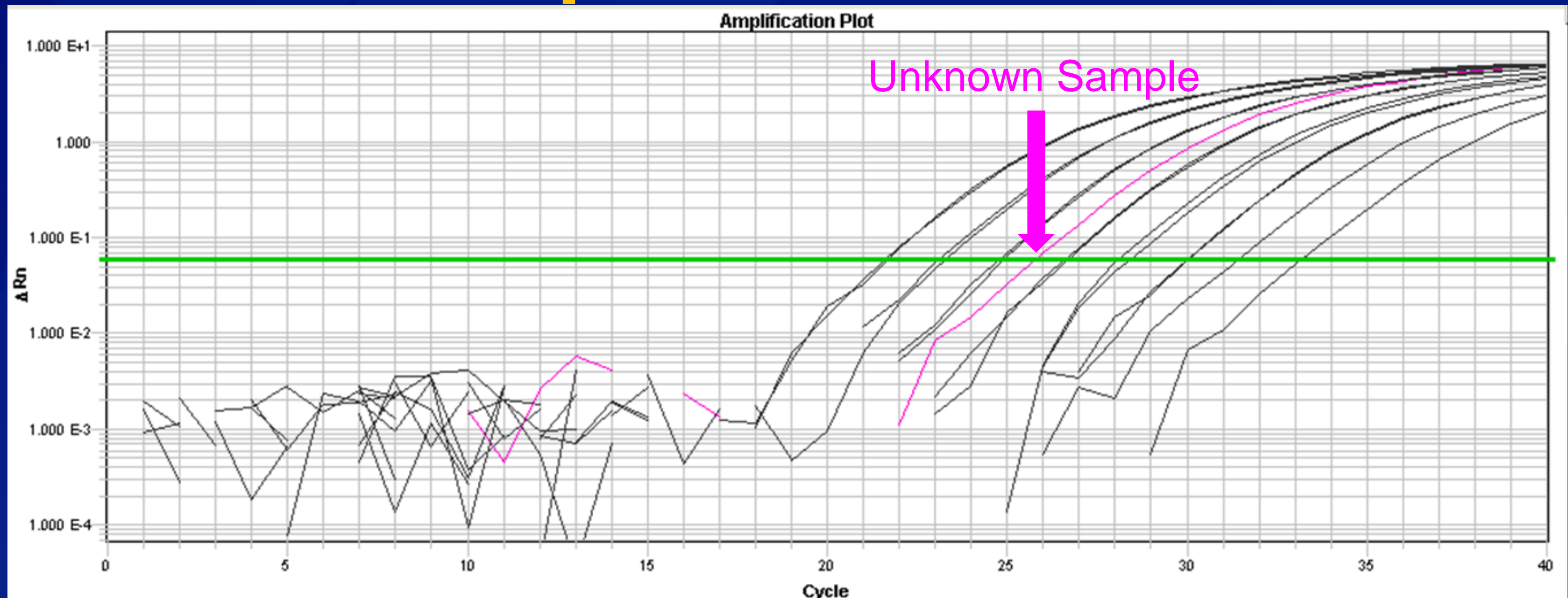
- Fluorescent dye binds to dsDNA
  - Absorbs light at 480 nm and emits light at 520 nm
- Light emitted is used to calculate DNA quantity by comparing to a known standard curve
  - Unincorporated dye does not absorb light at 480nm
- Contaminants typically do not impact this measure
- Since this assay uses a standard curve, the measure is only as good as the standard!

DNA Quantitation: Real Time PCR



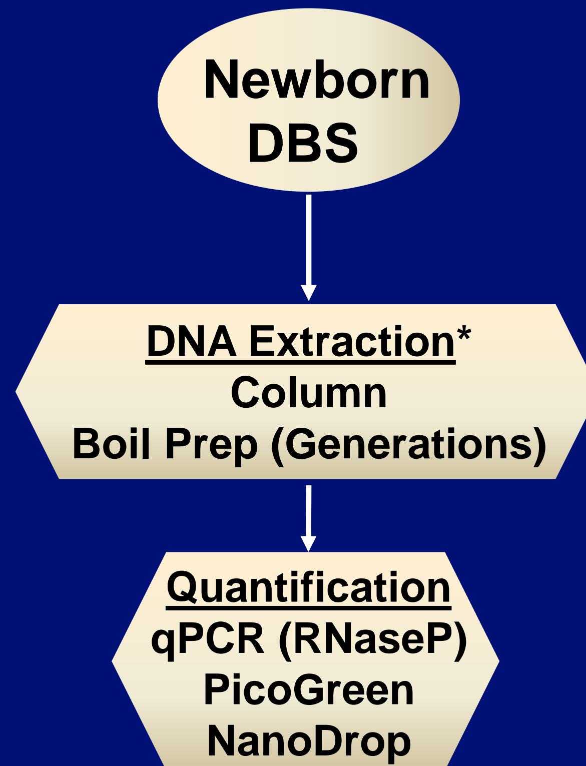
- **A fluorescent labeled probe binds to DNA**
  - The label is quenched when the probe is intact
- **Taq polymerase synthesizes a new DNA strand**
- **When Taq encounters the bound probe, exonuclease activity chews up the probe**
  - florescence can now be detected
- **The florescence generated at each cycle is measured**

Real time PCR of RNaseP Amplification Plot



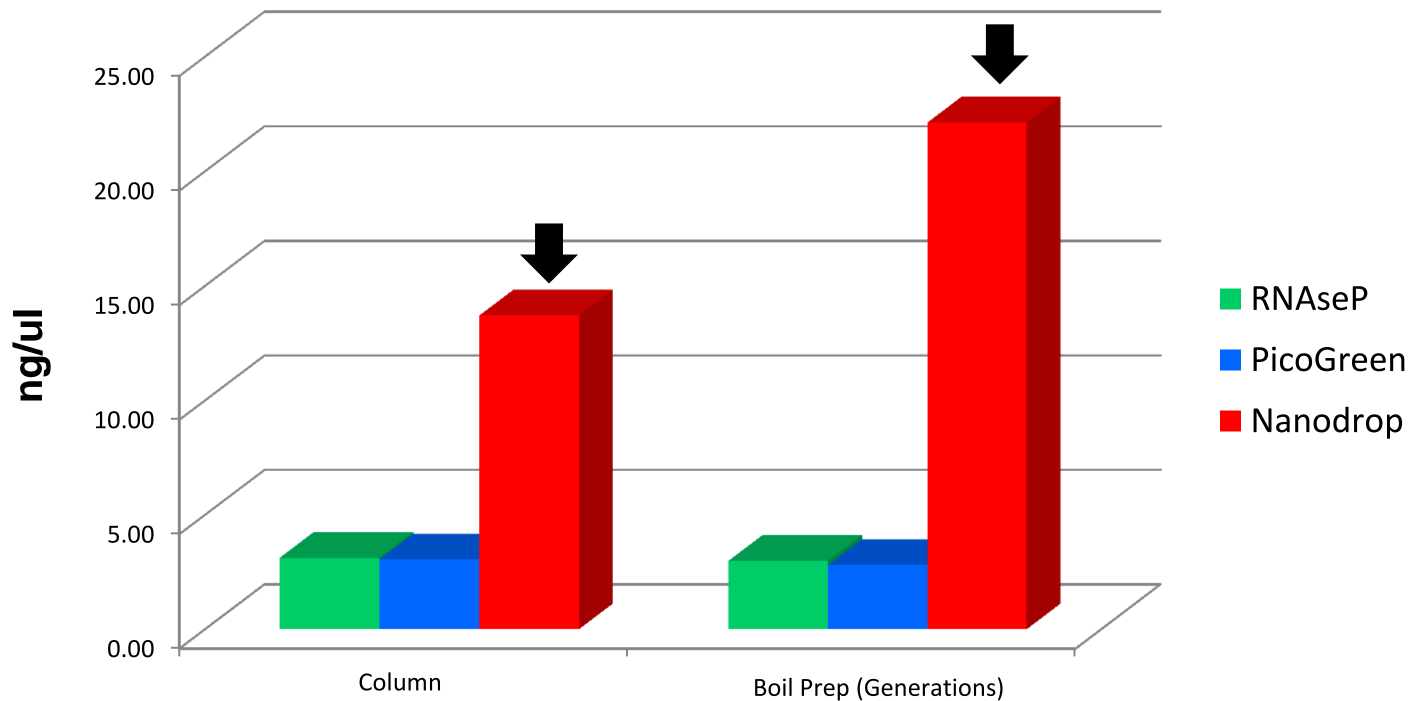
- Unknown concentrations are calculated based on a standard curve
  - Note: this measure is only as good as the standard curve!
- Concentration represents amplifiable DNA

Comparing DBS DNA Quantitation Methods



*DNA was extracted from one 3mm punch

DBS DNA Quantitation Methods: qPCR, PicoGreen and NanoDrop



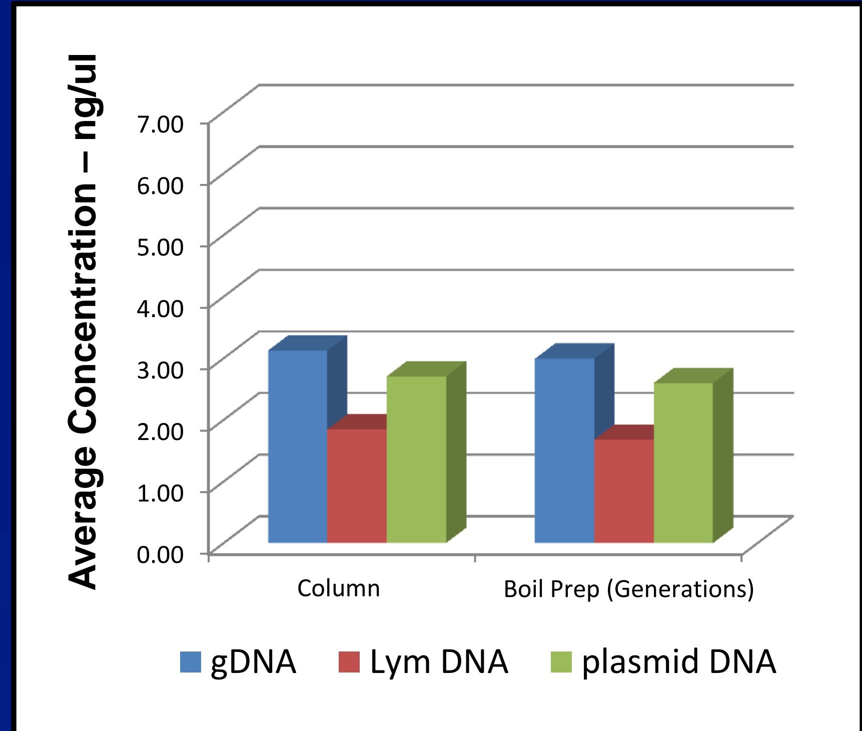
Average DNA Yield
Determined by Each Quantitation Method

Extraction method	N*	qPCR (RNASeP) (ng)	PicoGreen (ng)	NanoDrop (ng)
Column	20	188	183	8[illegible]2
Boil Prep (Gen)	20	180	169	1[illegible]8

*DNA was extracted from DBS that had been stored for 6 months at -20°C for 6 months

qPCR DNA Quantitation
Standard Curve Source Materials

- **Standard curve sources:**
  - DNA from liquid blood (gDNA)
  - DNA from transformed lymphocytes (LYM DNA)
  - Plasmid DNA containing gene to be amplified (pDNA)
- **Results are Different!**
  - LYM DNA standard is 0.41 fold lower than gDNA
  - pDNA standard is 0.13 fold lower than gDNA



DNA concentrations cannot be compared if measured with different standard curve sources!

DNA Yields from Common NBS DNA Extraction Methods

(measured by qPCR)

	Boil (Gen)	Boil	Methanol Boil
Sample	DNA yield (ng)	DNA yield (ng)	DNA yield (ng)
Adult PT Sample 1*	44.50	6.05	4.05
Adult PT Sample 2*	122.50	32.51	8.75
Adult PT Sample 3*	289.50	54.59	19.60

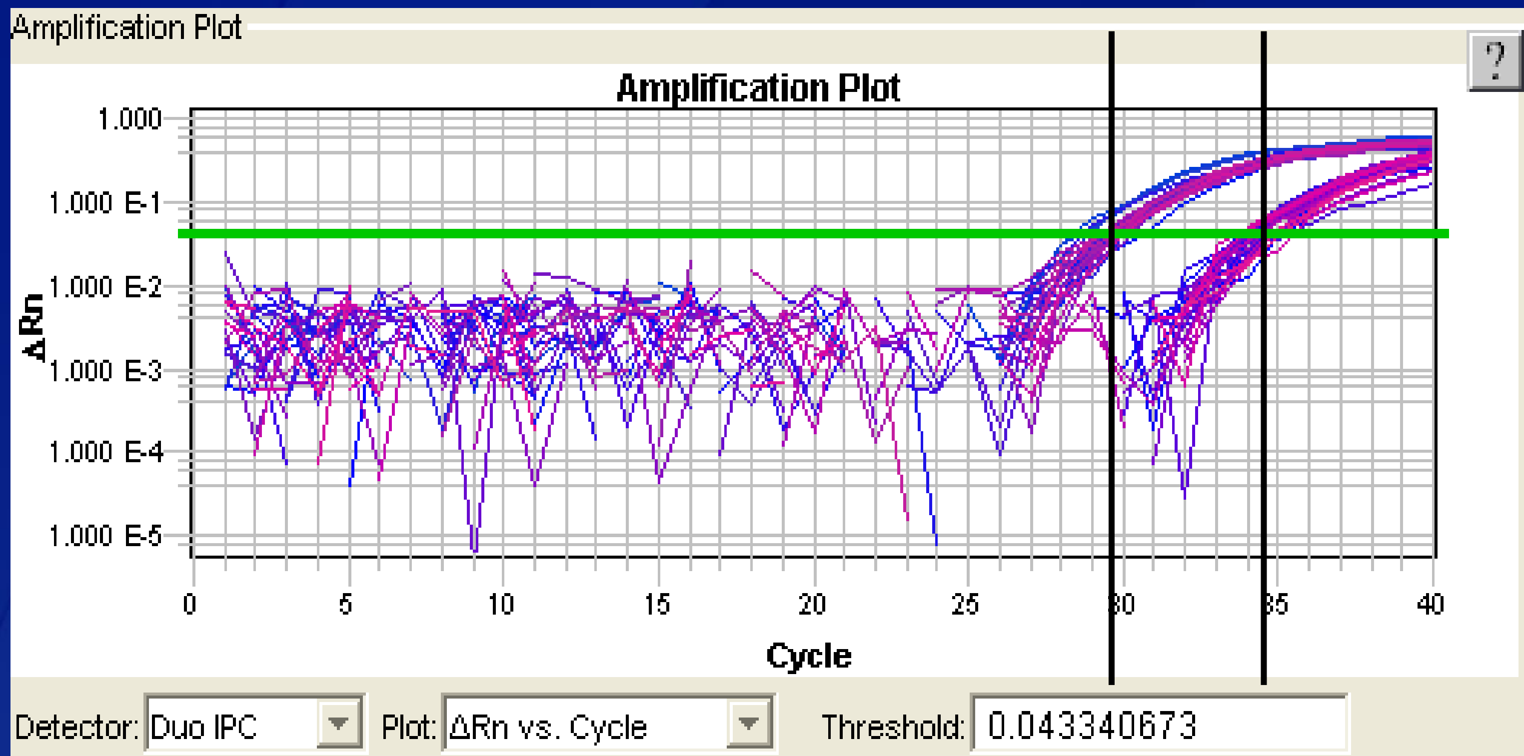
* Extracted from NSQAP's Adult Cystic Fibrosis PT specimens with known high, medium and low concentrations

- **Boil Prep**
  - ~5 fold lower than Boil Prep Generation
- **Methanol Boil Prep**
  - ~13 fold lower than Boil Prep Generation

qPCR to Detect Inhibitors
Quantifiler Duo Assay

- Detect PCR inhibitors using an internal positive control (IPC)
- IPC is an artificial template simultaneously amplified with human DNA
- IPC C^T values ≥ 31 indicate an extract may be inhibited

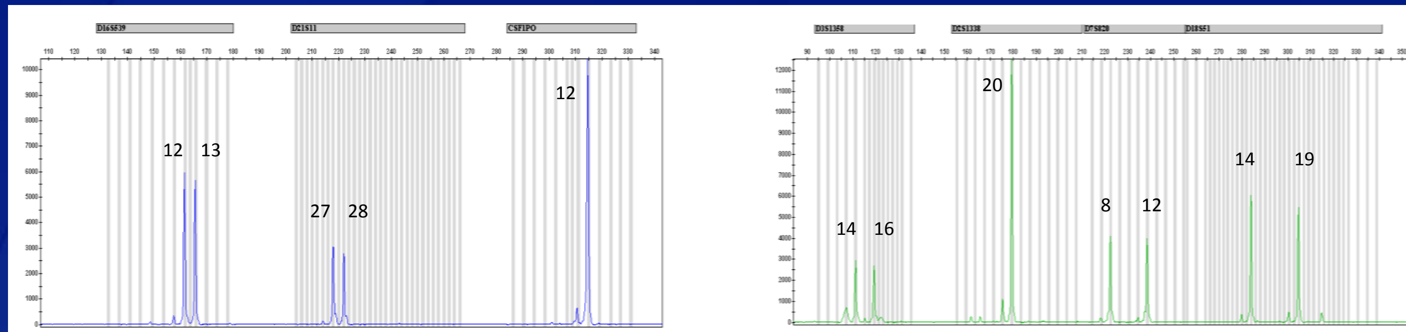
Internal Positive Control Amplification Plot



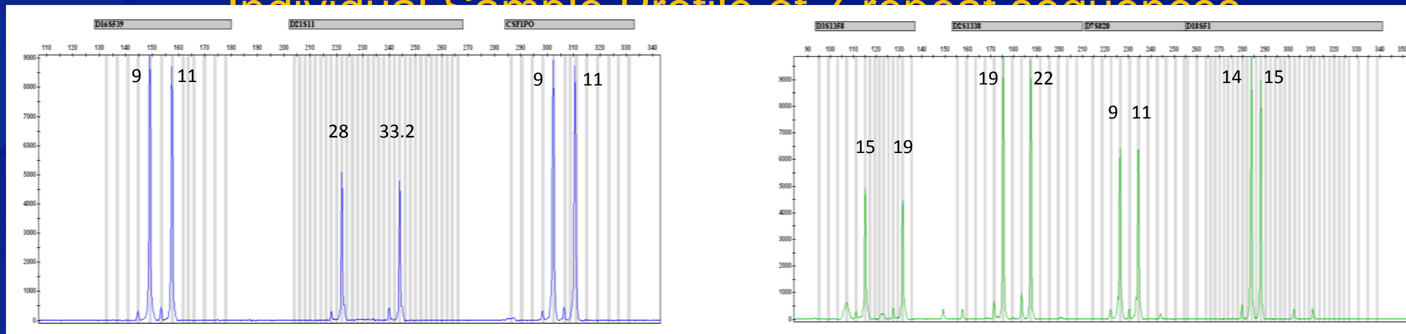
- First cluster amplifies as expected (IPC Ct<31)
- Second cluster amplifies later indicating inhibition (IPC Ct>31)

Testing for Sample Identity

- Tandem repeated sequences (units of 2-6 bp) are widespread in genome
- Number is variable from person to person and is used as “DNA fingerprint”

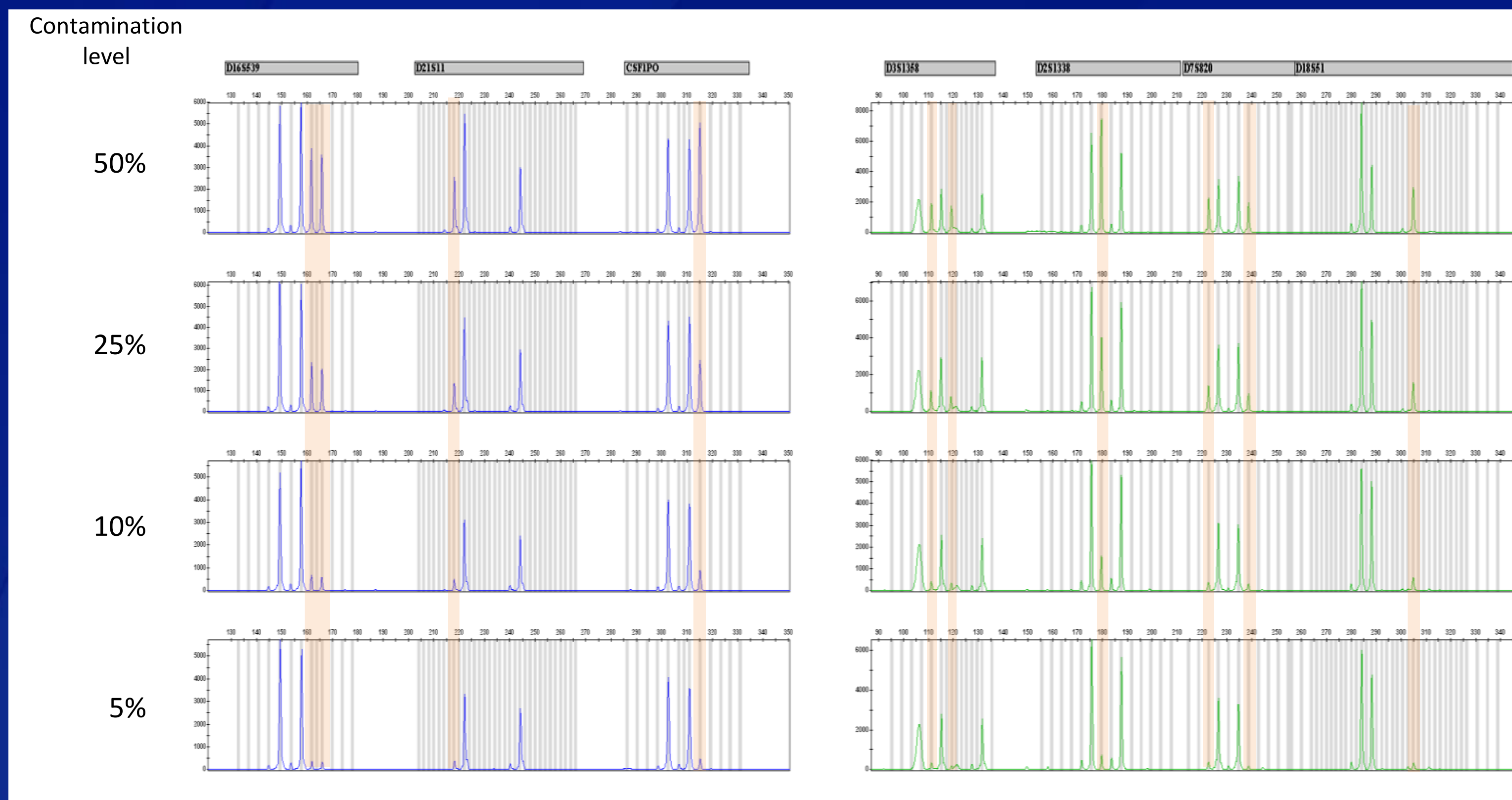


Individual Sample Profile of 7 repeat sequences



Testing DNA for Contamination

Highlighted areas show contamination of primary DNA source



Note: If a normal primary sample is contaminated with 5% F508del mutation, it does not test positive with NBS mutation detection assays

Take Home Messages

- **Highly purified DNA extractions are expensive and not typically necessary for NBS assays**
- **Commonly used methods to extract DNA from DBS:**
  - Boil prep Generation method - affordable commercial method
  - Homebrew boil prep method - results in ~5 fold lower yield than boil prep Generation
  - Methanol boil prep - results in ~13 fold lower yield than boil prep Generation
- **DBS extracted DNA should not be quantitated used spectrophotometer!**
  - Results in a significant overestimation

Take Home Messages - Continued

- **Real time PCR quantifies amplifiable DNA**
  - Standard curve source can introduce variability
- **Once an assay is validated, DNA quantitation is typically not necessary**
- **Real time PCR can detect PCR inhibitors**
- **DNA fingerprinting is a useful assay for NBS molecular validation**
  - Resolves discrepant results in duplicate samples
  - Can be used to detect sample contamination

The findings and conclusions in this presentation are those of the author and do not necessarily represent the views of the Centers for Disease Control and Prevention.

For more information please contact Centers for Disease Control and Prevention
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