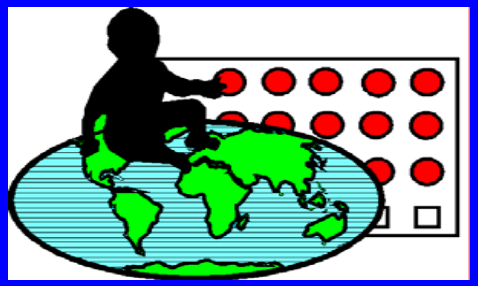

Newborn Screening
Quality Assurance Program

Development of Matched Phenotypic and Genotypic Control Materials in Dried Blood Spots

Joanne Mei
National Center for Environmental Health
Centers for Disease Control and Prevention

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Advantages of Dried Blood Spot Samples

- Collection simple
- DNA and most analytes are stable
- Transportation simple
- Storage easy/compact
- Whole blood matrix – includes white cells
- Safety/handling exposure
- Centralized technology/laboratory

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*Anal. Chem.***2002,** *74*, 1863-1869

Polymerase Chain Reaction Amplification of DNA from Aged Blood Stains: Quantitative Evaluation of the "Suitability for Purpose" of Four Filter Papers as Archival Media

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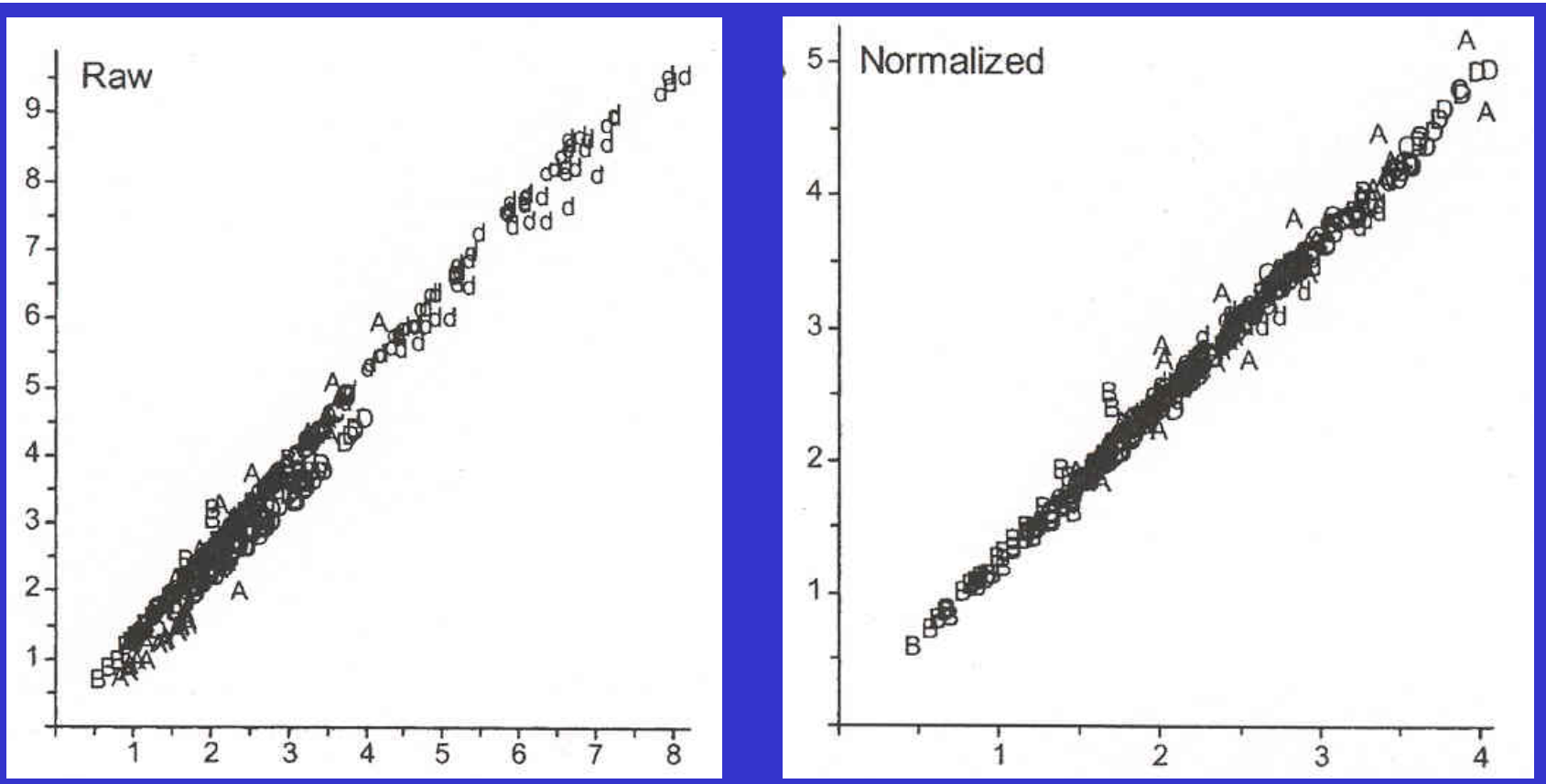
In collaboration with the Armed Forces Institute of Pathology's Department of Defense DNA Registry, the National Institute of Standards and Technology recently evaluated the performance of a short tandem repeat multiplex with dried whole blood stains on four different commercially available identification card matrixes. DNA from 70 stains that had been stored for 19 months at ambient temperature was extracted or directly amplified and then processed using routine methods. All four storage media provided fully typeable (qualitatively identi-cal) samples. After standardization, the average among-locus fluorescence intensity (electropherographic peak height or area) provided a suitable metric for quantitative analysis of the relative amounts of amplifiable DNA in an archived sample. The amounts of DNA in Chelex extracts from stains on two untreated high-purity cotton linter pulp papers and a paper treated with a DNA-binding coating were essentially identical. Average intensities for the aqueous extracts from a paper treated with a DNA-releasing coating were somewhat lower but also somewhat less variable than for the Chelex extracts. Average intensities of directly amplified punches of the DNA-binding paper were much larger but somewhat more variable than the Chelex extracts. Approximately 25% of the observed variation among the intensity measurements is shared among the four media and thus can be attributed to intrinsic variation in white blood count among the donors. All of the evaluated media adequately "bank" forensically useful DNA in well-dried whole blood stains for at least 19 months at ambient temperature.



Area vs height of Ampf/STR COfiler signals for a series of blood stains stored on four different storage media. The left plot displays the average height and areas of the unique allelic signals for each unique DNA over 10 genetic loci. The right plot displays the height and areas after standardization to the signals from the control DNA amplified at the same time as the sample extracts. (Kline et al. Anal Chem 2002:74;1863-1869)

Newborn Screening Disorder Detection

- Biomarkers of metabolic and inherited disorders screened – Phenotype
- Phenotype confirmed with genotypic analysis (for a limited number of disorders)
  - Sickle Cell Disease
  - Cystic Fibrosis
  - MCAD
  - Others

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Number of Laboratories in Genetic EQA Programs

Disorder	Domestic	International	Total
Hemoglobinopathies	53	18	71
Cystic Fibrosis	10	30	40

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External Quality Assurance for Newborn Screening

- DBS for EQA
  - Purchased patient blood (anonymous)
    - Biotinidase deficiency
    - Galactose-1-phosphate uridyltransferase deficiency
  - Prepared in-house with washed, intact red cells
    - Amino acidopathies
    - Endocrinopathies
    - CF/IRT
    - Others
  - Umbilical cord blood
    - Hemoglobinopathies
    - Phenotype and Genotype

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DBS Materials for Genetic Analysis Whole Blood Matrix

- CDC DBS materials primarily used for phenotype
  - Exception – Hemoglobinopathies
    - Used for External Quality Assurance ***only***
    - Hemoglobin proteins have poor long term stability
    - DNA in filter paper matrix very stable
- Challenge: Prepare DBS that express phenotype and genotype

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Hemoglobin Dried Blood Spot EQA Material

- Hemoglobin Phenotype Screening
  - Isoelectric Focusing
  - HPLC
- Hemoglobin Genotype Confirmation
  - PCR methods
    - –RFLP
    - –DNA sequencing

Preparation of Hb EQA Materials

- Pre-screened umbilical cord blood
  - Obtained from Alabama
  - Spotted on S&S Grade 903 paper
  - Phenotype re-screened by IEF and HPLC
  - Genotype confirmed by RFLP analysis
    - AA
    - AS, AC
    - SS
    - SC
    - AD, AE, other variants

RFLP Analysis

- Allows for discrimination between two alleles without sequencing
- Sample preparation
  - 3mm DBS punch
  - Fix with methanol, dry
- PCR reaction, 125 bp fragment
  - Primers for HbS, C, and E
    - 0.6 µM XcmIA forward (5'CCATGGTGCCCATGACTCC 3')
    - 0.6 μM XcmIB reverse (5'CTTAAACCTGTCTTGTAACCTTG 3')
  - Each PCR reaction had a final volume of 50 µl

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RFLP Analysis cont.

- Amplified DNA restriction digestion (25µl)
  - DdeI
  - Hpy181III
  - XcmI
- Reactions incubated at 37°C (1 hour)
  - 10 µl pre-cast 10% polyacrylamide gel (1 hour)
  - Gels stained (ethidium bromide, 20 min)
  - Gels destained in water (10 min)

Mutations

Mutation	Location	Change
HbS	Codon 6	GAG → GTG
HbC	Codon 6	GAG → AAG
HbE	Codon 26	GAG → AAG

Sequence

Codon 6 Codon 26

5’ ACT CCT **GAG** GAG…….GGT **GAG** GCC 3’

3’ TGA GGA **CTC** CTC……. CCT **CTC** CGG 5’

Enzyme Recognition Sequence

DdeI - 5’ CTNAG 3’

XcmI – 5’ CCANNNNNNNNTGG 3’

Hpy181III – 5’ TCNNGA 3’

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Wild Type, HbS, and HbC Mutations
DdeI digestion

AA SS AS SC AC AD ϕχ174 HinfI marker

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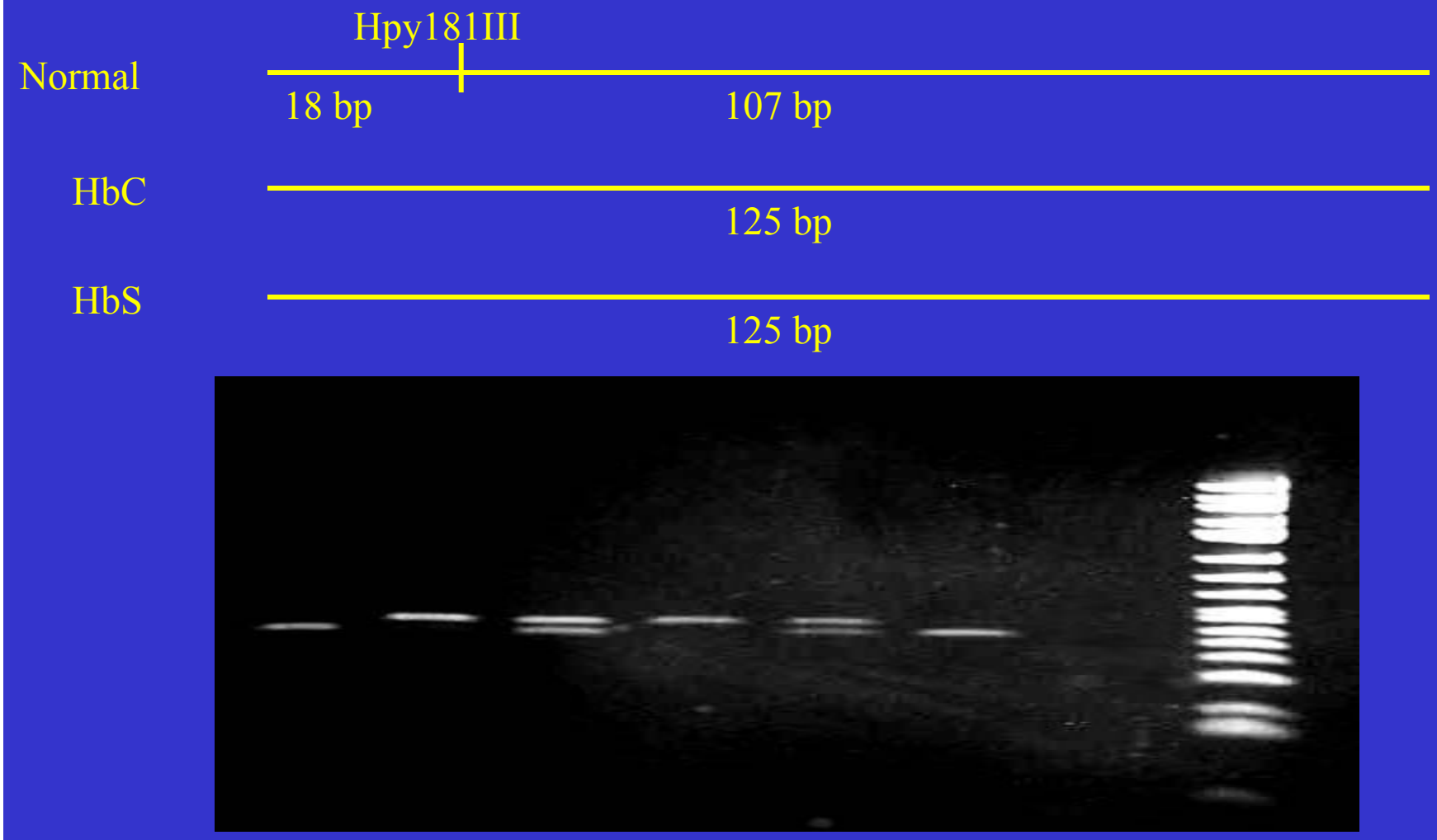
Wild Type, HbS, and HbC Mutations
Hpy181III digestion



Wild Type, HbS, and HbC Mutations
XcmI digestion

Normal

125 bp

HbC

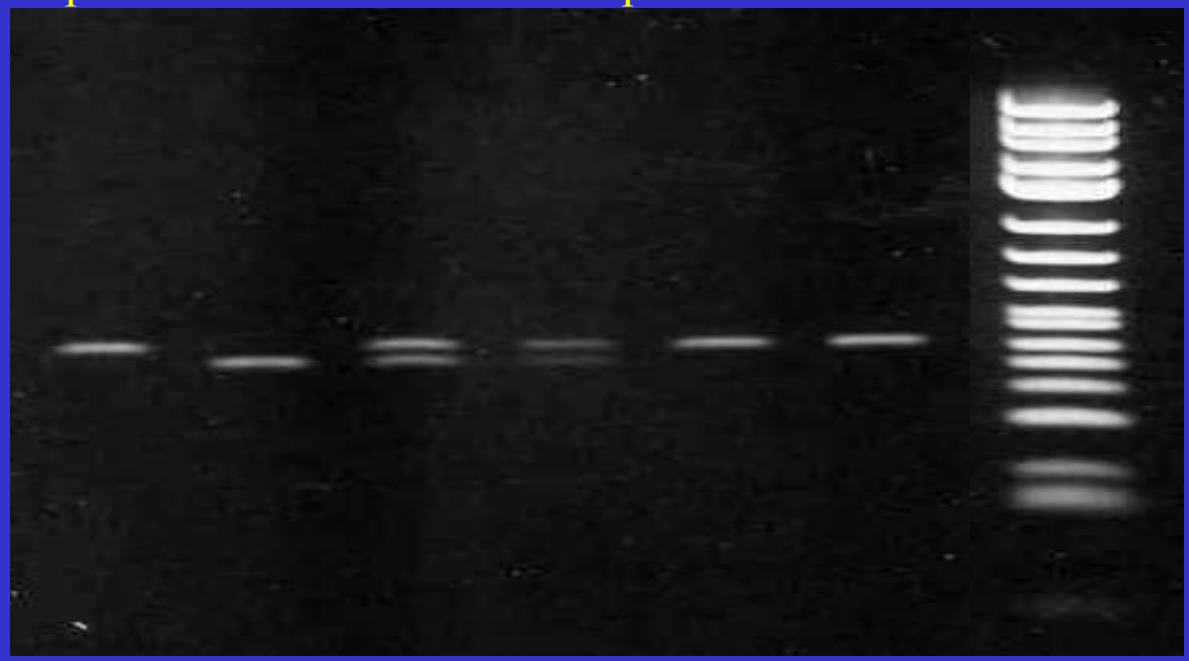
125 bp

XcmI

HbS

17 bp

108 bp



AA SS AS SC AC AD ϕχ174 HinfI marker

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EQA for Cystic Fibrosis Phenotype

- IRT-enriched whole blood made into blood spots
- IRT stability assessed
- Blinded IRT-DBS sent to domestic and foreign participants

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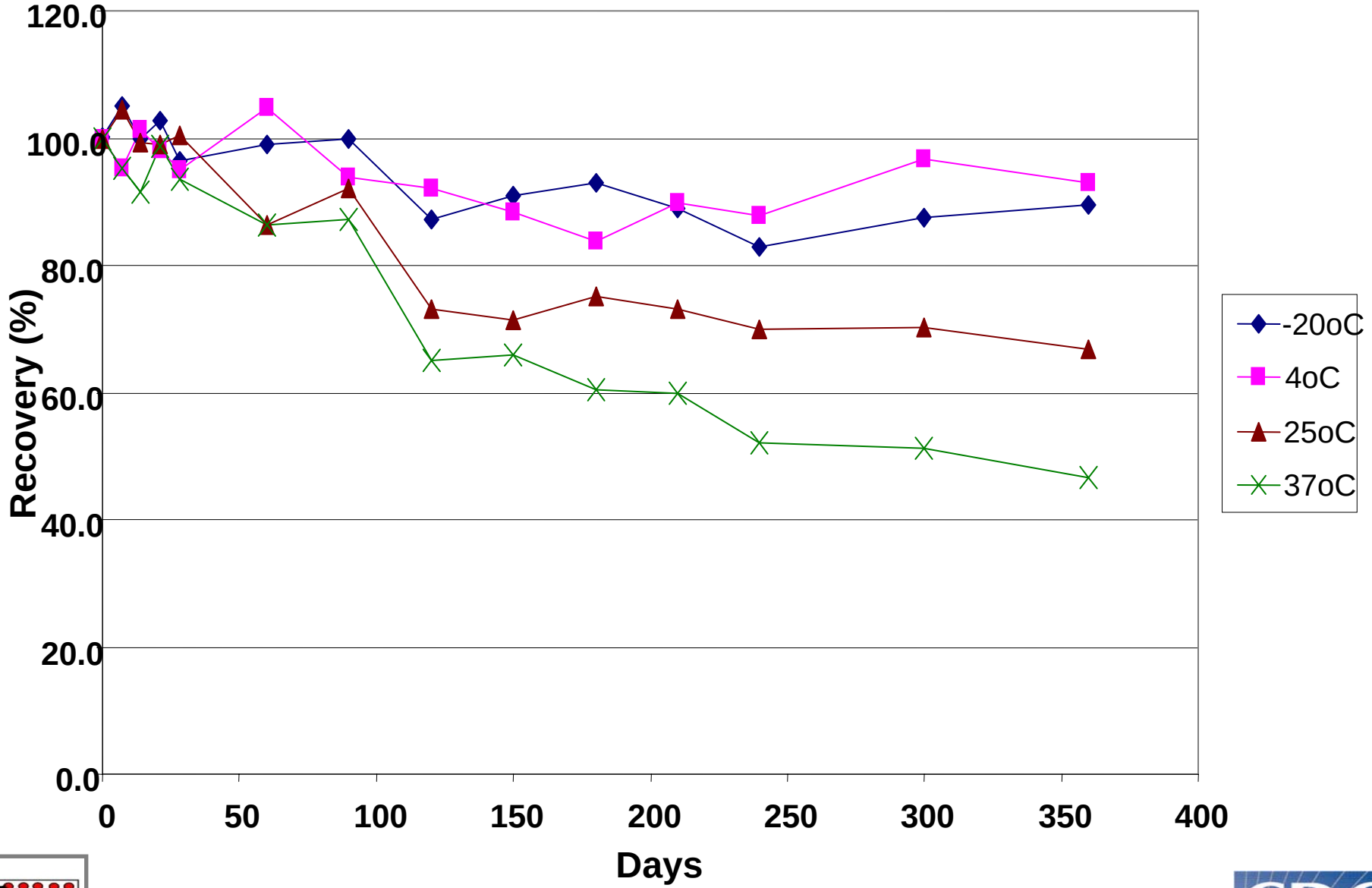
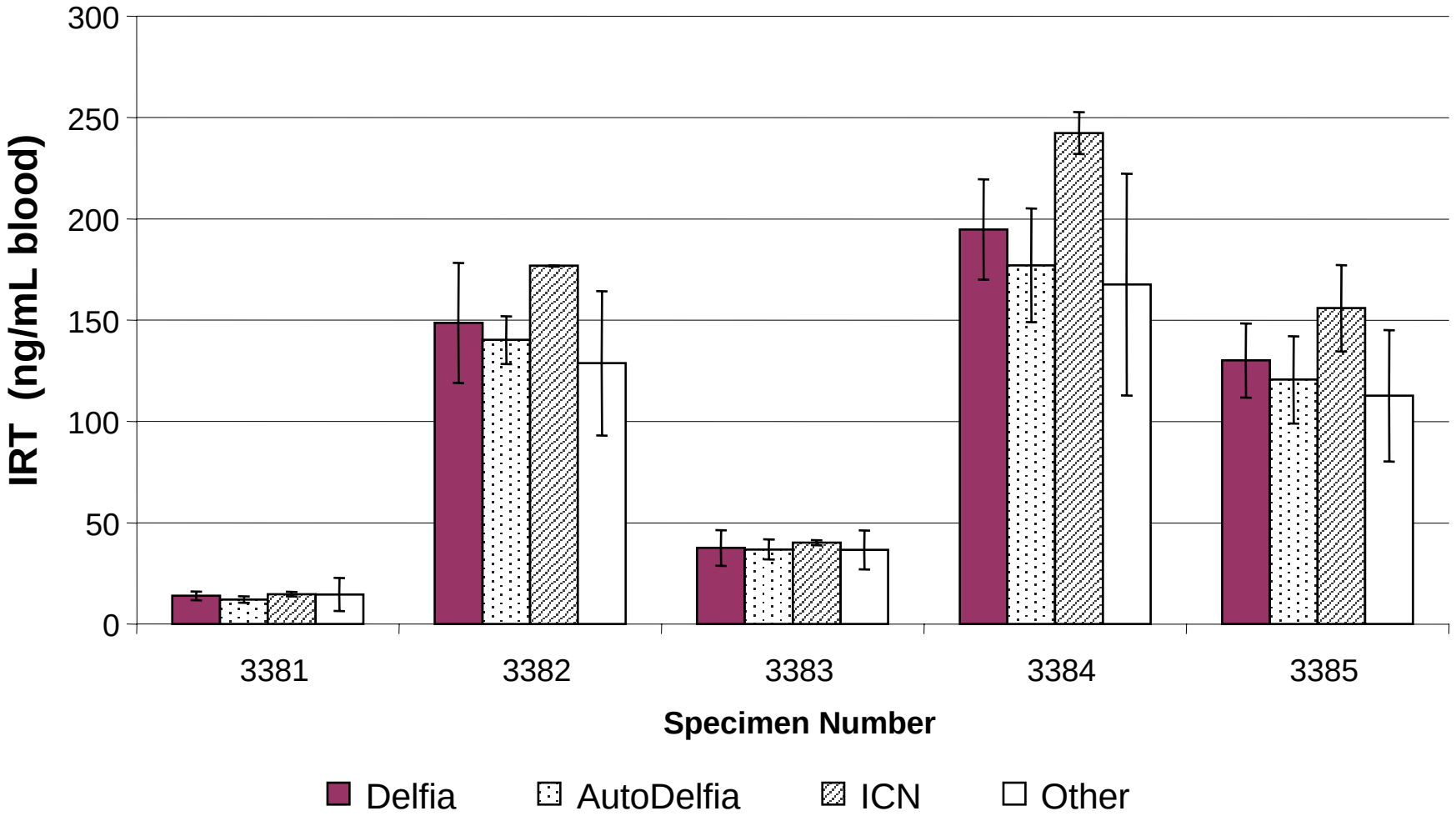
One year Stability of IRT in Dried Blood Spots



Comparison of IRT measurements by method.
N = 31 Laboratories.



Error bars = ± 1 SD

EQA for Cystic Fibrosis Genotype

- To avoid potential contamination with human DNA
  - ΔF508 cells (Coriell) were added to sheep whole blood matrix
  - Epstein Barr virus (EBV) transformed lymphoblastoid cell lines homozygous and heterozygous for ΔF508
  - Spots tested by 2 reference labs
    - Wild type (human donor pre-screened for 32 mutations)
    - ΔF508 homozygote
    - ΔF508 heterozygote

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CF Genotype Blood Spots

- Process
  - Cell lines are grown to appropriate concentration
  - At least 25 X 10^7 cells/mL blood is required
    - Takes us 2-3 weeks to grow enough cells for a 100 mL pool
  - Sheep serum is added to the lymphoblasts
  - Sheep red blood cells are recombined with serum and lymphoblasts
  - Blood is spotted on filter paper

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ΔF508 Mutation Detection

- Fluorescence polarization (Victor^2, Perkin Elmer Life and Analytical Sciences)
  - Adapt method for dried blood spots
    - PCR ΔF508 region (220 bp)
    - Clean-up (follow PE kit protocol)
    - Anneal and extend second primer using fluorescently labeled ddNTP's
      - Tamara (ddGTP)
      - R110 (ddTTP)
    - Read on instrument

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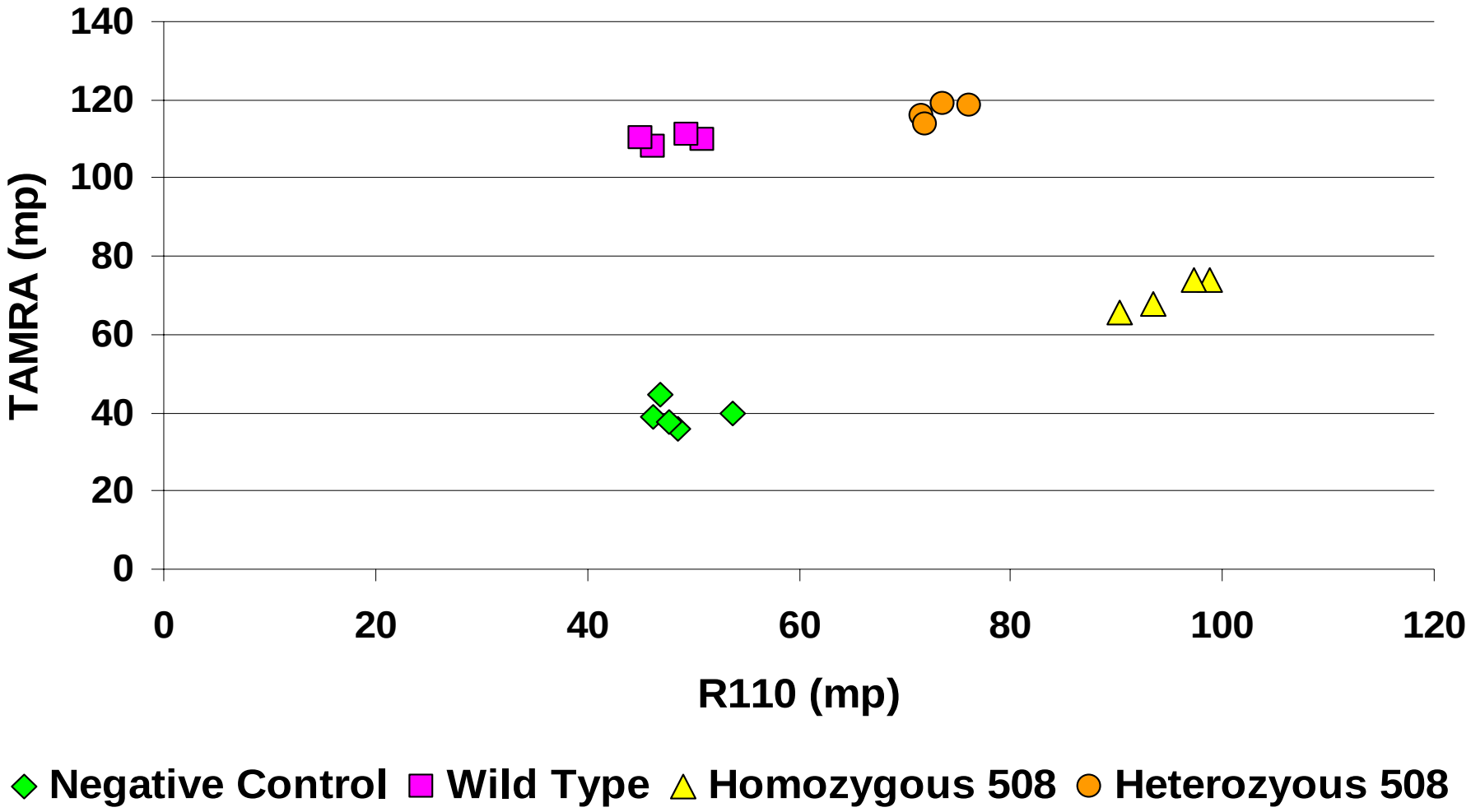
Delta F508 Mutation Analysis by Fluorescence Polarization



EQA for CF Newborn Screening To Do List

- Optimize FP assay for mutation detection
- Incorporate physiologically significant IRT levels into blood spots
  - IRT/ΔF508 spots will be sent out October 2003
- Move into human matrix
  - Assess potential for wild type DNA contamination from serum or cells
- Add other mutations to panel

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Conclusions

- Dried blood spots can be used for both phenotypic and genotypic EQA
- DNA on filter paper is stable
- CDC will continue to investigate methods for producing phenotypic/genotypic materials
- CDC will offer the Newborn Screening community EQA challenges for phenotype and genotype testing.

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