Establishment of Stably EBV-Transformed Cell Lines from Residual Clinical Blood Samples

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Background and Hypothesis

- >600 different genetic tests performed in US.
- Relatively few have readily available, sustainable positive control material.
- Residual clinical samples could be a source.
- B lymphocytes from these residual clinical samples could be transformed with EBV to create a cell line bank with readily available, stable, and sustainable samples.

CDC's "General Recommendations for Quality Assurance Programs for Laboratory Molecular Genetic Tests" - Top Recommendations

- Conduct pilot research to develop positive controls and test samples for pilot performance evaluation (PE) programs.
- The lack of positive controls/samples was identified as having the utmost urgency in the field of MGT for both routine testing and QA/PT programs.

Advantages of Using EBV-Immortalized Cell Lines

- EBV is a tried and true method of transformation
- Yields essentially an unlimited amount of cells and/or DNA
- Easily banked
- Relatively stable
- Closely mimic lymphocytes obtained from whole blood samples
- It is the same sample type used by the ACMG/CAP proficiency testing program for genetics

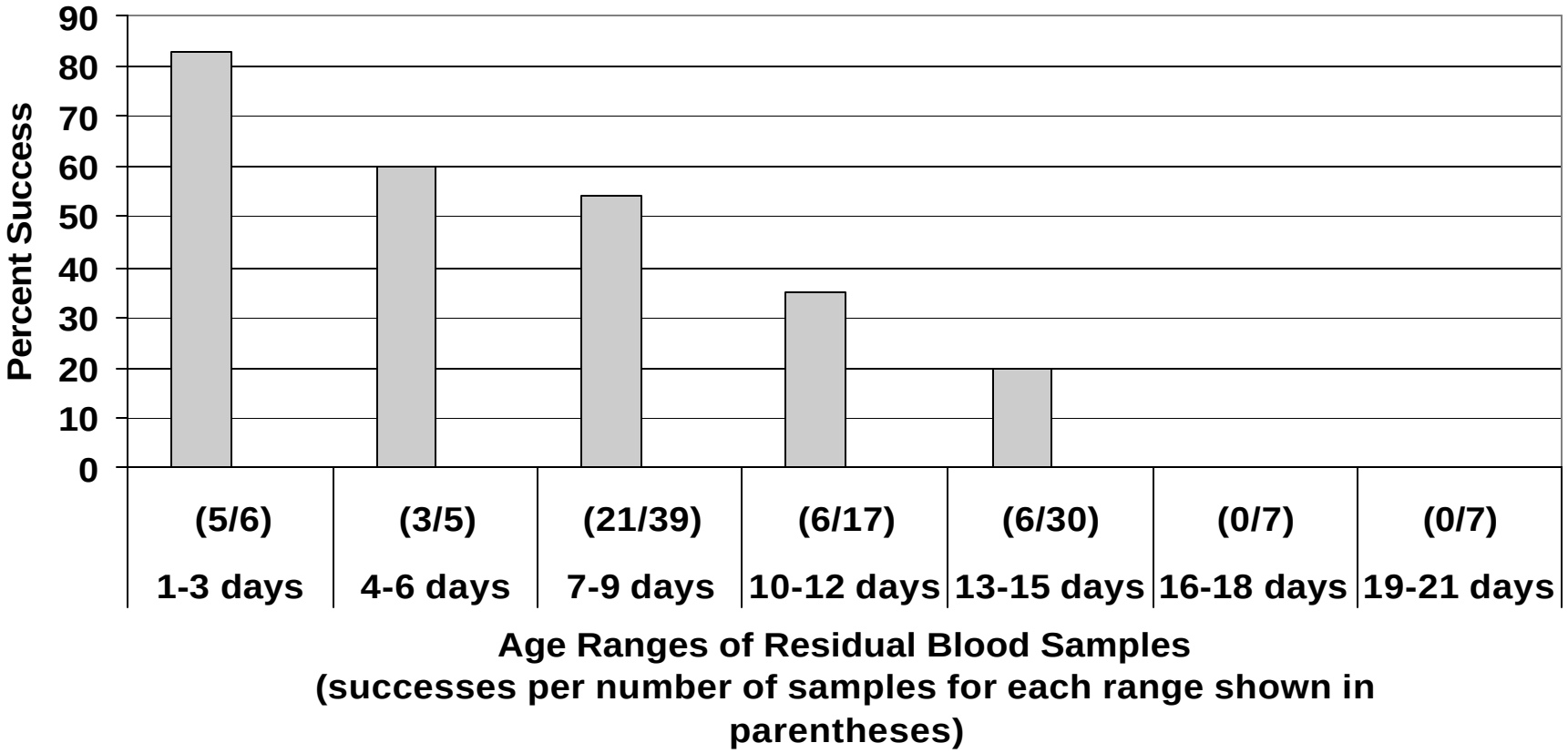
Major Milestones

- Convened panels of experts to prepare, implement and evaluate the pilot plan
- Recruited labs to submit samples and to perform confirmation and pilot proficiency testing
- Implemented the process of sample collection and transformation and verified the stability and presence of the mutations
- Sent samples to at least 5 labs for confirmation testing and later to at least 5 labs for pilot proficiency testing

Effect of Conditions

Sample Age	Anticoagulant and Storage Temperature				
Days Post-Draw	ACD	EDTA	4°C	Ambient	Overall
0-7 Days	85% (11/13)	58% (14/24)	68% (19/28)	67% (6/9)	68% (25/37)
8-14 Days	56% (5/9)	24% (11/46)	31% (16/51)	0% (0/5)	31% (16/56)
15-21 Days	0% (0/5)	0% (0/14)	0% (0/14)	0% (0/5)	0% (0/19)

The Effect of Sample Age on Transformation Success



Univariate relationships between sample variables and transformation success

SAMPLE VARIABLE	# OF SUCCESSFUL TRANSFORMATIONS/# OF ATTEMPTS (%)	P-VALUE
Age of Sample (Days from venipuncture to addition of EBV):		0.002**
1-7 Days	6/ 9 (67%)	
8-14 Days	3/ 8 (38%)	
>14 Days	0/11 (0%)	
Anticoagulant:		
EDTA	3/17 (18%)	0.095*
ACD	6/11 (55%)	
Storage Temperature:		
4C	6/14 (43%)	0.420*
RT	3/14 (21%)	
Hemolysis:		
No	8/18 (44%)	0.098*
Yes	1/10 (10%)	
Sex ***:		
Male	5/15 (33%)	>0.999*
Female	4/12 (33%)	
Age of Subject ***:		
<20	2/ 8 (25%)	
20-49	4/10 (40%)	>0.999**
50+	2/ 9 (22%)	
Sample Volume:		
<3	3/ 8 (38%)	
3 – 5.99	4/12 (33%)	0.794**
6+	2/ 8 (29%)	

Guidelines for Residual Blood Samples Acceptable for EBV Transformation

- Age of Sample: 0-14 Days
- Anticoagulant: ACD or EDTA
- Storage Conditions:
  - 0-7 Day Old Samples: Ambient or 4°C
  - 8-14 Day Old Samples: 4°C Only
- Minimum Sample Volume: 1.0 ml
- 41 (36%) cell lines were established from the 113 transformation attempts. The success rate for was 47% for the 88 samples that conformed to the submission guidelines.
- No successful transformations were achieved with samples that did not conform to the guidelines.

First Set

DUK19061	Cystic Fibrosis	3120+1G>A/621+1G>T
DUK63683	Cystic Fibrosis	DF508/R117H
	Hemochromatosis	H63D/H63D
DUK90919	Factor V Leiden	R506Q/WT
	Hemochromatosis	C282Y/H63D
DUK89614	Prothrombin	G20210A/G20210A
	Hemochromatosis	H63D/WT
	MTHFR	C677T/WT
DUK11305	MTHFR	C677T/WT
	Prothrombin	G20210A/WT
	Factor V Leiden	R506Q/WT
	Hemochromatosis	S65C/WT
DUK46668	Sickle Cell/Hb C Disease	HbS/HbC
DUK53834	Hemochromatosis	H63D/WT
DUK29765	Hemochromatosis	C282Y/WT
DUK32053	Hemochromatosis	H63D/H63D
DUK87691	Hemochromatosis	S65C/WT

Second Set

DUK15765	Alpha-Thalassemia	Type 1 Het (SEA)
DUK40878	Cystic Fibrosis	S1235R/WT
DUK13521	Fragile X (FRAXA)	57/WT CGG repeats
DUK69915	Huntington Disease	31/18 CAG repeats
DUK60302	Craniosynostosis	FGFR3 C749G Het
DUK19946	Connexin 26	35delG/WT
DUK61832	MTHFR	C677T/C677T
DUK21185	MTHFR	C677T/C677T
DUK34385	Hemochromatosis	H63D/S65C
DUK11538	Hemochromatosis	C282Y/WT
DUK22472	Hemochromatosis	S65C/WT

Third Set

DUK82747	Cystic Fibrosis; I148T heterozygote
DUK62150	Cystic Fibrosis; I148T heterozygote
DUK54732	Cystic Fibrosis; I148T heterozygote
DUK15576	Cystic Fibrosis; 394delTT heterozygote
DUK65584	Cystic Fibrosis; 1078delT heterozygote
DUK58698	Cystic Fibrosis; 1898+1G>A, heterozygote
DUK10464	Cystic Fibrosis; 1898+1G>A, heterozygote
DUK99211	Cystic Fibrosis; 1898+1G>A heterozygote
DUK64169	Cystic Fibrosis; 1898+1G>A heterozygote
DUK54361	Cystic Fibrosis; 2184delA heterozygote
DUK66652	Alpha-thalassemia type 1; SEA heterozygote
DUK84629	MTHFR; C677T/C677T homozygote

Confirmation and Pilot Proficiency Testing Results

The reference labs confirmed all mutations in 33 cell lines

With few exceptions, genotypes were correctly identified in pilot proficiency testing

- **A total of three results from different cell lines were incorrectly reported**
- **A total of twelve results from different cell lines were not reported due to technical difficulties**

Conclusions

EBV-transformed B-lymphocyte cell lines carrying mutations of public health importanc can be derived from residual clinical blood samples up to 14 days post-draw.

We established a total of 27 new viable cell lines with mutations of interest from residual clinical samples.

We developed guidelines to help determine whether a particular residual sample would be a good candidate for transformation.

Conclusions

33 different point mutations, one 1-bp deletion, one bp deletion, one large deletion, and four repeat regions were stable in B-lymphocyte cell lines through 20 population doublings.

21 cell lines were successfully piloted to outside genetic testing labs as potential positive control material for PE/QA applications and have been shown to be excellent control material.

EBV transformation of residual clinical samples appears to be a very good way to sustain this effort.

Future Directions

- Fragile X and other triplet diseases
- Funding for sustaining this effort
- Depositing these cell lines in a bank

Acknowledgements

- Susan Bernacki, PhD
- Jeanne Beck, PhD
- Eugene C. Cole, DrPH
- 32+ Testing labs
- Ana Stankovic, MD, PhD
- Laurina Williams, PhD
- D. Joe Boone, PhD
- Ira Lubin, PhD
- Others at the CDC

Expert Panelists

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- Victoria Pratt, PhD
- Thomas Prior, PhD
- Antony Shrimpton, PhD
- Karen Snow, PhD
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IRB-Approved Submitting Labs

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Laboratory Corporation of America	Vicky Pratt, Ph.D
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H.A. Chapman Institute	Frederick Schaefer, Ph.D.
S.U.N.Y. Upstate Medical University	Antony Shrimpton, Ph.D.
Mayo Clinic	Karen Snow, Ph.D.
Duke University Medical Center	Timothy Stenzel, M.D., Ph.D
University of California	Linda Wasserman, MD, Ph.D
Quest Diagnostics	Feras Hantash, PhD
Specialty Laboratories	Jean Amos, PhD

End

Procedure for the Establishment of a Lymphoblastoid Cell Line from Residual Blood

Receive blood collected with ACD or EDTA as the anticoagulant.

Isolate lymphocytes on a Histopaque®-1077 gradient.

After washing, resuspend in cell culture medium (RPMI 1640, 20% FBS) and add Epstein Barr virus and PHA to initiate transformation

When cells have transformed, collect by centrifugation.

After washing, resuspend in cryopreservation medium (RPMI 1640, 30% FBS, 6% DMSO) and dispense into glass ampules, each containing 1 ml of medium with approximately five million cells.

Cryopreserve using controlled rate freezing.

Store in liquid nitrogen.

Cell Culture Quality Control Standard

- Cell lines must be viable, i.e., recover after cryopreservation.
- Cell lines must be free from contamination.
- Cell lines, “original sample,” and DNA must have the same DNA fingerprint.

Disease Requested as Positive Controls

	Cells	DNA
Cystic fibrosis	141	902
Fragile X	104	347
BRCA1	29	101
Hemochromatosis	28	90
Factor V	16	56
Myotonic dystrophy	20	39
Huntington disease	11	38
BRCA2	14	13
Muscular dystrophy	11	2
MTHFR	0	1

Diseases Requested Through Surveys

Disease	total	%
Fragile X	132	49.6
Cystic Fibrosis	98	36.8
Muscular Dystrophy	67	25.2
BRCA1/BRCA2 Hereditary Breast Cancer	55	20.7
Spinal Muscular Atrophy	54	20.3
Factor V	53	19.9
Hemochromatosis	49	18.4
Myotonic Dystrophy	46	17.3
Huntington Disease	46	17.3
Connexin 26	45	16.9
MTHFR	44	16.5
APC	37	13.9
HNPCC	36	13.5
Friedreich Ataxia	30	11.3
Gaucher Disease	26	9.8
Prothrombin	25	9.4
Apolipoprotein E	25	9.4
Spinocerebellar Ataxia	21	7.9
Tay Sachs	19	7.1
Hemoglobin S	17	6.4
Rhesus Blood Group, D Antigen	9	3.9

“Failed Searches”

- Reviewed 20,751 records entered since March, 2000
- 866 listed specific mutations or genes
- 296 of those 866 (34.2%) were for mutations in cystic fibrosis

CFTR Mutations Requested

Mutations Requested	Number
2184DEL A	43
I148T	39
1898+1 G>A	27
1078 DEL T	27
I507 V	20
3849+4 A>G	16
2183AA >G	16
3876DEL A (Hispanic)	9
3120+1 G>A	8
2789+5 G>A	7
711+1 G>T	7
2143DEL T (German)	5
5T/7T/9T	4
1812G >A	2