

Genetic Diseases Showing Molecular Heterogeneity

- Cystic fibrosis
- Duchenne muscular dystrophy
- Phenylketonuria
- β -thalassemia
- Familial cancers
(BRCA1, p53, etc.)
- Gaucher disease
- Neurofibromatosis
- Marfan syndrome
- α 1-antitrypsin deficiency
- Ataxia-telangiectasia
- Tay-Sachs disease
- Mitochondrial disorders

Table 1

RECOMMENDED CORE MUTATION PANEL FOR GENERAL POPULATION CF CARRIER SCREENING

Standard Mutation Panel:

DF508	DI507	G542X	G551D	W1282X	N1303K
R553X	621+1G>T	R117H	1717-1G>A	A455E	R560T
R1162X	G85E	R334W	R347P	711+1G>T	1898+1G>A
2184delA	1078delT	3849+10kbC>T	2789+5G>A	3659delC	I148T
3120+1G>A					

Sequence Analysis of BRCA1 and BRCA2 Can Find the Needle in the Haystack

- BRCA1: 22 coding exons, > 5,500 bp

GGCTTTAAGTATCCAT

- BRCA2: 26 coding exons, > 11,000 bp

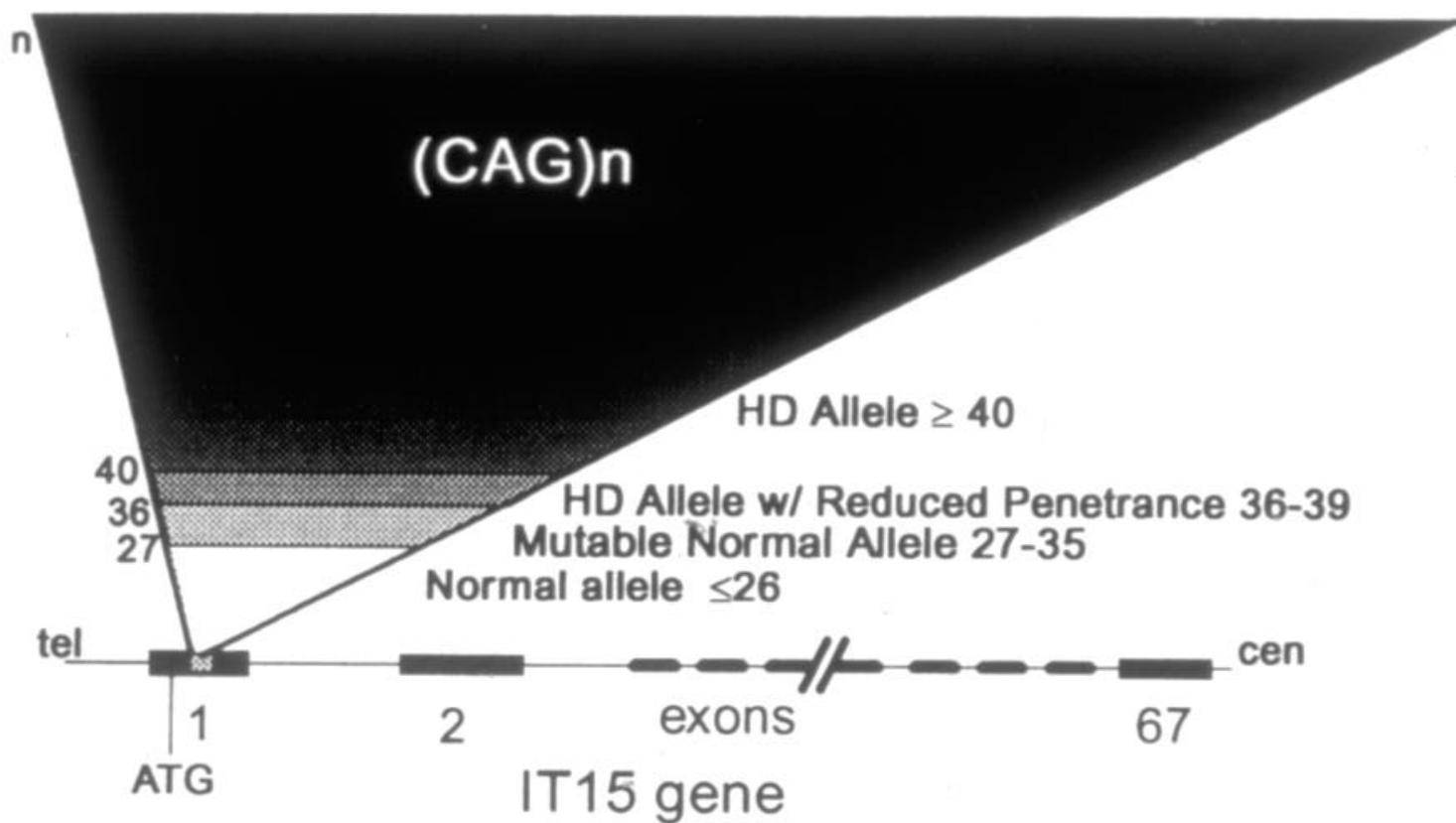
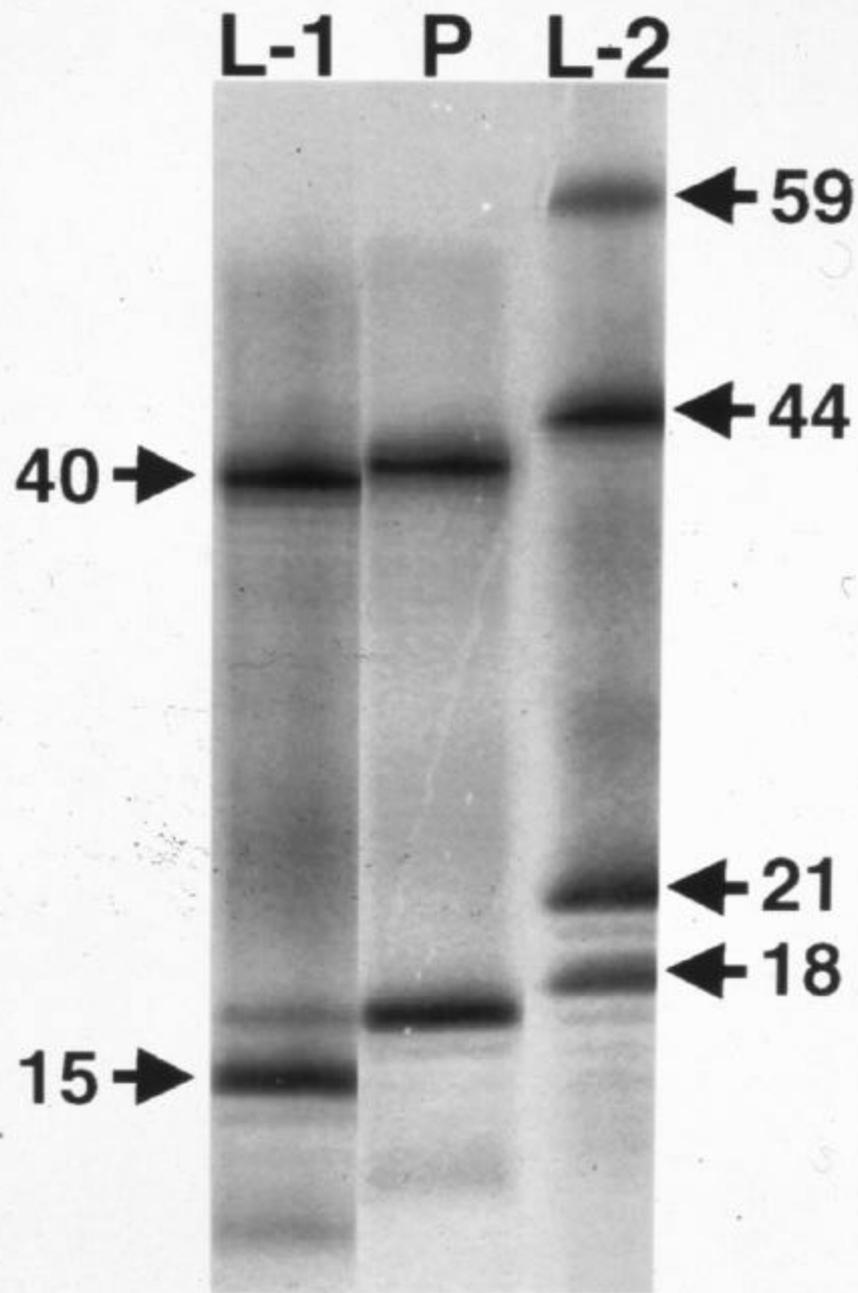


Figure 1 Diagram of the IT-15 gene, showing the location of the CAG-repeat sequence and the boundaries defining the ranges of normal, mutable normal, HD allele with reduced penetrance, and HD allele with full penetrance, based on the worldwide published experience and the experience of the US Huntington Disease Genetic Testing Group.



GENETIC TESTS OFFERED BY RESEARCH vs. CLINICAL LABS

(compiled by D. Ledbetter from *GeneTests* website, 10/01)

866 genetic diseases listed

511 offered by clinical labs (CLIA-certified)

355 offered by research labs only (**41% of total**)

PROPOSED MOLECULAR PATHOLOGY CHECKLIST ITEM REVISION ON REQUIREMENT FOR POSITIVE MUTATION CONTROLS

Old version:

MOL.31560 (Phase II). For direct mutation detection tests (other than by sequencing), are positive and negative controls for each of the mutations in the test panel included?

New version:

MOL.31560 (Phase II). In assays for which multiple analytes are tested simultaneously in a panel or array format, are analyte-specific controls included in each run or in an appropriate proportion of runs?

Note: For test panels or arrays, analyte-specific positive controls may be run in a rotating manner. The periodicity of each control should be appropriate for the frequency of the analyte in the patient population and for the risks posed by a false negative result. In no case should the periodicity exceed 6 months nor should it be less than once in every N runs, where N equals the number of analytes in the panel.



College of American Pathologists

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CAP SURVEYS

PROFICIENCY
TESTING
PROGRAM

Approved by

Health Care Financing
Administration (HCFA)

MGL Survey History

9/19/2000

Disease	1995	1996	1997	1998	1999	2000	2001
CF	A/B	B	A	A	A/B	A/B	A/B
DMD/BMD	A	A	A	A	A	A/B	A/B
Freidreich's Ataxia			B			A	B
FVL			A	A	A	A/B	A/B
Fragile X	B	A	B	B	A/B	A/B	A/B
Hb S/C	B	B				A	A
Hemochromatosis				B	B	A/B	A/B
HD		A/B	B	B	B	B	B
Myotonic Dystrophy			A			A	A
Prothrombin					B	A/B	A/B
PWS/AS			B	B	B	B	B
RhD				A		A	A
SCA					A	B	B
SMA					A	A/B	A
MTHFR						A/B	A/B
BRCA1/2							A
MEN2							B

PRESENT SOURCES OF MUTATION SAMPLES

- Existing CAP samples
- Specimens submitted by committee members
- Coriell Institute
- ATCC

OBSTACLES TO MUTATION SAMPLE PROCUREMENT

- Rarity of patients with unusual mutations
- Onerous informed consent requirements
- Ethical issues
- Expense of clinical collection (incl. sample ownership)
- Cumbersome bulk preparation/validation/distribution

STUDY DESIGN

Phase I -- Development of Mutation Samples

- a) Sample Development
- b) Sample Validation

Phase II -- Pilot Testing of Mutation Samples

- a) Sample Preparation
- b) Sample Shipment to Participant Labs
- c) Data Analysis

DISEASE CANDIDATES FOR SAMPLE DEVELOPMENT

<u>Disease*</u>	<u>Public Health Importance</u>	<u>Suitable for Multiplex</u>	<u>Rare Mutations</u>	<u>Suitable for Sequencing</u>
BRCA	++	+++	+++	+++
CF	+++	+++	++	-
DMD	+	++	+	-
FAP	+	+++	+++	+++
FMF	+	+++	++	+++
FRAX	++	-	+	-
FRDA	<u>+</u>	++	++	-
FVL	+++	-	-	-
HbS/C	+++	++	-	-
HD	+	<u>+</u>	+	-
HFE	+++	+	<u>+</u>	-
HNPCC	++	+++	+++	+++
MEN2	<u>+</u>	+++	+	+++
PWS/AS	<u>+</u>	-	-	-
SCA	<u>+</u>	+++	<u>+</u>	-
SMA	+	+	+	-
α -Thal	++	-	<u>+</u>	-
β -Thal	++	+++	+++	+++
Ashk. Panel	+	+++	-	-
Mito. Panel	+	+++	++	+++
Thromb. Panel	+++	++	-	-
Tri-NT Panel	+	++	+	-

POTENTIAL EXPERIMENTAL APPROACHES

- 1) Sample spiking/mixing
- 2) DNA preparation in bacteria
- 3) Transient transfection
- 4) Stable transformation
- 5) Homologous recombination

POTENTIAL EXPERIMENTAL APPROACHES

Sample Spiking

ADVANTAGES:

- easy
- cheap

DISADVANTAGES:

- requires access to desired mutations to mix
- need accurate 50-50% mixing
- in past surveys, labs have complained of "trick"

POTENTIAL EXPERIMENTAL APPROACHES

DNA Preparation in Bacteria

ADVANTAGES:

- gene manipulation easier than in eukaryotic cells
- relatively inexpensive to prepare many different clones
- can grow to unlimited amounts in bacterial culture

DISADVANTAGES:

- intact bacteria don't resemble human cells
- extracted DNA may be different from human DNA
- sending pure DNA doesn't test labs' complete procedures
- some human mutations may contain "suicide sequences"

POTENTIAL EXPERIMENTAL APPROACHES

Transient Transfection

ADVANTAGES:

- quicker and easier than permanent transformation
- could include multiple gene inserts on same plasmid
- autonomously replicating plasmid can grow to high copy number
- if no integration, less chance of deleterious effect on host cell

DISADVANTAGES:

- cannot maintain transgene for long periods
- would need to prepare fresh for each shipment
- impossible to control dosage of mutation
- transgene present in addition to diploid genome (3 alleles)
- transgene flanked by non-native RE sites (atypical Southern blot results)
- transiently transfected DNA can mutate or rearrange

POTENTIAL EXPERIMENTAL APPROACHES

Stable Transformation

ADVANTAGES:

- permanent mutant cell line
- transgene becomes part of host cell genome

DISADVANTAGES:

- integration of multiple copies at various locations
- transgene flanked by non-native RE sites (atypical Southern blot results)
- "triploid" (or more) appearance
- no way to create homozygous mutant sample, since endogenous gene still present

POTENTIAL EXPERIMENTAL APPROACHES

Homologous Recombination

ADVANTAGES:

- permanently transformed cell line
- realistic dosing, replacing native gene with mutant
- transgene in proper genomic location
- can create homozygous sample by repeating procedure on opposite allele
- should resemble real human mutant specimens

DISADVANTAGES:

- complex, laborious, expensive, tricky
- leaves residual "footprint" (*neo* cassette or *loxP* sequence) gene still present

Gene Targeting

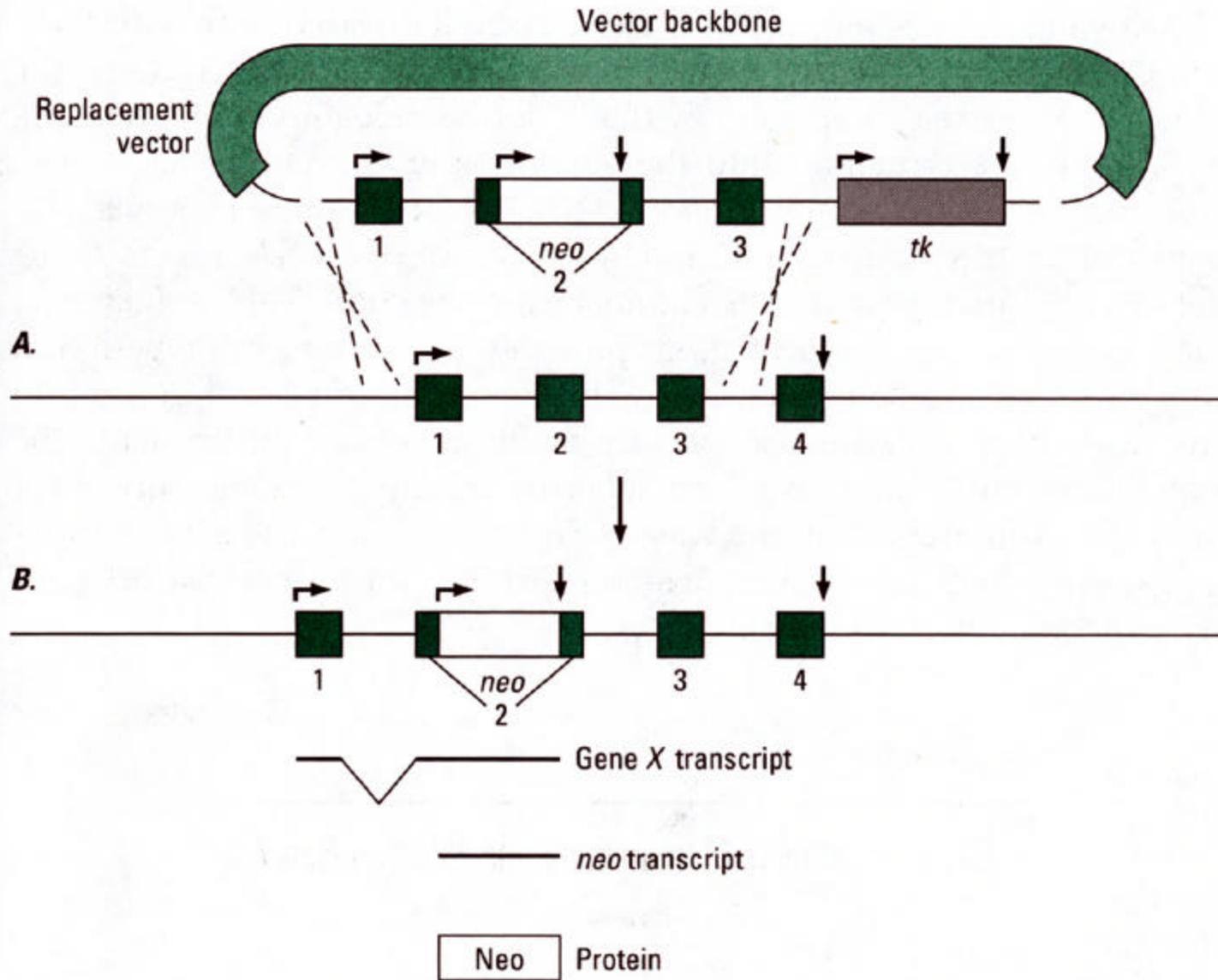


Table 1

CFTR Mutations: gene location and predicted BAC location.

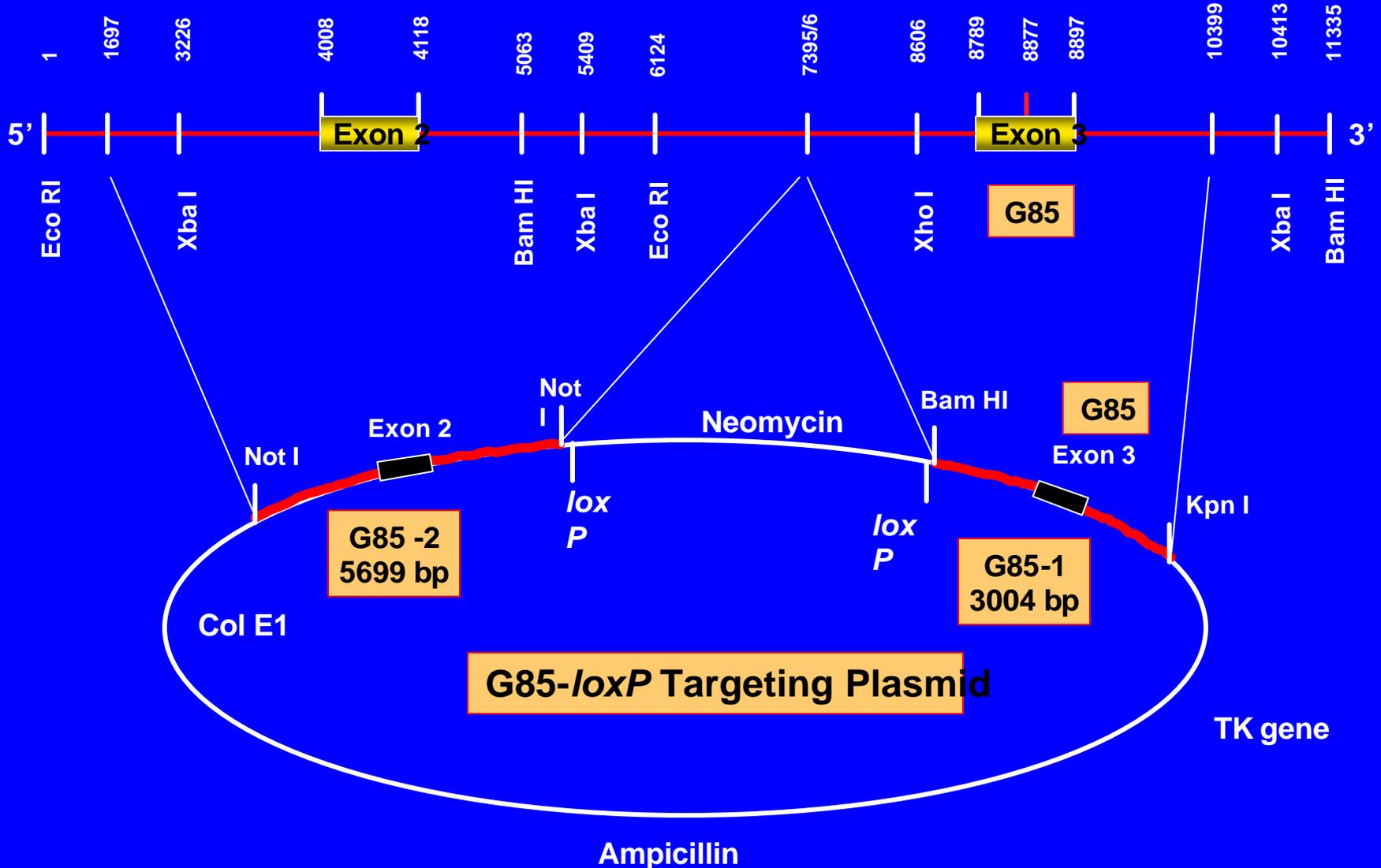
(From <http://www.genet.sickkids.on.ca/cftr-cgi-bin/FullTable>)

<u>Mutation</u>	<u>Gene Location</u>	<u>Predicted BAC</u>	<u>Consequence</u>	<u>Reference</u>
G85E	Exon 3	068P20	386G->A	Zielenski <i>et al</i> 1991
R117H	Exon 4	068P20	482G->A	Dean <i>et al</i> 1990
I148T	Exon 4	068P20	575T->C	Bozon <i>et al</i> 1994
R334W	Exon 7	068P20	1132C->T	Estivill <i>et al</i> 1991
R347P	Exon 7	068P20	1172G->C	Dean <i>et al</i> (NL#6)
A455E	Exon 9	068P20	1496C->A	Kerem <i>et al</i> 1990
ΔI507	Exon 10	068P20	del ILE	Kerem <i>et al</i> 1990
ΔF508	Exon 10	068P20	del PHE	Kerem <i>et al</i> 1989
G542X	Exon 11	068P20	1756G->T	Kerem <i>et al</i> 1990
G551D	Exon 11	068P20	1784G->A	Cutting <i>et al</i> 1990
R553X	Exon 11	068P20	1789C->T	Cutting <i>et al</i> 1990
R560T	Exon 11	068P20	1811G->C	Kerem <i>et al</i> 1990
R1162X	Exon 19	133K23	3616C->T	Gasparini <i>et al</i> 1991
W1282X	Exon 20	133K23	3978G->A	Vidaud <i>et al</i> 1990
N1303K	Exon 21	133K23	4041C->G	Osborne <i>et al</i> 1991
621+1G->T	Intron 4	068P20	mRNA splicing defect	Zielenski <i>et al</i> 1991
1717-1G->A	Intron 10	068P20	mRNA splicing defect	Kerem <i>et al</i> 1990
711+1G->T	Intron 5	068P20	mRNA splicing defect	Zielenski <i>et al</i> 1991
1898+1G->A	Intron 12	068P20	mRNA splicing defect	Strong <i>et al</i> 1992
2184delA	Exon 13	068P20	Frameshift	Dork <i>et al</i> 1994
1078delT	Exon 7	068P20	Frameshift	Claustres <i>et al</i> 1992
3849+10kbC->T	Intron 19	133K23	+splice acceptor site	Highsmith <i>et al</i> 1994
2789+5G->A	Intron 14b	068P20	mRNA splicing defect	Highsmith <i>et al</i> 1990
3659delC	Exon 19	133K23	Frameshift	Kerem <i>et al</i> 1990
3120+1G->A	Intron 16	068P20	mRNA splicing defect	Macek <i>et al</i> 1997

I506V, I507V, F508C, 5T/7T/9T

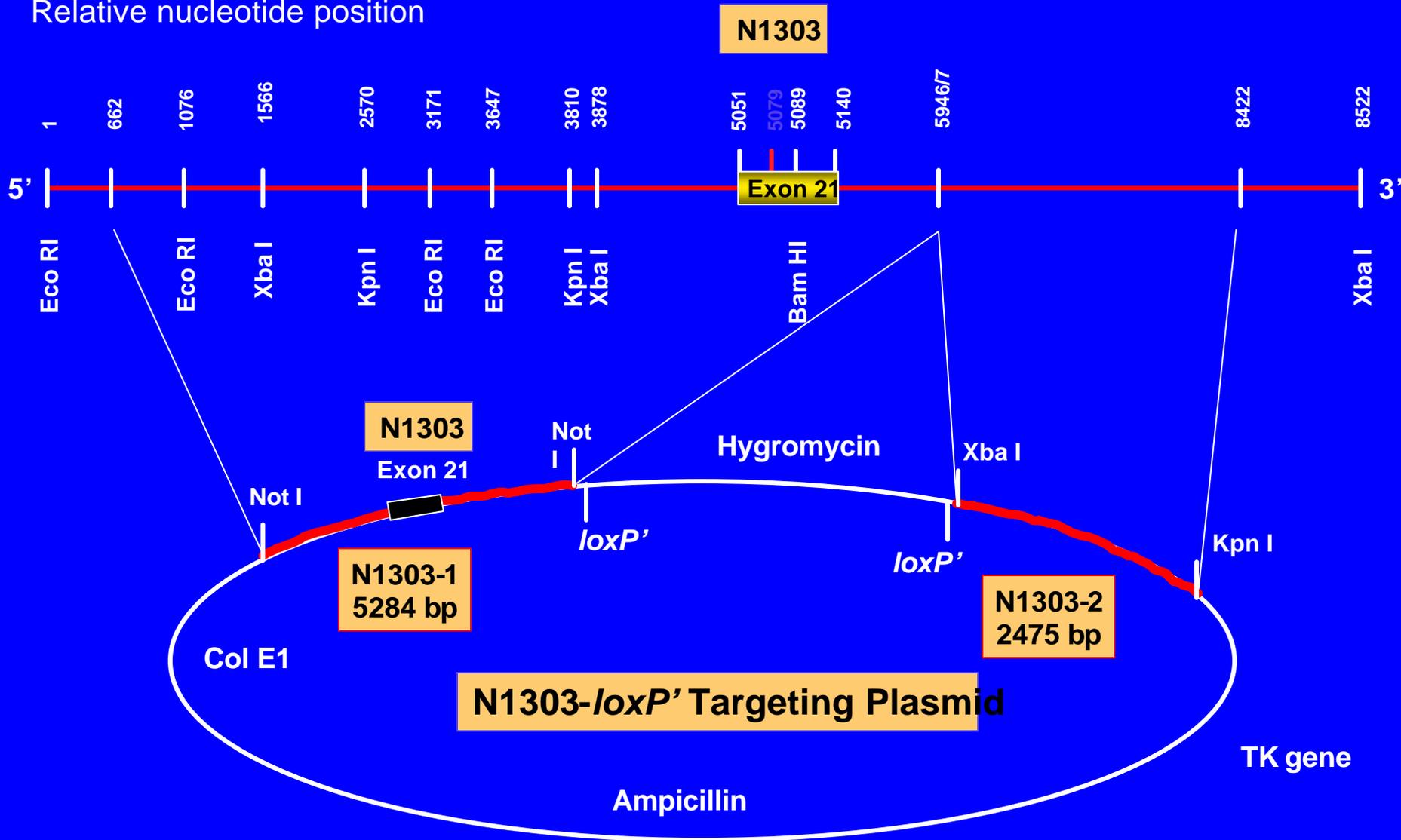
CFTR G85 Cloning Strategy

Relative nucleotide position

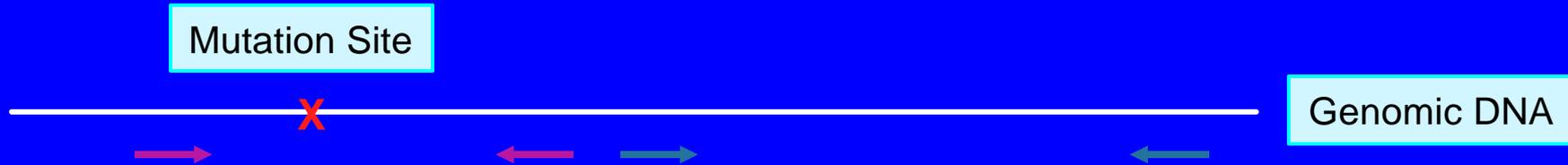


CFTR N1303 Cloning Strategy

Relative nucleotide position



Sample Construction



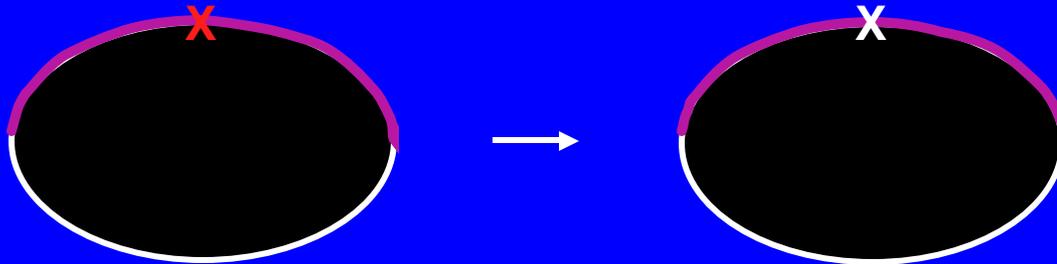
A: PCR Amplify Target Arms

Short Arm



Long Arm

B: Sub-Clone Short Arm & Mutagenesis



Sample Preparation

Salmon Sperm DNA

Human BAC DNA covering CFTR gene sequence

Plasmid constructs containing genomic DNA +/- mutation

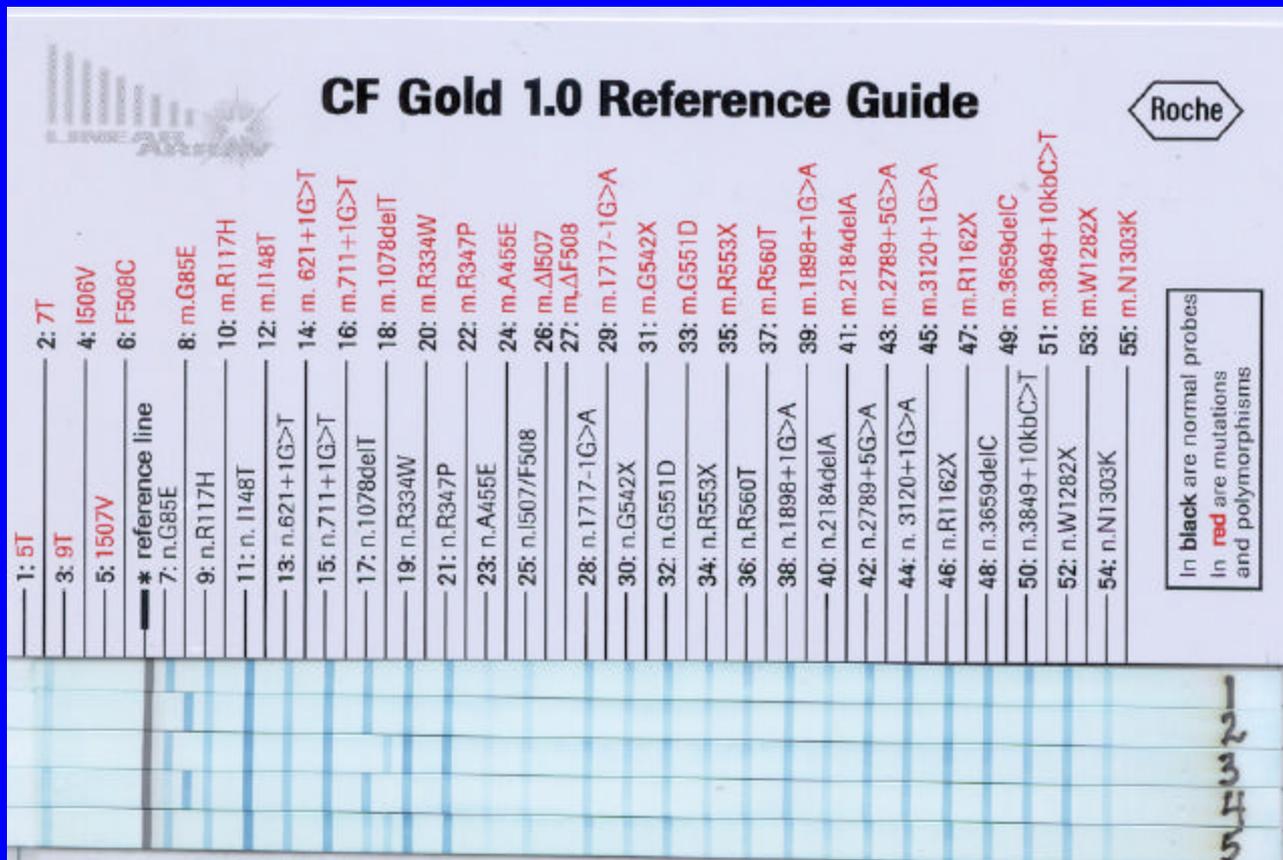
Optimized DNA concentrations:

SS DNA : BAC : Plasmid

Final concentration: 50 ug DNA/ 20 uL sample

ARTIFICIAL SAMPLES USED FOR PILOT TESTING

1. Wild Type
2. Heterozygous G85E
3. Homozygous G85E
4. Heterozygous 1078delT
5. Homozygous 1078delT



Roche strip analysis for five CFTR test samples:
(UCLA verification results)

- 1 WT/WT
- 2 G85E/G85E
- 3 1078delT/1078delT
- 4 WT/G85E
- 5 WT/1078delT

TECHNIQUES USED BY PILOT TESTING LABORATORIES

Reverse ASO Line Blots (strips) (4)

ABI Genotyper (1)

Microarray (2)

Proprietary Multiplex Assay (1)

Sequencing (1)

ARMS (1)

PILOT TESTING RESULTS

<u>Sample</u>	<u>Lab Analysis (Correct Results/Total Results)</u>	<u>Unable to Analyze</u>
Normal (w.t.)	8/9	1
G85E, heterozygous	9/10	
G85E, homozygous	9/10	
1078delT, heterozygous	9/10	
1078delT, homozygous	8/10	

PILOT PERFORMANCE BY METHOD

<u>Method</u>	<u>No.of Labs</u>	<u>Results</u>	<u>Comments</u>
Reverse ASO Line Blots	4	OK	Exon 7 partial PCR failure w/Innogenetics
ABI Genotyper	1	OK	Het. rates not exactly 50:50
Microarray	2	Mostly OK	Trace w.t. in homo. 1078delT
Proprietary Multiplex Assay	1	OK	
Sequencing	1	OK	M470V polymorphism also detected
ARMS	1	Equivocal	Several extraneous mutations detected

ONGOING AND FUTURE DIRECTIONS

- Additional CFTR mutations/combinations
- Homologous recombination
- BRCA1/2
- Trinucleotide repeat expansions
- Cancer markers
- Infectious diseases

