Reference reagents for genetic testing: development of plasmid based mutation detection reagents by the UK National Genetics Reference Laboratory (Wessex)

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Salisbury

UK

UK National Genetics Reference Laboratories

- Established in 2002 by the Department of Health
- Two laboratories based in Manchester and Salisbury (Wessex)

- Aim to Develop reference and control reagents for genetic testing
- Other functions of the laboratories include:
  - Horizon Scanning and Technology Assessment
  - Developing information systems for genetics
  - Evaluate technologies and systems that are close to service and assess their applicability to genetic testing within the National Health Service
  - Providing advice to government and other professional bodies

Requirements for Control Reagents for Genetic Testing

- NHS genetic diagnostic laboratories in the UK (and labs worldwide) perform thousands of mutation detection assays every month using diverse technologies

- Laboratories generally use locally sourced controls as standards to confirm that an assay is working correctly

- Widespread variation in the number and type of controls used in different laboratories

- Can potentially compromise quality assurance

Outline of talk

- Mutation Scanning and Genetic Testing requirements in the UK
- Development of plasmid reagents for mutation scanning of:

  BRCA1, BRCA2, hMLH1 and MSH2
- Development of generic mutation scanning controls
- Development of quantitative material for Standardisation of BCR/ABL RQ-PCR

Mutation Scanning

- Detecting ‘unknown’ sequence variation at any position within an amplicon:

e.g. single base substitutions (point mutations)

deletions

insertions

- Over half (51 – 75%) of all genetic test performed in the UK involve mutation scanning for ‘private’ mutations e.g. hereditary breast cancer (9%) and colorectal cancer (4%), Marfans etc.

- In the UK the results of mutation scanning of large genes are now required to be reported within 6-8 weeks of sample receipt

- Many labs now use ‘pre-screening’ techniques rather than direct sequencing to reduce costs of genetic tests and improve reporting times

Development of plasmid reagents for mutation scanning of BRCA1, BRCA2, hMLH1 and MSH2

Advantages

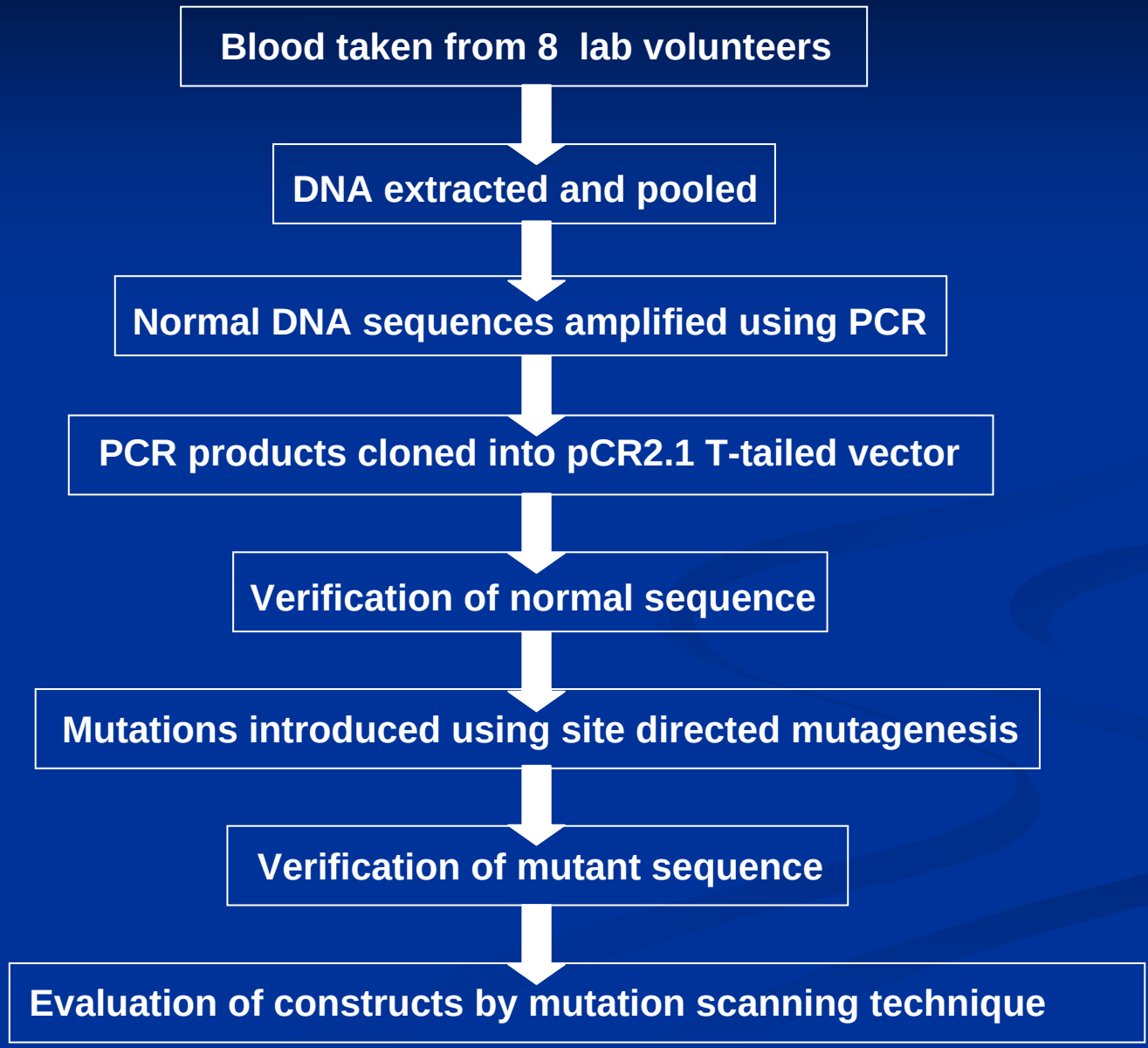
- Non cell line based and therefore patient consent not required
- Cost effective and renewable resource
- Can accommodate rapidly changing technology platforms
- Easy to prepare in large batches
- Many mutations can be combined into single control material
    - Useful for multiplex assays and large gene screens
    - Can customise mutations

Disadvantages

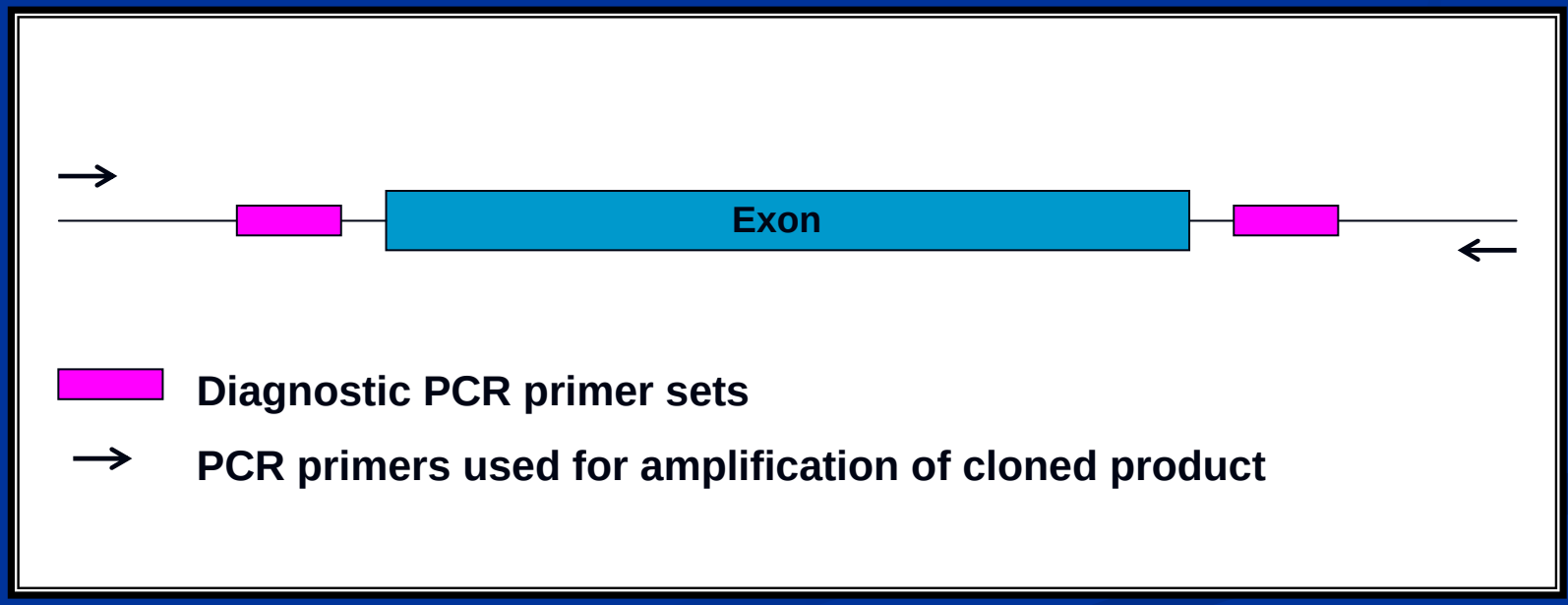
- May not resemble patient specimens
- Cannot be used to monitor certain steps of test procedure

  e.g. PCR optimisation
- No established certification route for production as certified reference material

Production of Plasmid Constructs for mutation detection in cancer predisposition genes

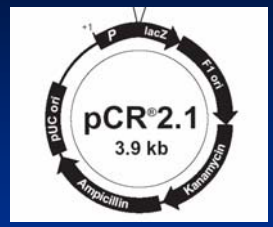


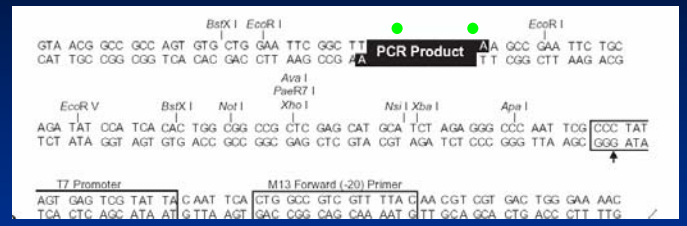
Cloned fragment should be large enough to contain PCR primer sites used by all diagnostic laboratories



Site Directed Mutagenesis

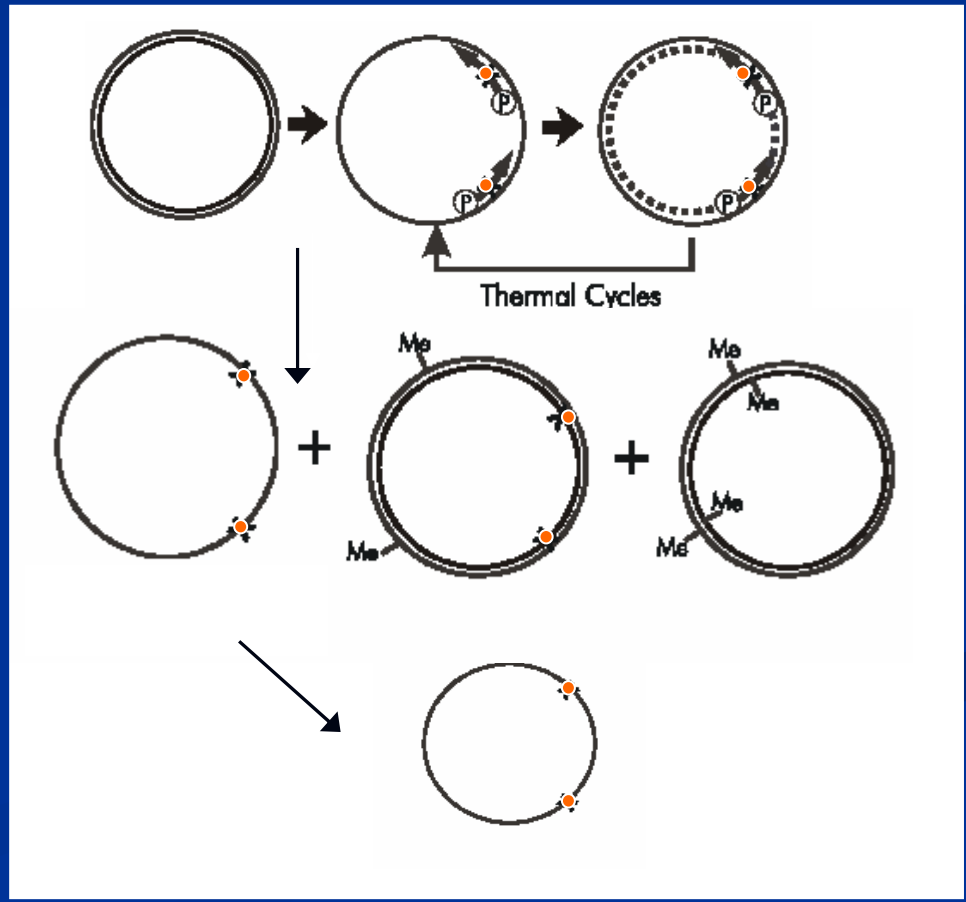
1. Clone wt PCR product & sequence





2. Design mutagenic oligos

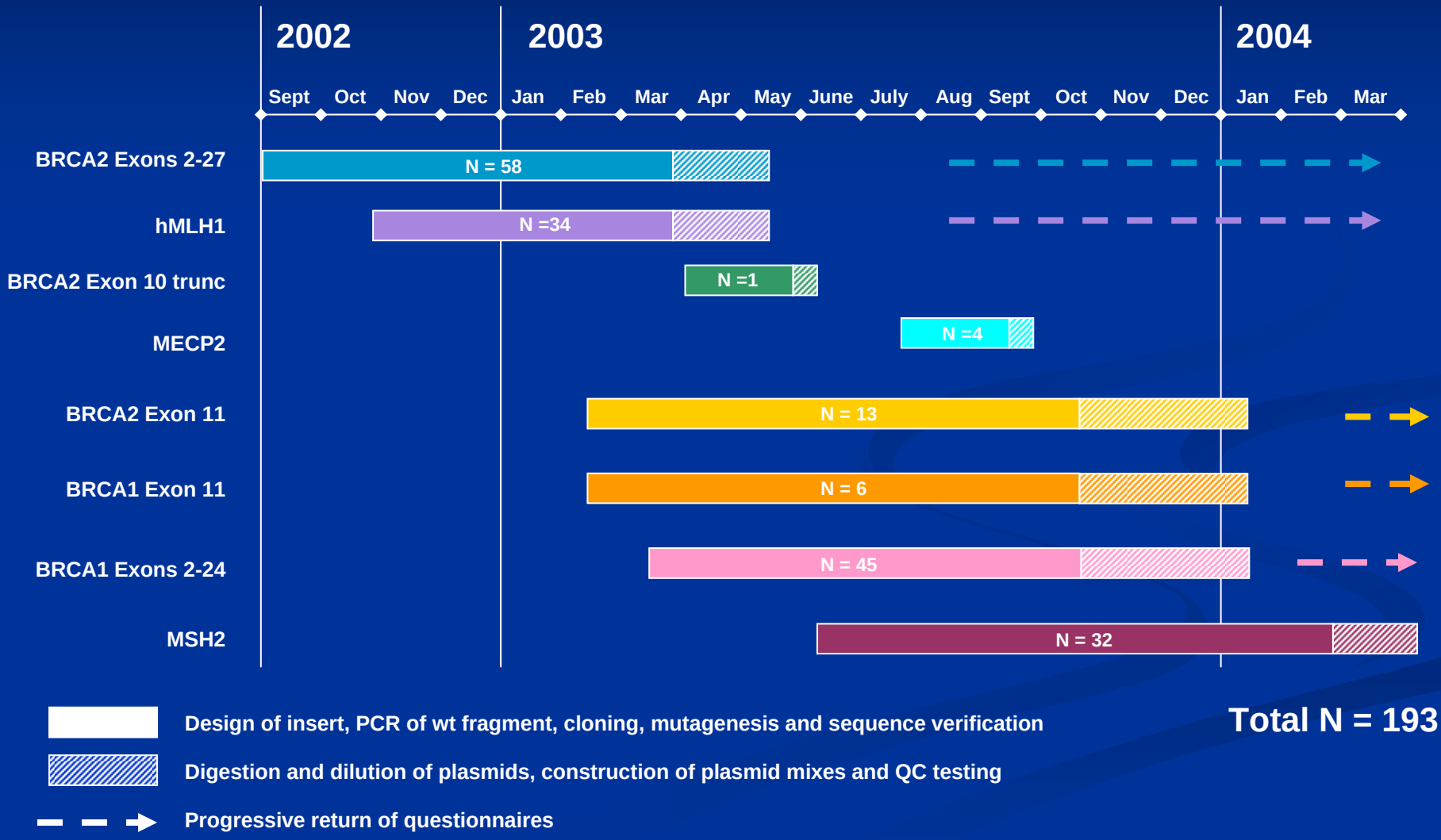
3. Synthesize mutagenic strand



4. Digest Template DNA using Dpn I

5. Transform E.Coli

Production and Evaluation of Reference Reagents for hereditary breast cancer and HNPCC Mutation Detection 2002 – 2004…



hMLH1

Exons 1-19 :

34 plasmids

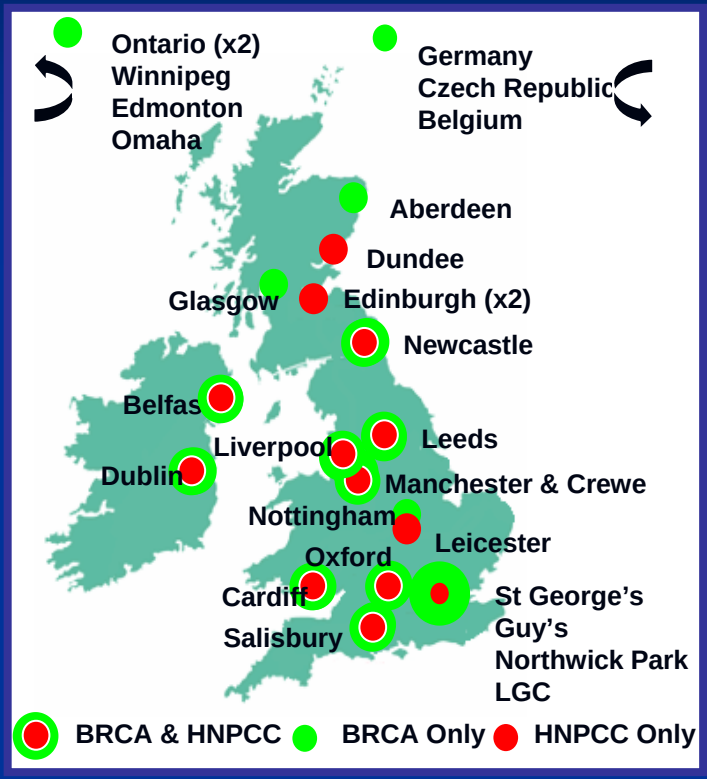
17 wild type (all exons)

17 mutant (19 mutations)

Exon	Nucleotide Change	Amino acid Change
1	62 C>G	A21A
2	207+1 G>C	Splice
3	280 A>T	I94F
4	367 A>T	K123X
5	418 A>T	K140X
6	497 T>A	L166X
7	588+1 G>A	Splice
8	645 T>A	N215K
9	725 T>A	M242K
10	868 C>A	P290T
11	911 A>T	D304V
12	1376 C>G	S459X
13	1486 C>A	P496T
14	1661 A>C	K554T
15	1717 G>T	V573F
16	1846 A>T	K616X
17	1897-2 A>G	Splice
18	2008 A>T	K670X
19	2176 T>A	S726T

Performance Indicator Field Trial of Plasmid Based Reagents for Mutation Scanning of BRCA1, BRCA2, hMLH1 and hMSH2

(May 2003 – October 2005)



- Distributed to 34 individuals in 26 labs
- Six mutation scanning techniques
  - dHPLC
  - Sequencing
  - SSCP/HD
  - CSCE
  - PTT
  - MALDI-TOF

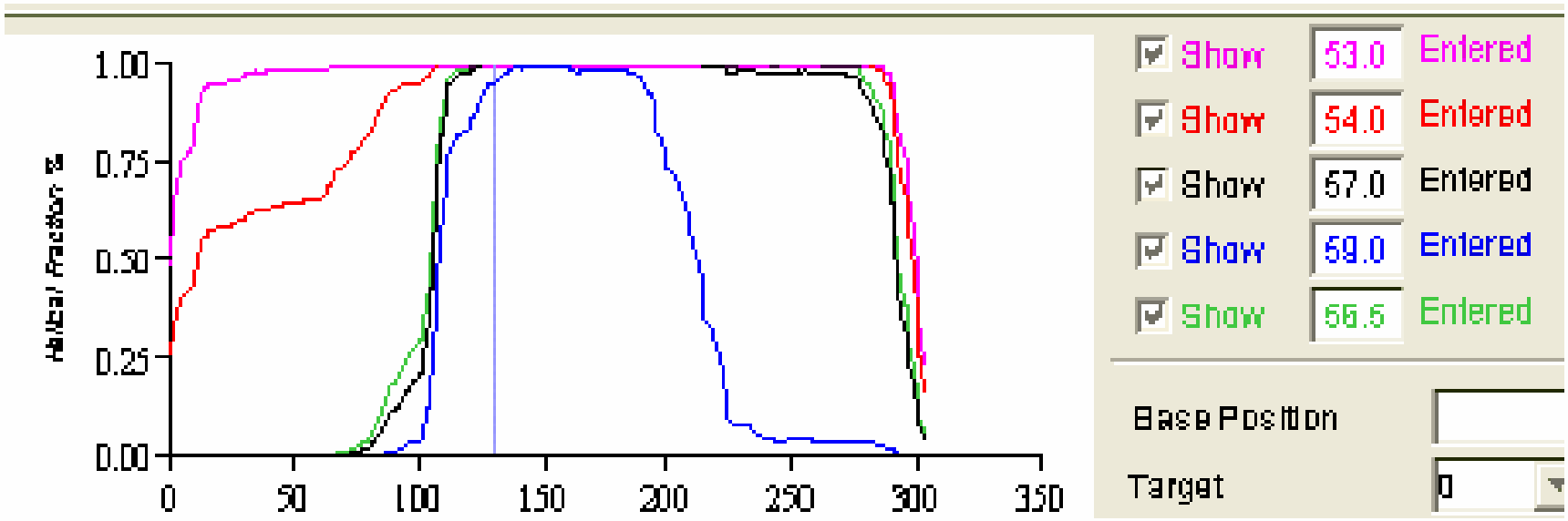
Feedback from Field Trial

- Data returned from 20 field trial participants
  - 80% used the controls in routine testing
  - 65% used the reagents to develop new assays or validate existing screens
  - 30% altered diagnostic protocols as a results of using the controls
  - 95% found plasmid DNA to be an acceptable alternative to genomic DNA
  - 100% thought that the reagents were a useful resource
  - 85% agreed that the reagents should be produced as reference material

- BRCA Plasmids have undergone modification
  - compatible with standardised primers (NGRL designed and tested)
  - acceptable format for certification (pUC18)
  - additional ‘polymorphism controls’ also produced

WAVE Melt profiles (WAVE MD Software)

BRCA2 Exon 21: 8909A>G

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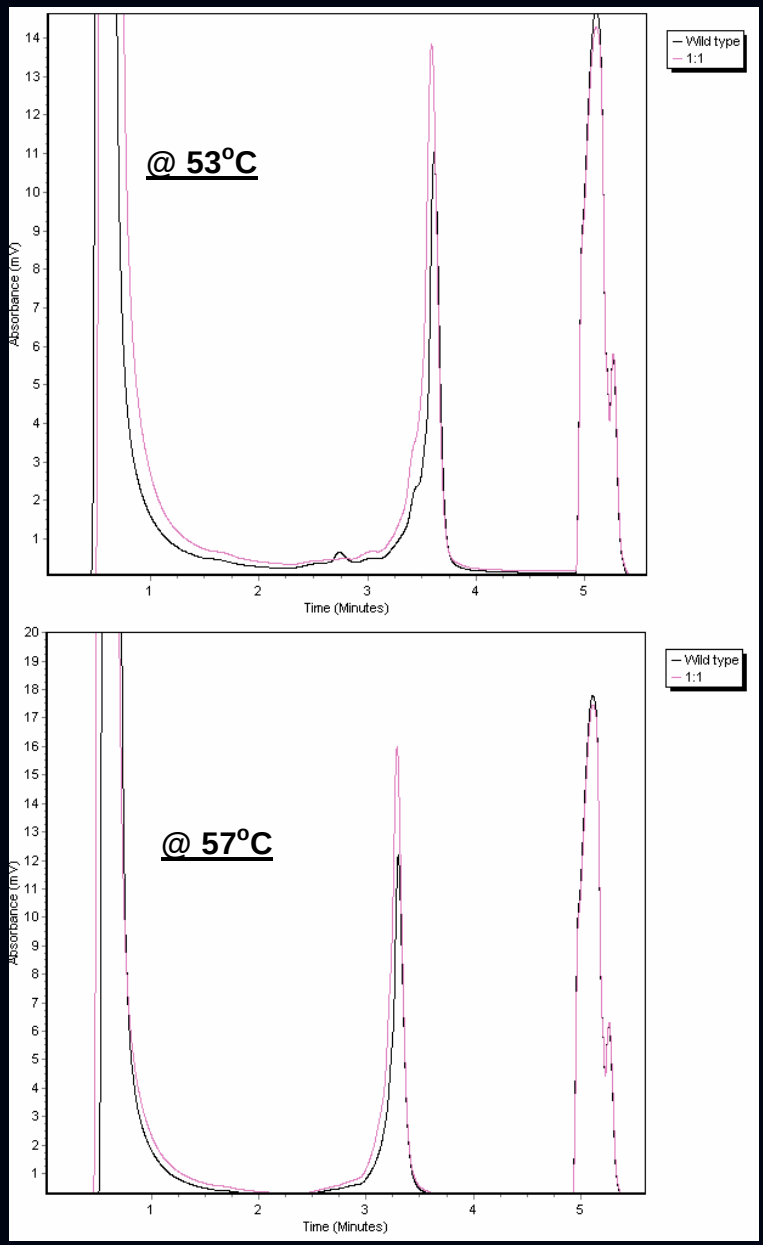
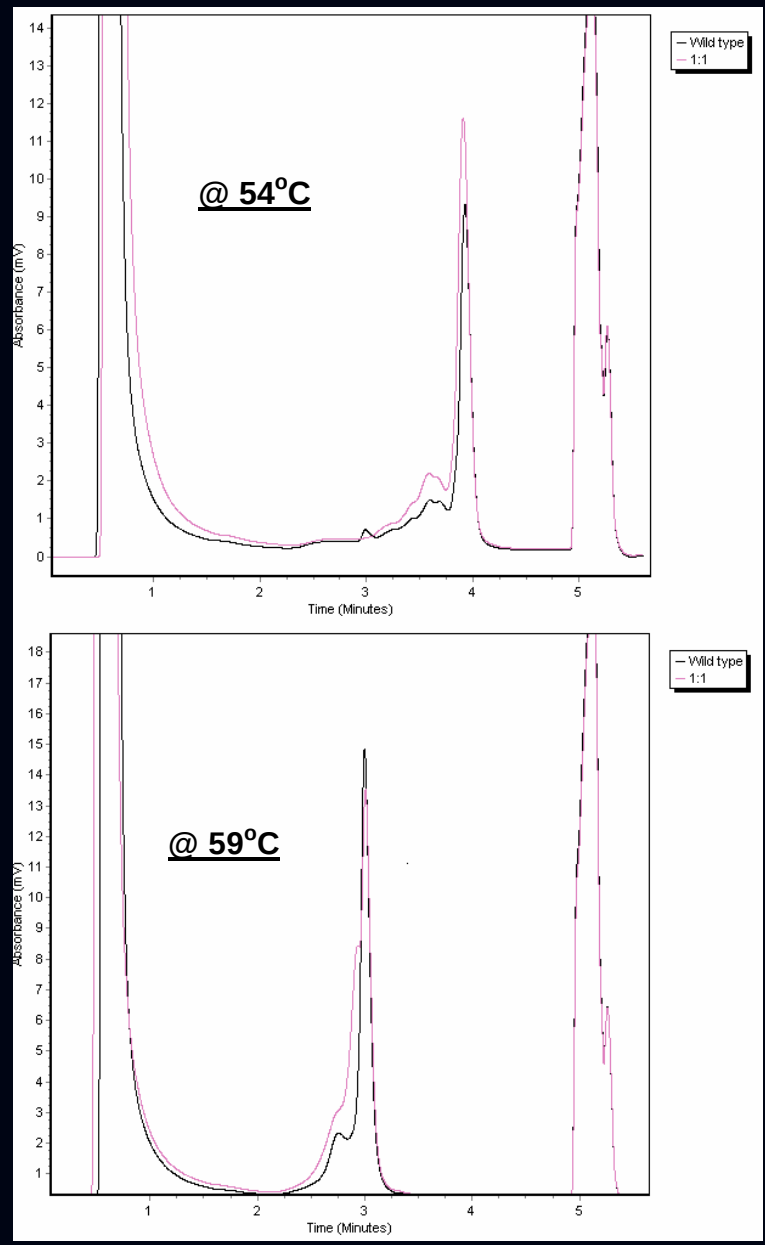
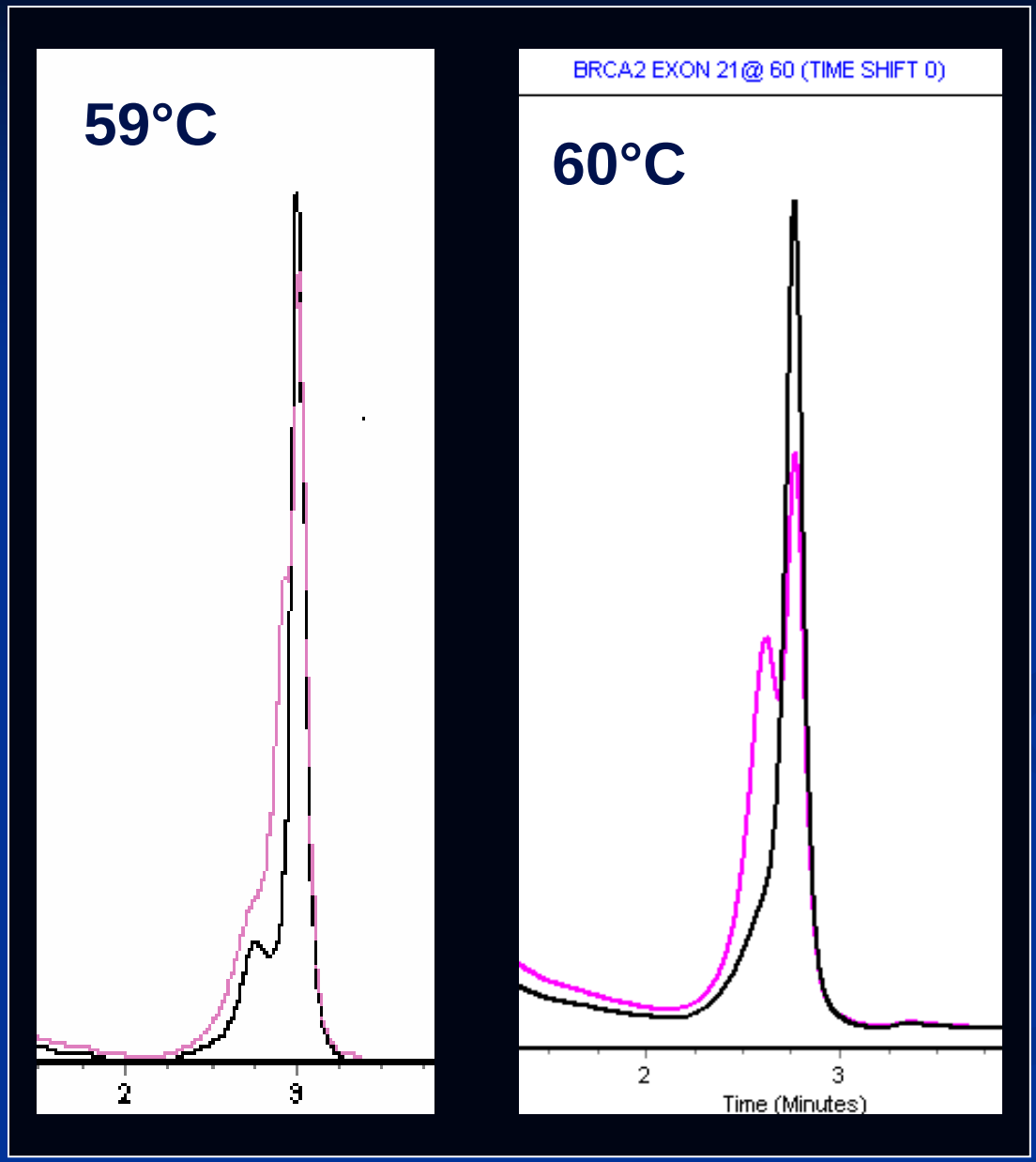
000001-000060 gggtgttttatgcttggttctttagttttagttgcttttgaatttacagtttagtgaatt
000061-000120 aataatccctctgttttctcagaaaacacaacaaaaccacacttaccatccacgtgcacta
000121-000180 acaagacagctcagttcgtgctttgcaagatggtgcagagctttatgaagccagtgaagaat
000181-000240 gcagcagacccagcttaccttgaggtgagagagtaagaggacatataatgaggcttgatg
000241-000300 attattcaaggtgagaagctgttttagactctctggccatcacaggaaggagtatgttga
000301-000304 aatg
```

BRCA2 Exon 21 A8909G







BRCA2 Exon 21 A8909G

Detected at 60°C

Consider altering current diagnostic protocols

Development of generic mutation scanning controls

A Different Approach

- Not feasible to produce controls for every exon of every gene

- To facilitate the evaluation of high throughput mutation detection strategies NGRL (Wessex) has developed a **generic set** of reference reagents which can be used to assess both new and existing mutation detection techniques.

- Plasmid controls have been produced which can be used to determine the sensitivity and specificity of these techniques by analysing factors that are of general importance for all technologies including:

  - the type of base substitution
  - the GC content of the amplicon
  - the location of the mutation in the fragment

- The controls can be used to amplify 52 fragments:

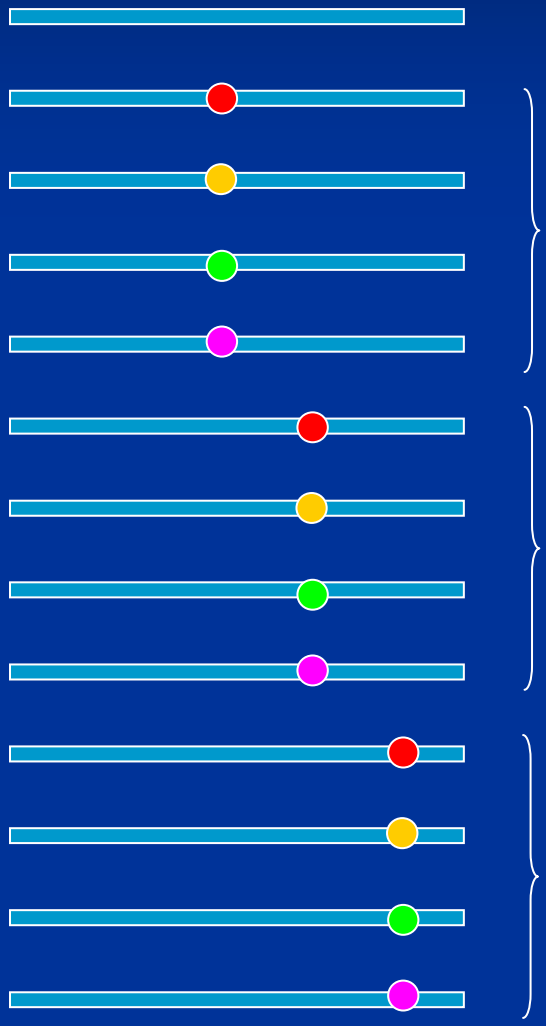
  - ranging from 400-450bp
  - average GC sequence content of 20%, 40%, 60% and 80%.
  - every possible heteroduplex at three positions within the amplicon

Generic Mutation Detection Reference Reagents

Mutation created	Sequence generated	Heteroduplex produced
A > C	nnnCGnnn	C:T & G:A
A > T	nnnTGnnn	T:T & A:A
G > A	nnnAAnnn	A:C & G:T
G > C	nnnACnnn	C:C & G:G

Panel of 52 amplicons for mutation scanning

Each % GC rich template (n=13)



Wild type

Position 3

Position 2

Position 1

A > C	C:T	G:A
A > T	T:T	A:A
G > A	A:C	G:T
G > C	C:C	G:G

Performance Indicator Field Trial of Generic Reference Reagents (May – November 2005)

Distributed to 16 labs and tested with 5 mutation detection techniques:

- Sequencing
- dHPLC
- TGCE
- CSCE
- MALDI-TOF

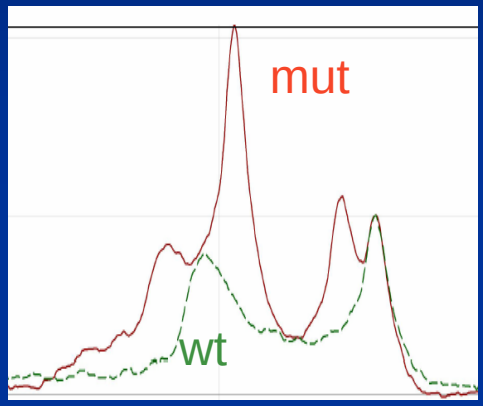
Results from Field Trial

- Reagents performed well in most laboratories
- Comments from participants have confirmed that the reagents are a useful resource
- Sensitivity and specificity of mutation detection showed marked variation ranging from 75% - 100% and 68 – 100% respectively

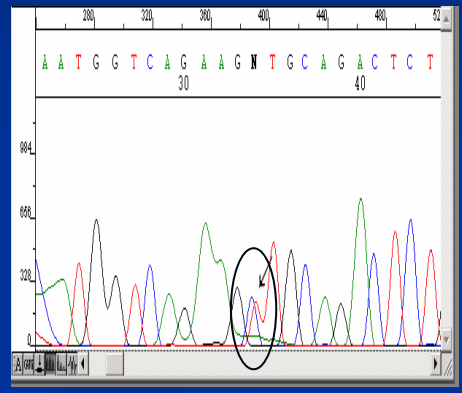
Lab ID	Method	Sensitivity (%)	Specificity (%)	Failure rate (%)
1	dHPLC	100	100	0
3	dHPLC	98	93	0
4	dHPLC	100	100	1
7	dHPLC	89	97	3
8	dHPLC	74	99	7
4	CSCE	98	100	0
5	CSCE	86	79	2
9	CSCE	93	92	1
10	CSCE	97	95	3
6	Seq	99	99	9
8	Seq	98	99	1
9	TGCE	94	68	5
2	MALDI	80	94	11

60.1 G>A

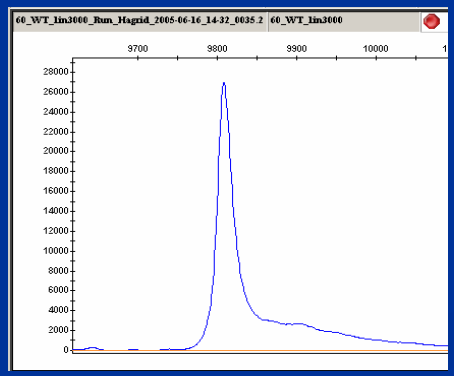
dHPLC

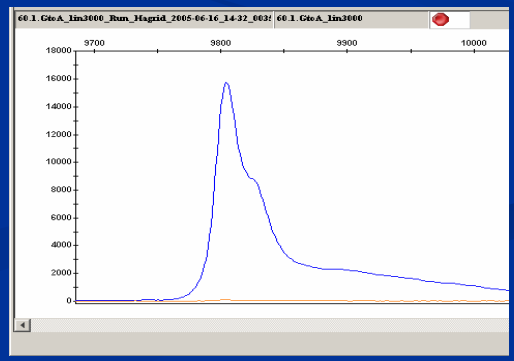


Sequencing



CSCE





Laboratory Quality Control

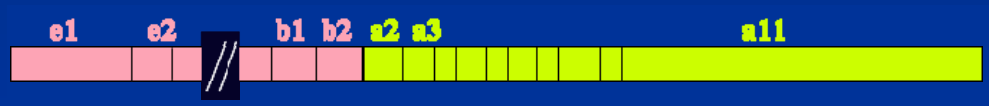
TECHNIQUE-SPECIFIC EQA SCHEMES

MSCAN Mutation scanning			
Scheme code	**Application deadline**	**Sample dispatch date**	**Survey period**
MSCAN-07	20.07.07	01.10.07	02.10.07 – 14.01.08
Target			
• Mutation scanning techniques			
Sample material			
• Lyophilised DNA			
Scheme format			
• Pilot –restriction on number of participants (limited to 30) • Open to laboratories from ALL countries • Three to five samples for mutation scanning • Assessment of ability to detect mutations			
Additional Information			
• Suitable for all DNA based mutation scanning techniques			
EQA scheme fee[22]			
Fee period 1: **Free** Fee period 2: 250 €			

EMQN
The European Molecular Genetics Quality Network

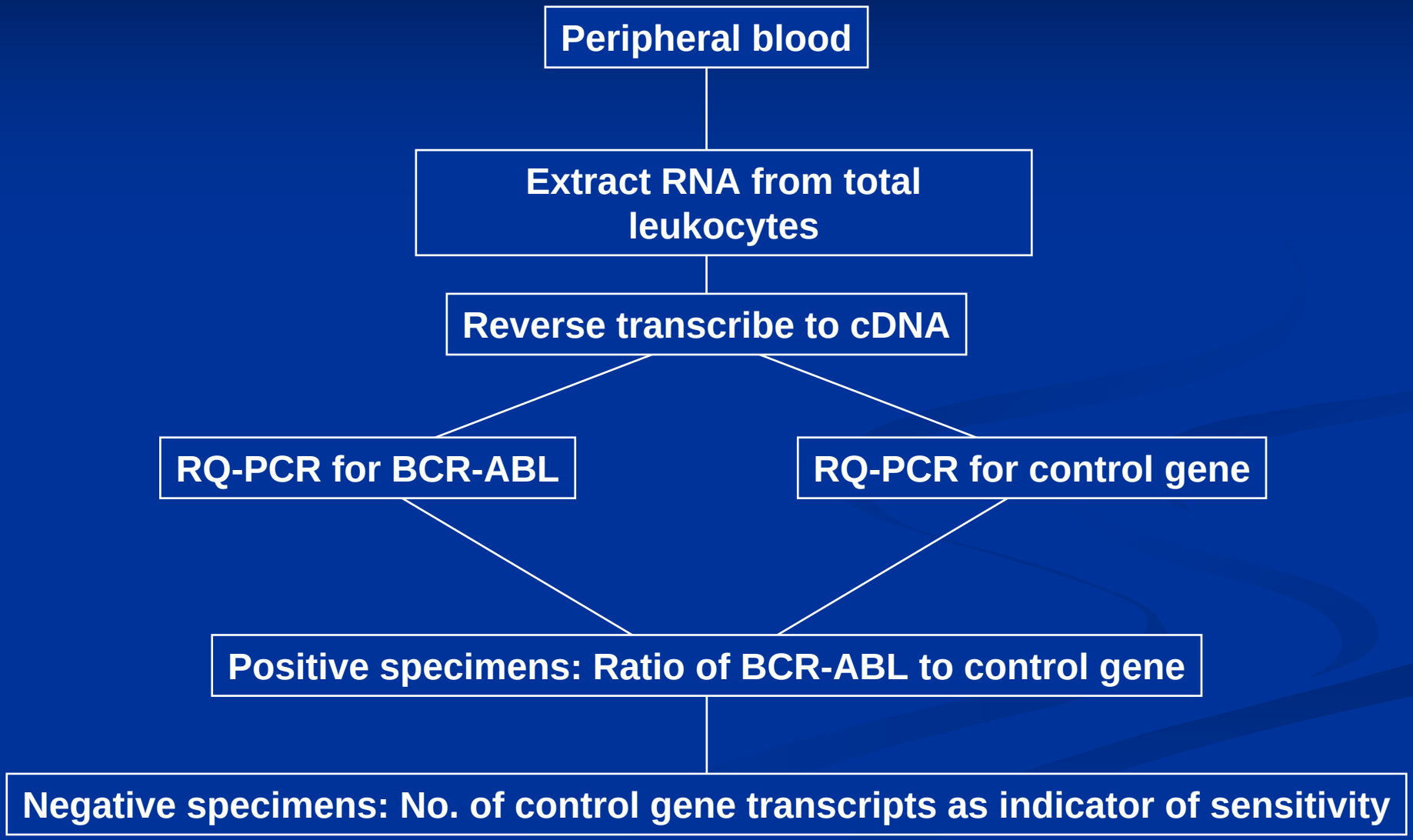
Project for the Standardisation of *BCR-ABL* RQ-PCR Methods

BCR-ABL transcripts

		CML	Ph+ALL
p210 *BCR-ABL*			
b2a2 (e13a2)		98%	35%
b3a2 (e14a2)			
p190 *BCR-ABL*			
e1a2		1%	65%

- Diagnosis
- Assessment of the response to treatment
- Early detection of relapse
- Surrogate endpoint in the evaluation of new treatments

Real time PCR for BCR-ABL



Measurement of BCR-ABL

- BCR-ABL/ABL
- BCR-ABL/BCR
- BCR-ABL/GUS

- Different primers/probes

- TaqMan
- LightCycler
- Corbett
- Others

- TMA (Japan)

Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells

BRIAN J. DRUKER[1], SHU TAMURA[1], ELISABETH BUCHDUNGER[2], SAYURI OHNO[1], GERALD M. SEGAL[1], SHANE FANNING[1], JÜRG ZIMMERMANN[2] & NICHOLAS B. LYDON[2]

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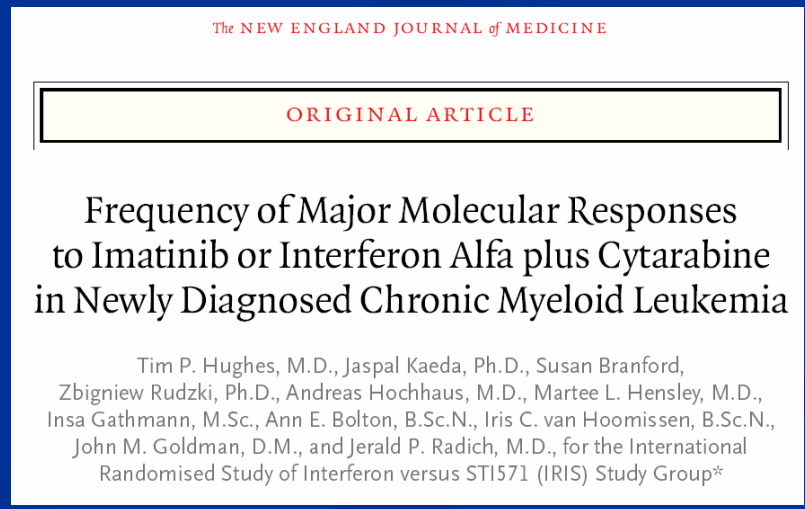
[2]Ciba Pharmaceuticals Division, Oncology Research Department, Ciba-Geigy Limited, CH-4002, Basel, Switzerland
Correspondence should be addressed to B.J.D.

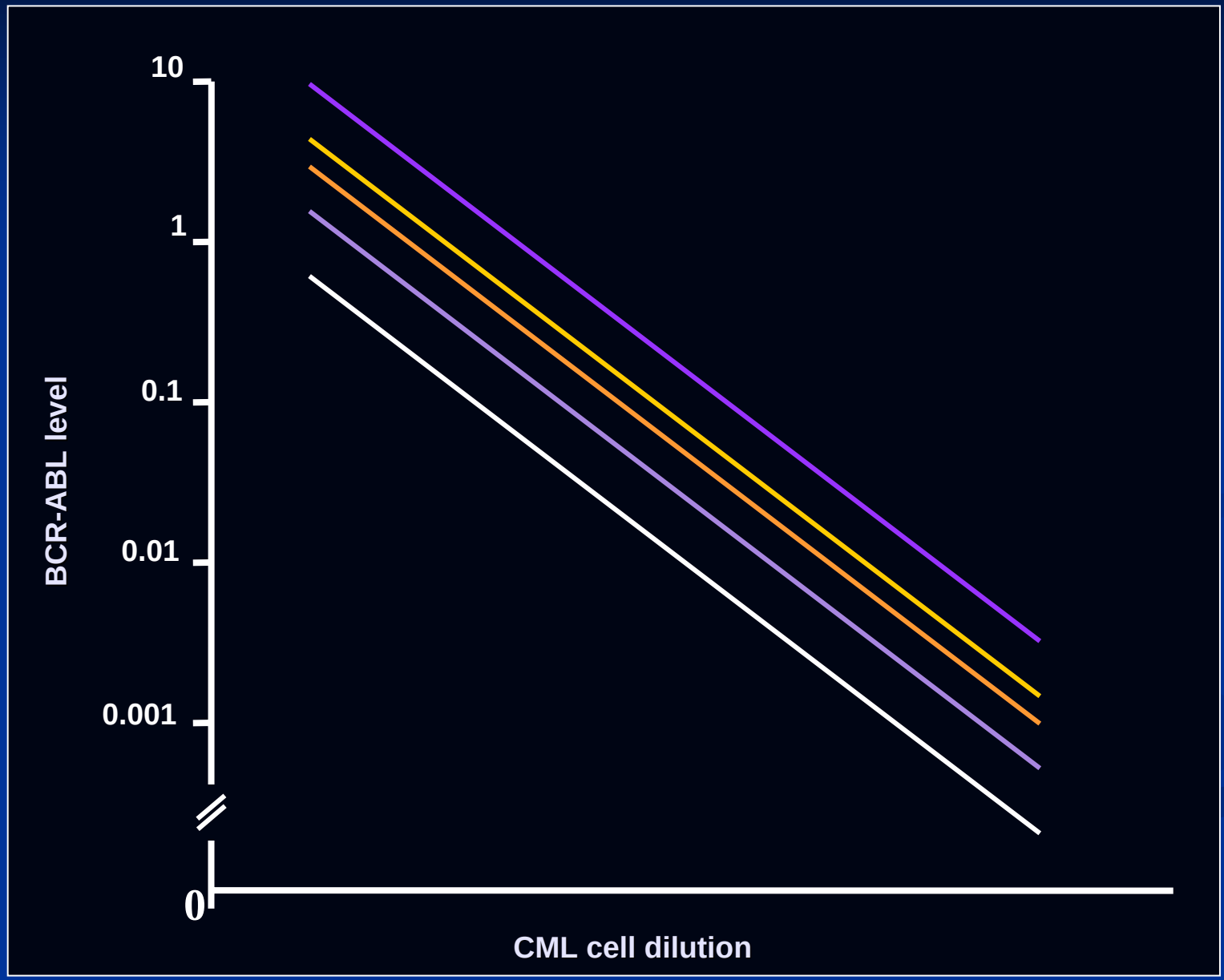

The NEW ENGLAND JOURNAL of MEDICINE

ORIGINAL ARTICLE

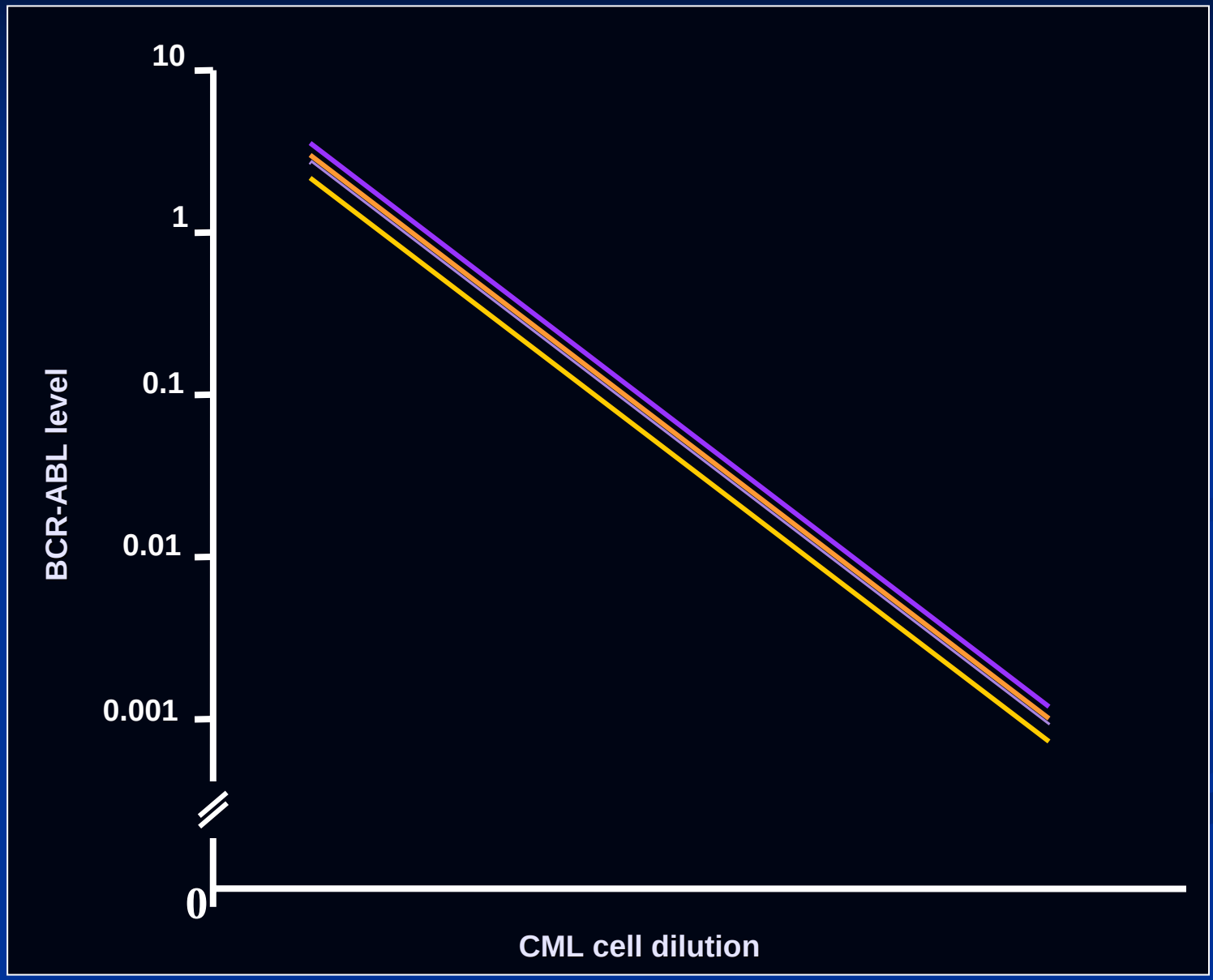
Frequency of Major Molecular Responses to Imatinib or Interferon Alfa plus Cytarabine in Newly Diagnosed Chronic Myeloid Leukemia

Tim P. Hughes, M.D., Jaspal Kaeda, Ph.D., Susan Branford, Zbigniew Rudzki, Ph.D., Andreas Hochhaus, M.D., Martee L. Hensley, M.D., Insa Gathmann, M.Sc., Ann E. Bolton, B.Sc.N., Iris C. van Hoomissen, B.Sc.N., John M. Goldman, D.M., and Jerald P. Radich, M.D., for the International Randomised Study of Interferon versus STI571 (IRIS) Study Group*

International standardisation of BCR-ABL measurements



International standardisation of BCR-ABL measurements



Formulation for primary and / or secondary reagents

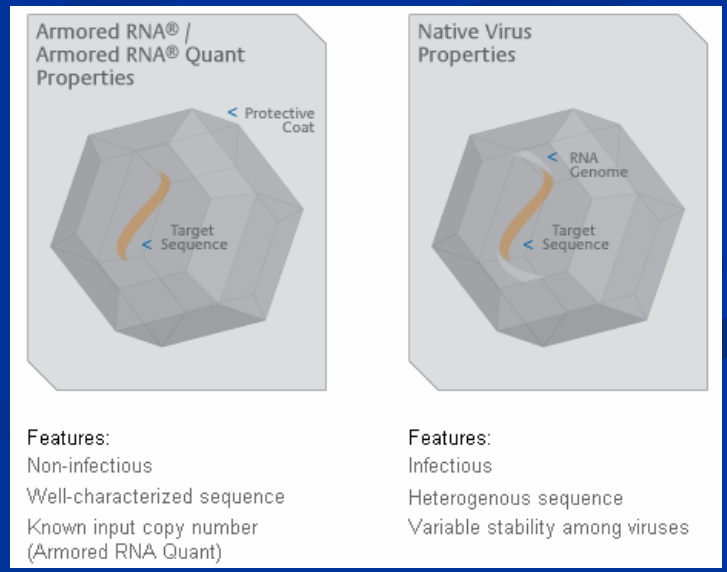
- CML cells (primary or K562) diluted in normal leucocytes
- Cell line mixtures
- Armored RNAs

Evaluation of cell lines

- K-562 is fine for BCR-ABL
- KG-1, HL-60 possible for non-BCR-ABL (at least the isolates we have tested).
- Tested pilot batch of freeze dried samples:
  - 4 dilutions spanning 10%-0.01%; $3x10^6$ cells/vial; 60 vials/dilution
  - K-562 diluted in two BCR-ABL negative lines (HL-60 and KG-1)
  - Grow ups and dilutions at NGRL (Wessex)
  - Freeze dried at National Institute of Biological Standards and Control
  - Initial testing performed by NGRL (Wessex) – BCR/ABL levels same before and after freeze drying
  - Preliminary testing and trial of resuspension protocols carried out by Mannheim and Marseille
  - Larger international trial completed Sept 2007 (14 labs worldwide)

Armored RNAs

- Pros
  - Easily available in large quantities
  - Stable
  - Good track record for calibration of RNA virus detection assays
  - Easy to adjust BCR:ABL:GUS ratio
  - Flexible: can use directly for reverse transcription or put through RNA extraction
- Cons
  - (? Unproven for BCR-ABL)



Armoured RNAs: current status

- Survey of primer sets performed July 2006
- Plasmids made (BCR, ABL, GUS, b2a2, b3a2) that cover the regions targeted by all members of the international group
- Potential for single plasmid containing b3a2/ABL/BCR/GUS to be used as quantitative standard
- Sequence verified; sent (essentially gifted) to Asuragen Nov 2006
- Armored RNAs available for initial testing May 2007
- Evaluation round
  - To be prepared in Salisbury; protocol to be agreed with Asuragen October 2007
  - BCR-ABL + BCR+ ABL+ GUS armored RNA mixtures alone (in Trizol or GTC)
- Potential for selection for development as primary reagents? How?
- Marketed as secondary reagents by Asuragen?

Current position

- NGRL (Wessex) funding was renewed April 2007 – end March 2012
- Workpackage covers production of controls material
- Lower level of funding and reduced staffing
- No infrastructure or staffing for certification of plasmid reagents
- Seeking commercial partners for production and certification of plasmid reagents

Future plans

- Completion of BCR/ABL programme
- Oversight committee
- Production of further plasmids for large gene screens
- Acquired abnormalities e.g. V617F *JAK2*

Acknowledgements and Further Information

Mrs Gemma Watkins

Mrs Vicky Hall

Mr Chris Mattocks

Prof Nick Cross

www.ngrl.org.uk/Wessex

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