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Multiplex SuperSelective PCR Assays for the Detection and Quantitation of Rare Somatic Mutations Associated with Cancer Diagnosis, Prognosis, and Therapy

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Diana Vargas-Gold
Sanjay Tyagi
Salvatore A.E. Marras

Public Health Research Institute
New Jersey Medical School
Rutgers Health

Rutgers docket number/s: **S12-040, S2016-121, 2019-188, and 2024-096**

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Rutgers Inventors

- Fred Russell Kramer
  - Diana Vargas-Gold
- Sanjay Tyagi
  - Salvatore A.E. Marras

https://techfinder.rutgers.edu/tech/SuperSelective_PCR_Primers

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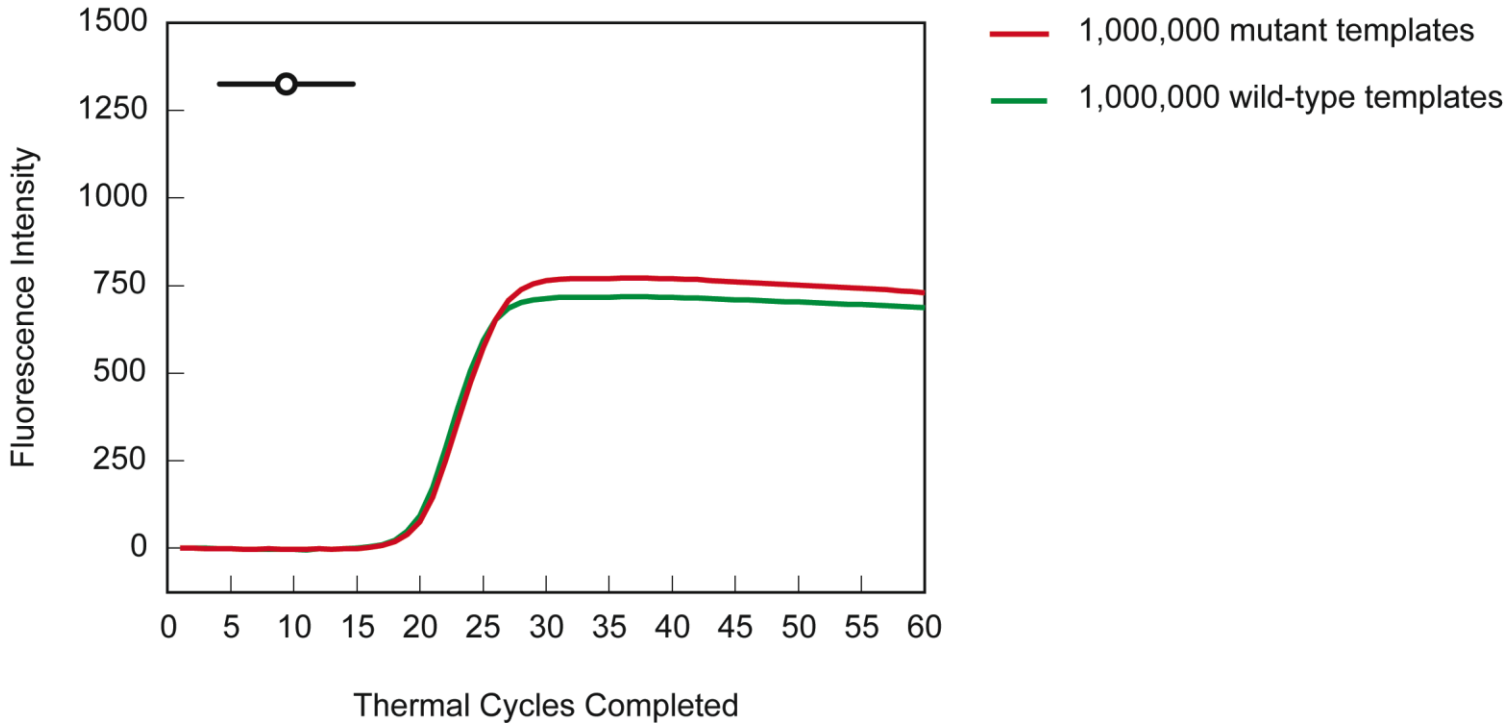
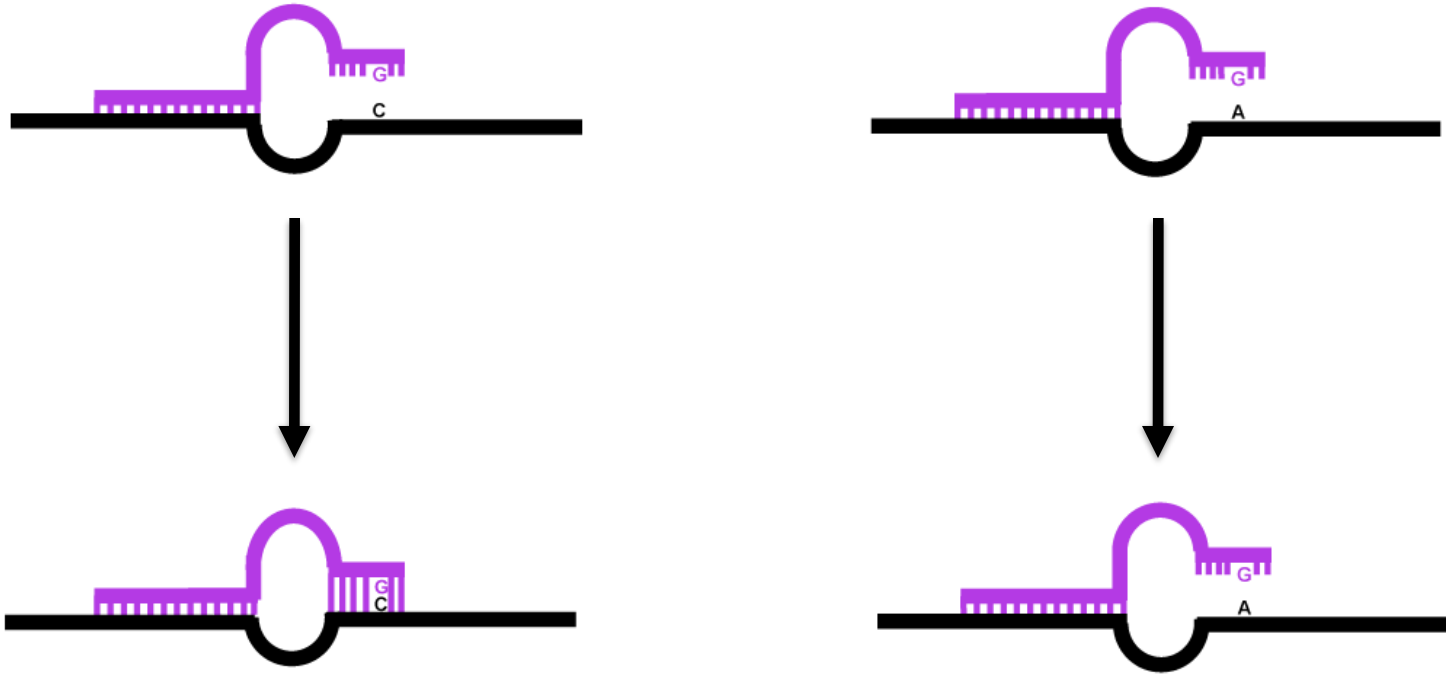
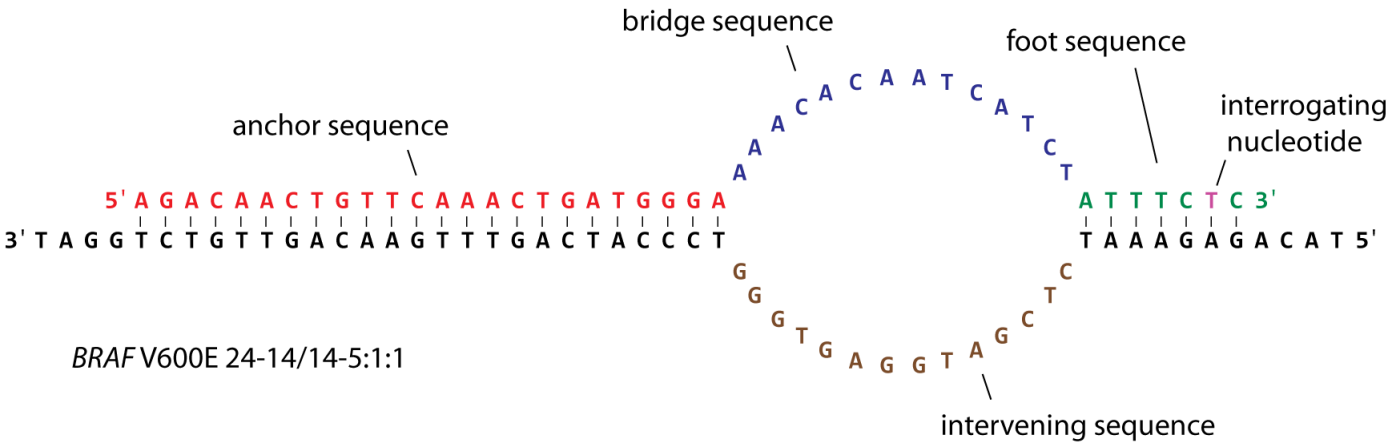
Conventional Linear Primers



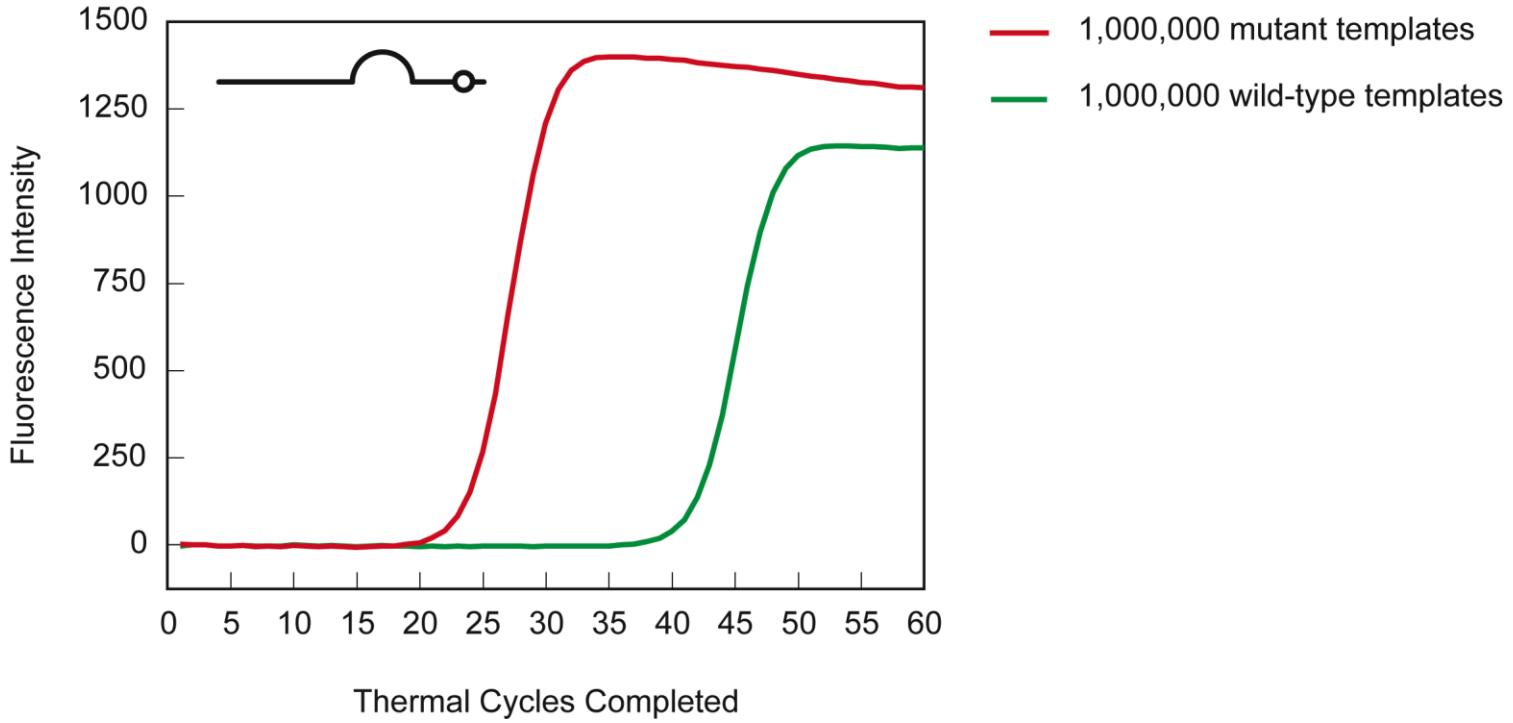
Operation of SuperSelective Primers



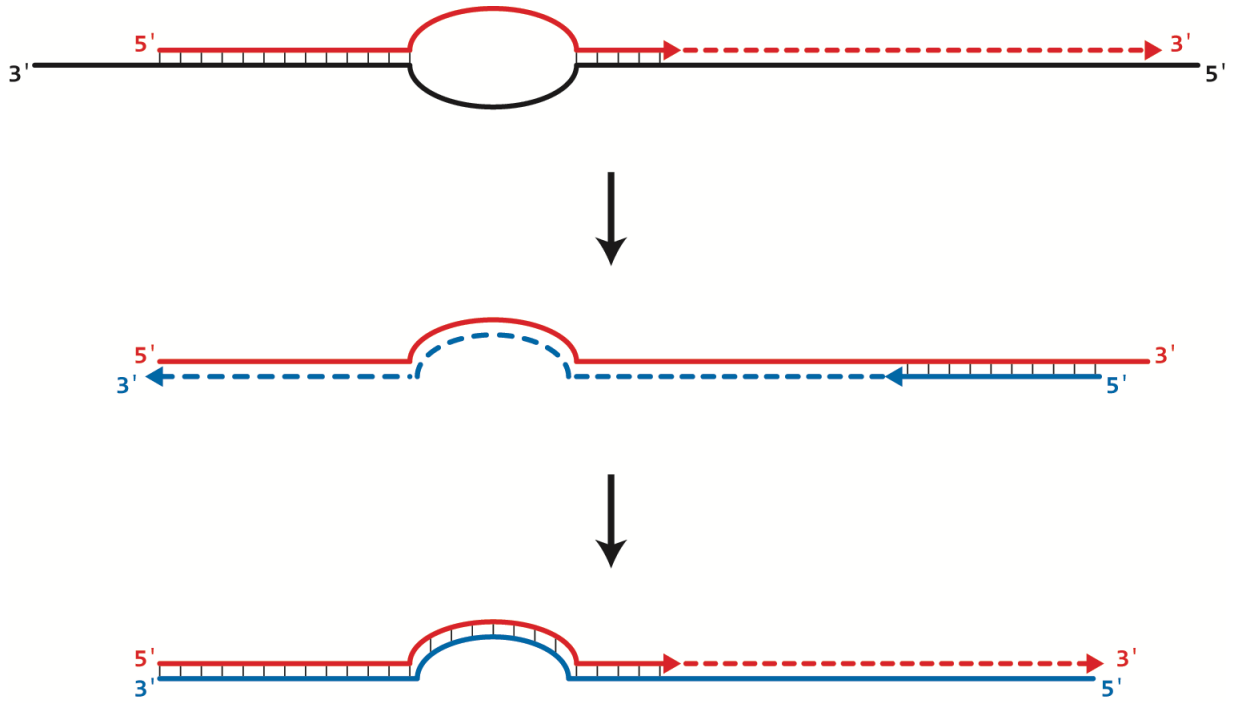


BRAF V600E 24-14/14-5:1:1

SuperSelective Primer

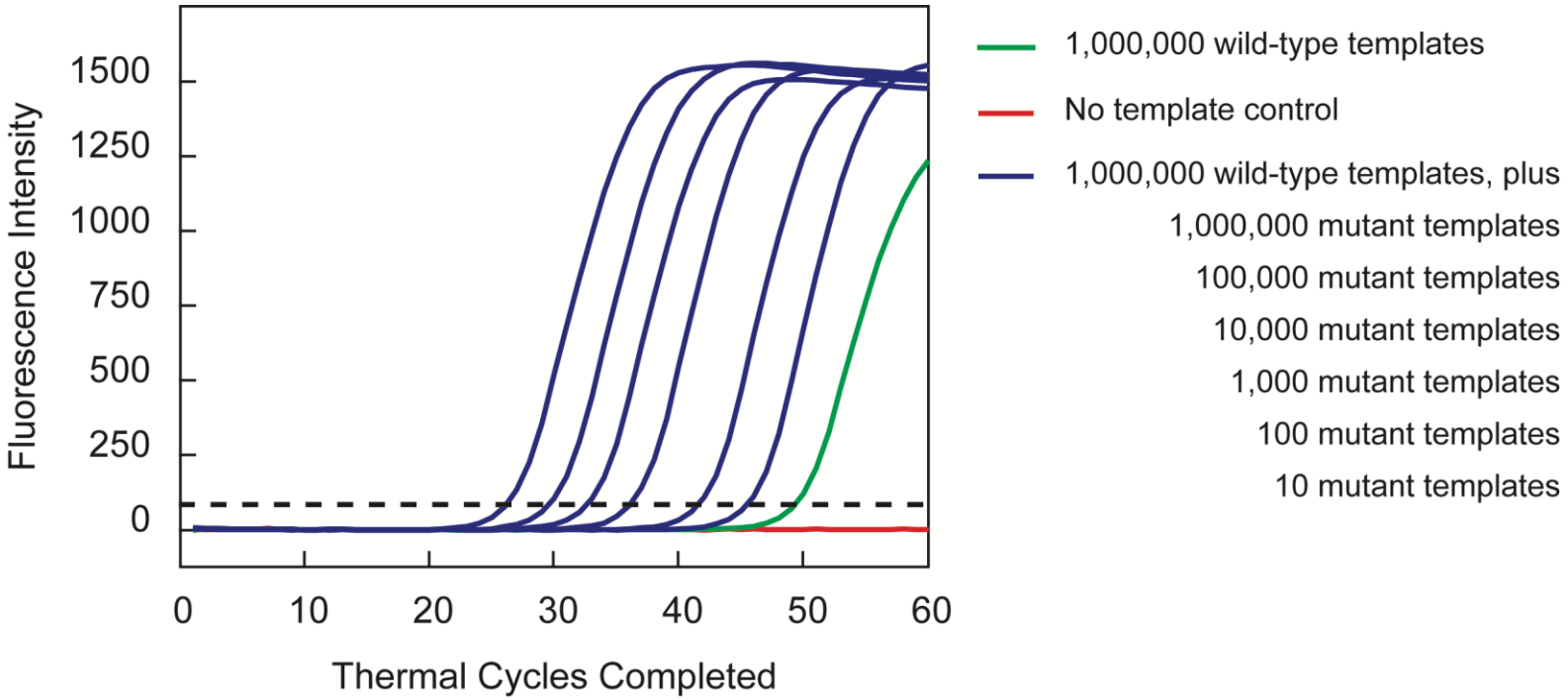


Synthesis of Amplicons



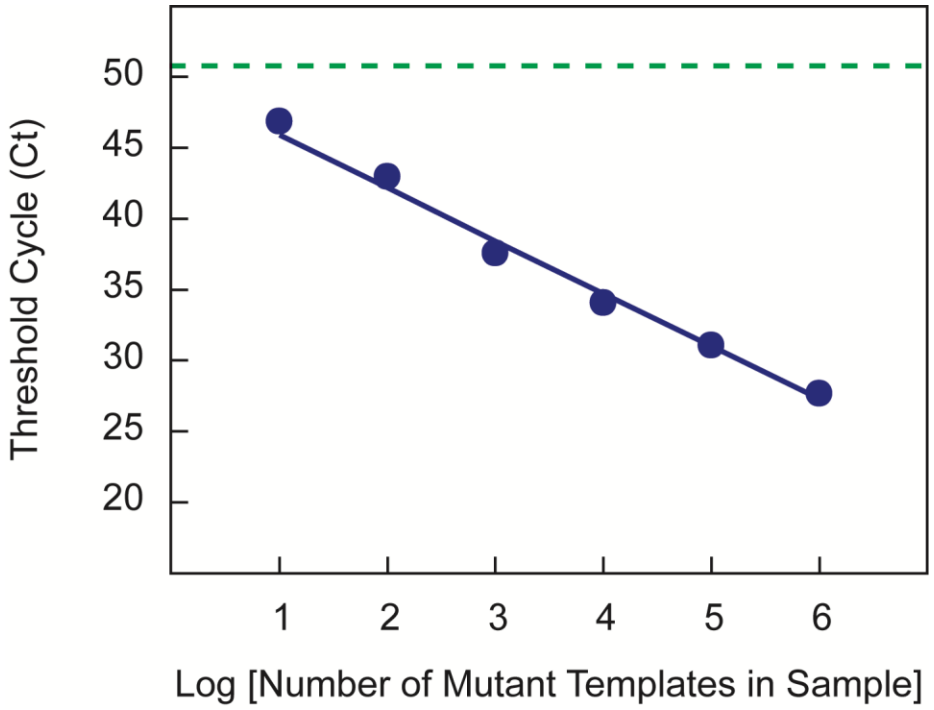
Selective Amplification of Templates Containing *BRAF* Mutation V600E in the Presence of 1,000,000 Wild-type DNA Templates

SuperSelective Primer 24-14/14-5:1:1

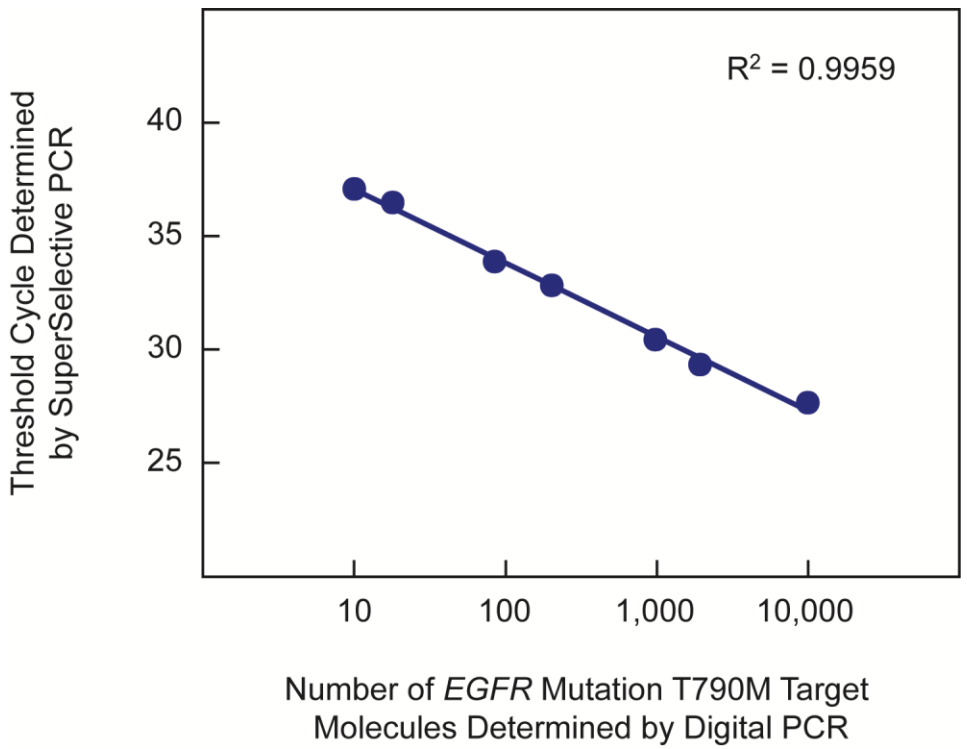


Selective Amplification of Templates Containing *BRAF* Mutation V600E in the Presence of 1,000,000 Wild-type DNA Templates

SuperSelective Primer 24-14/14-5:1:1

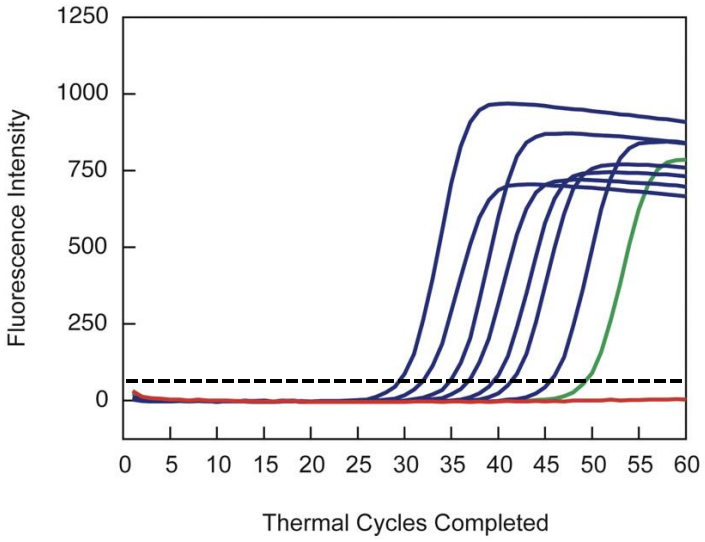


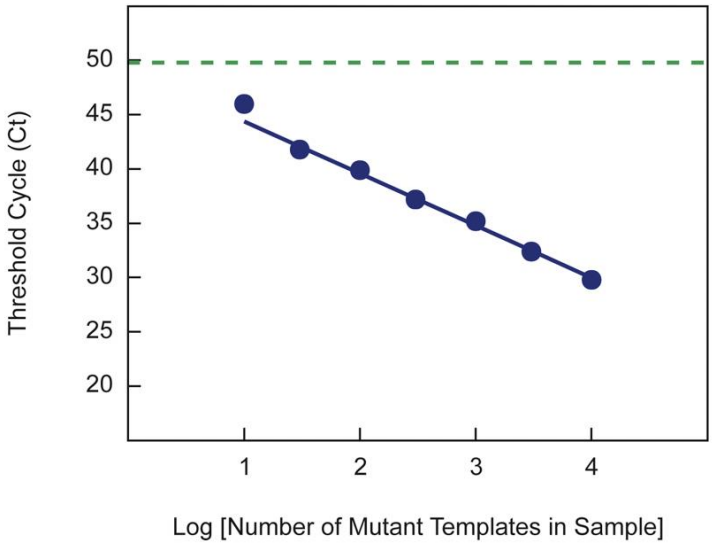
Digital PCR vs. SuperSelective PCR



Selective Amplification of Sequences Containing *EGFR* Mutation L858R (in H1975 cell-line genomic DNA) in the Presence of Wild-type Genomic DNA from 10,000 Cells

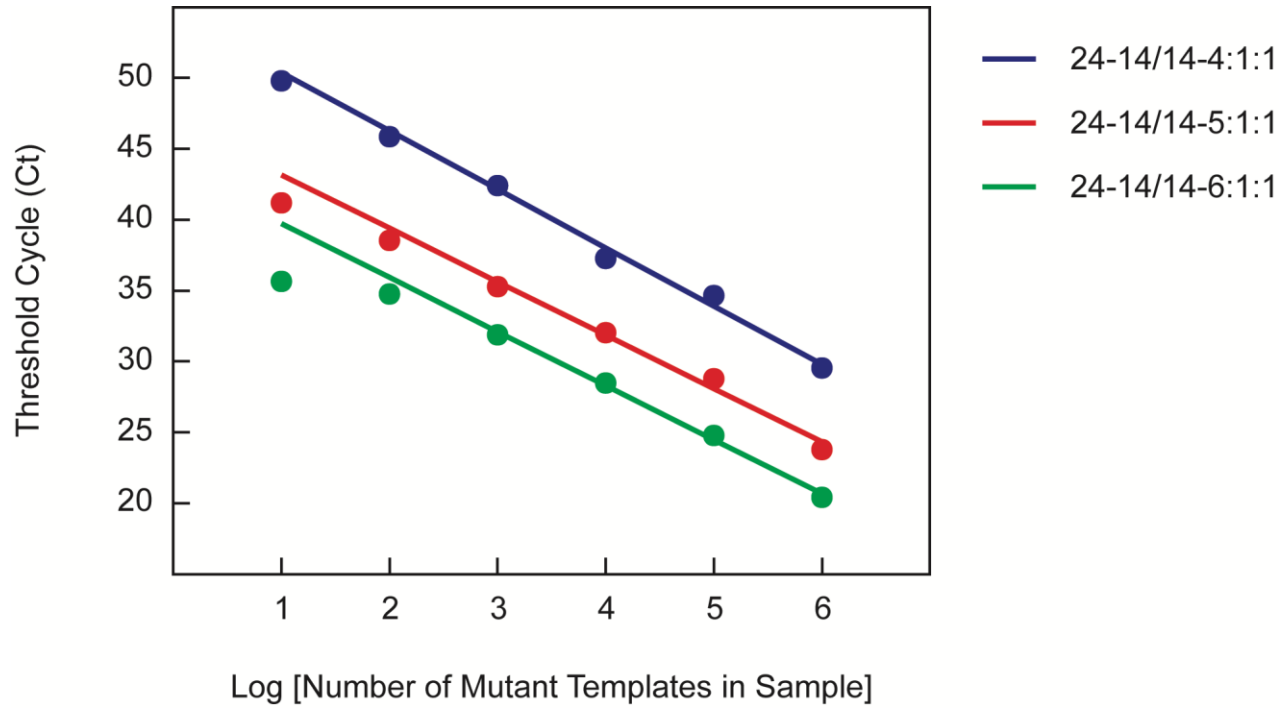
SuperSelective Primer 24-14/14-5:1:1





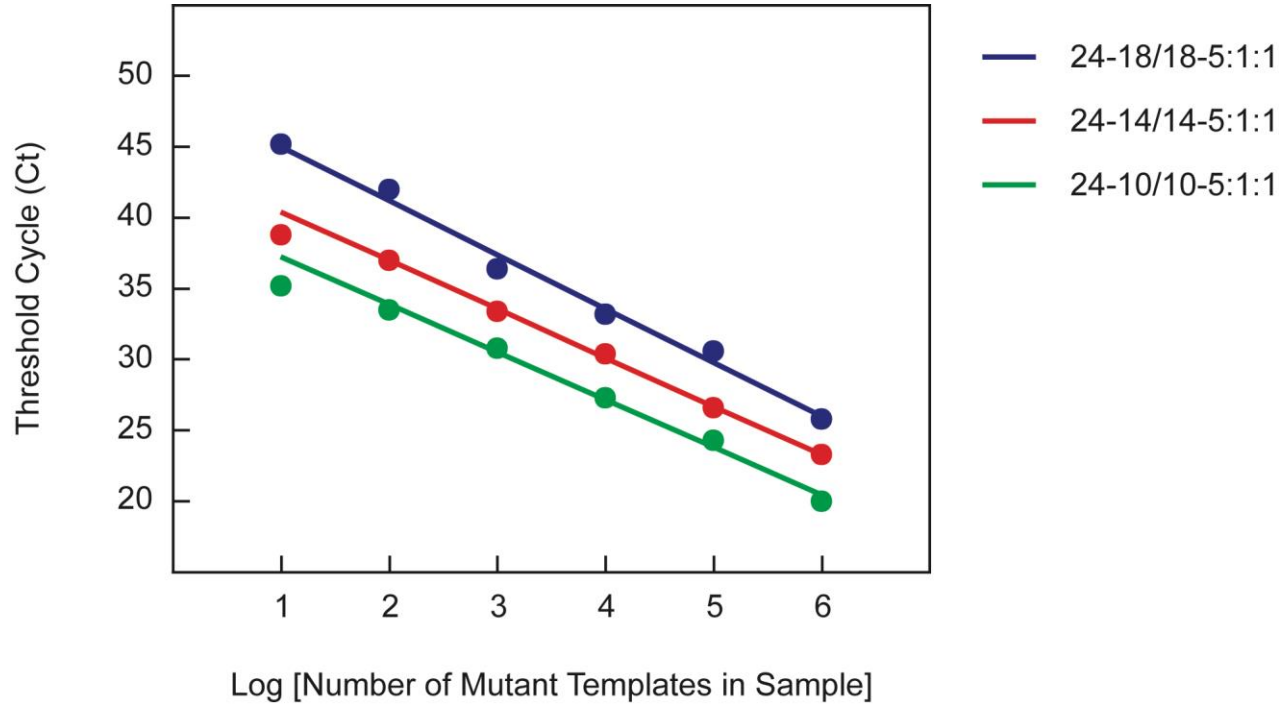
Selective Amplification of Templates Containing *EGFR* Mutation L858R in the Presence of 1,000,000 Wild-type DNA Templates

Comparison of Different Foot Lengths (24-14/14-foot)



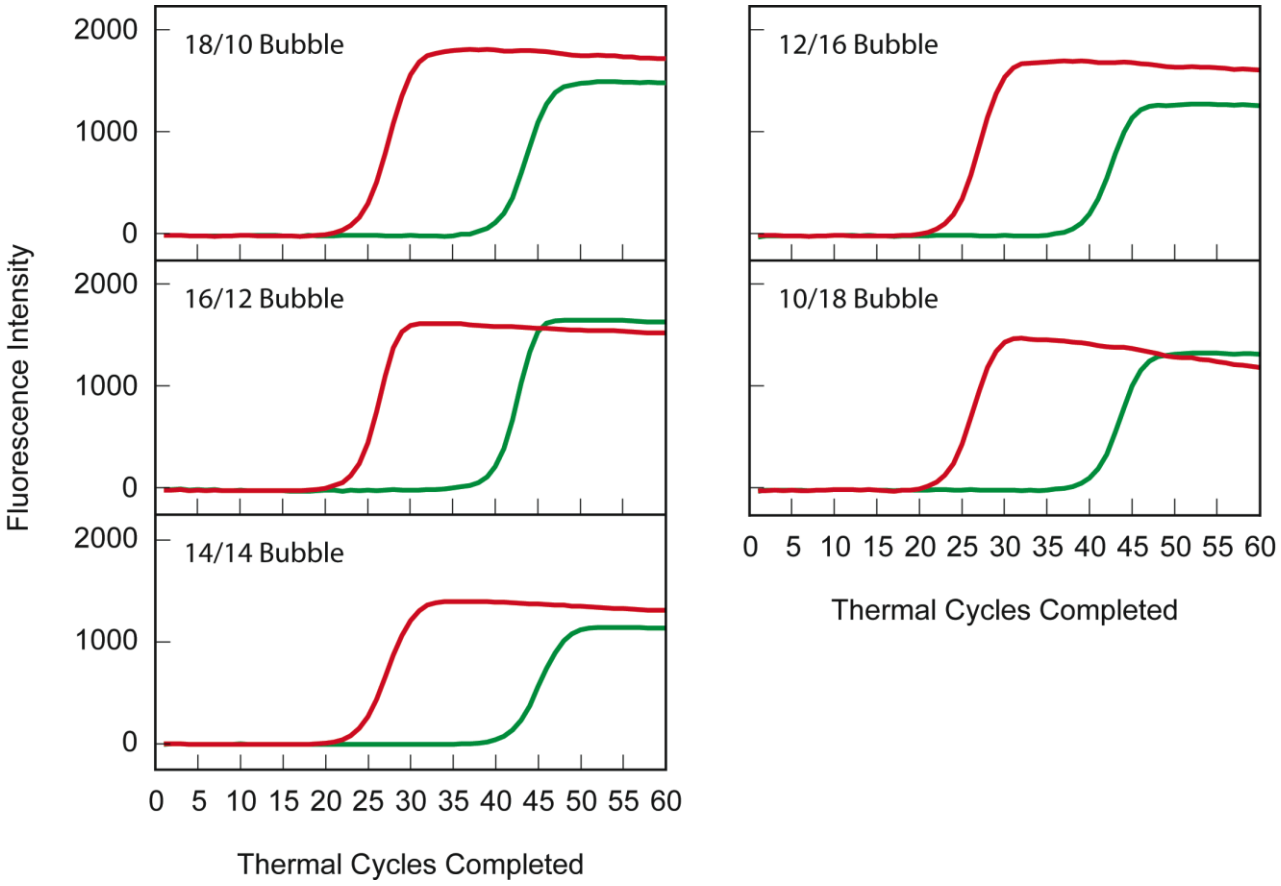
Selective Amplification of Templates Containing *EGFR* Mutation L858R in the Presence of 1,000,000 Wild-type DNA Templates

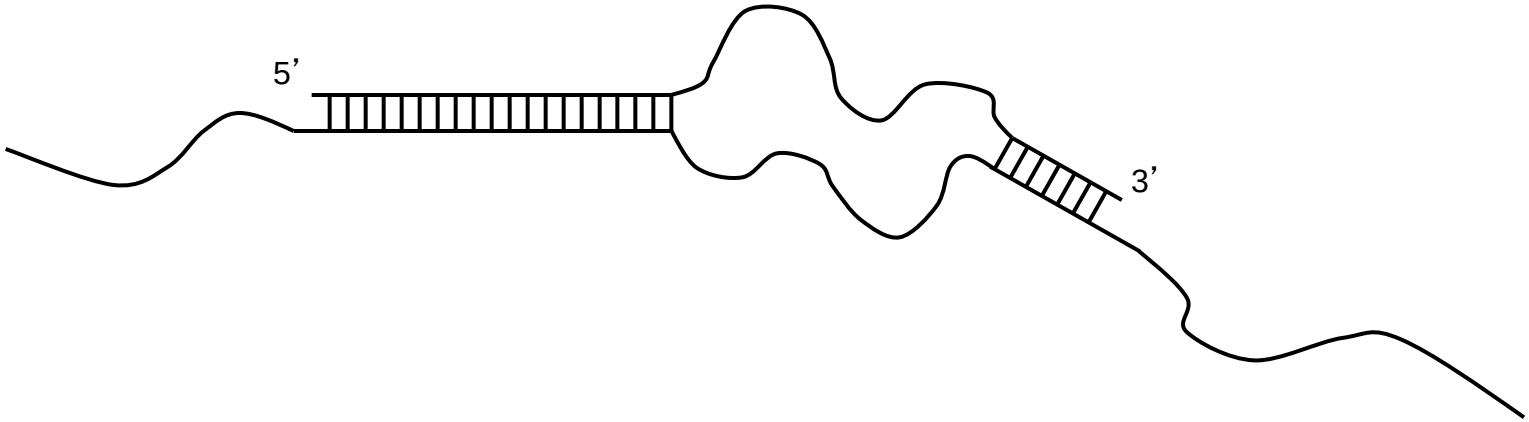
Comparison of Different Bubble Circumferences (24-bridge/intervening sequence-5:1:1)



Selective Amplification of Templates Containing *EGFR* Mutation L858R in the Presence of 1,000,000 Wild-type DNA Templates

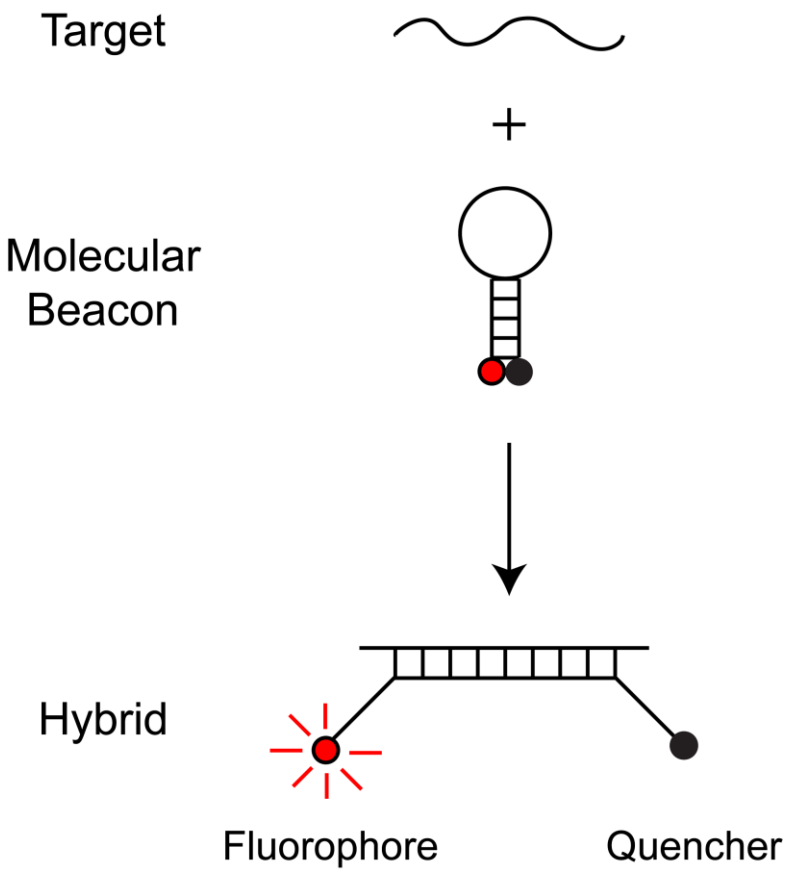
Comparison of Different Bubble Symmetries (24-bridge/intervening sequence-5:1:1)

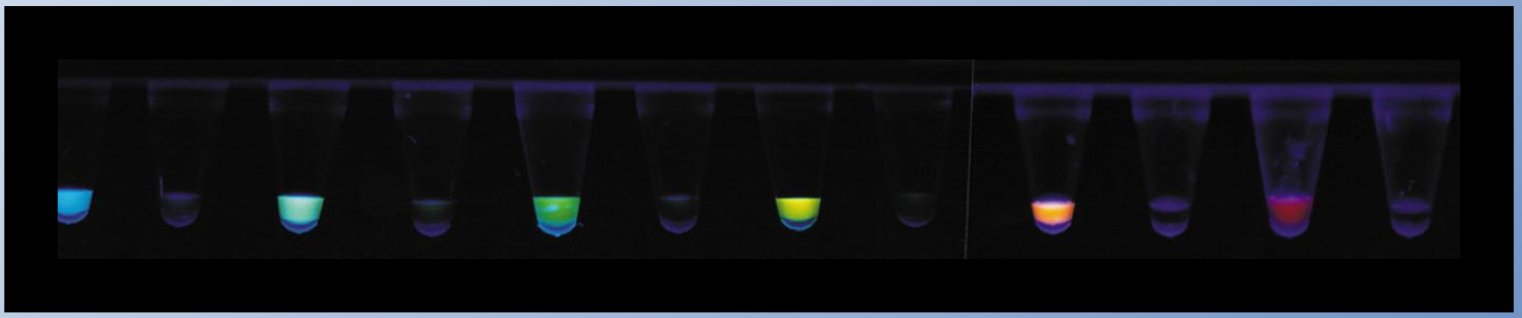


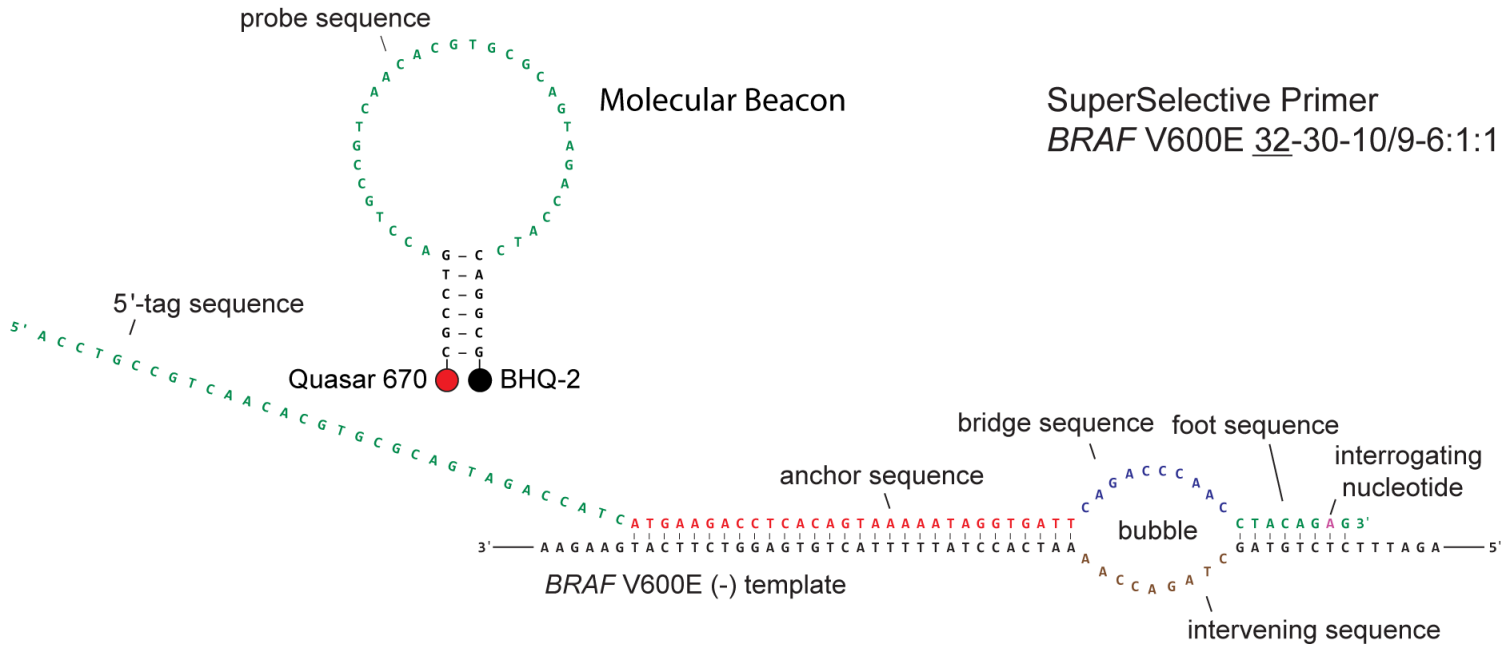

5’
3’

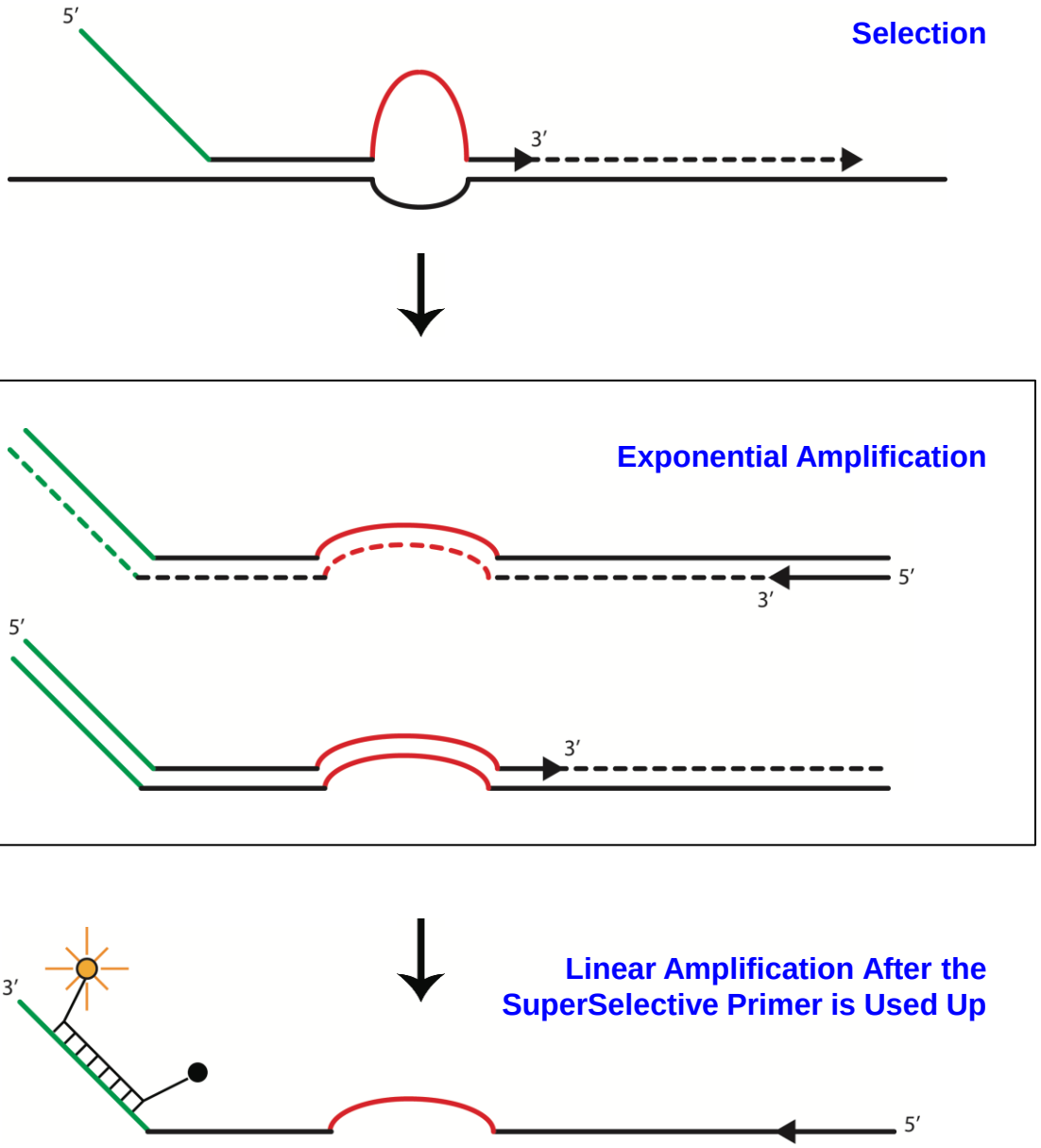
Multiplex PCR Assays for Rare Mutations Located in the Same or Adjacent Codon

1. Amplicon-specific fluorescent probes
2. Only “correct” primer copies each amplicon
3. Heteroduplex formation does not prevent synthesis
4. Amplification of reference gene for inter-comparability


Target
+
Molecular
Beacon
Hybrid
Fluorophore
Quencher

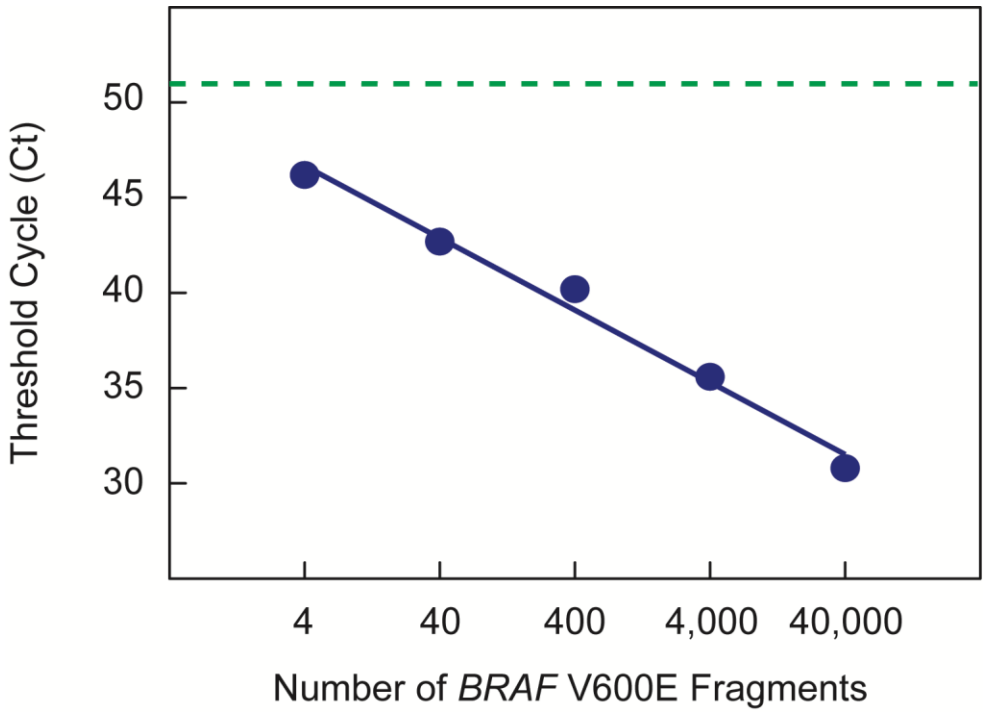



probe sequence
Molecular Beacon
SuperSelective Primer
BRAF V600E 32-30-10/9-6:1:1
5'-tag sequence
Quasar 670
BHQ-2
bridge sequence
foot sequence
interrogating nucleotide
anchor sequence
bubble
BRAF V600E (-) template
intervening sequence

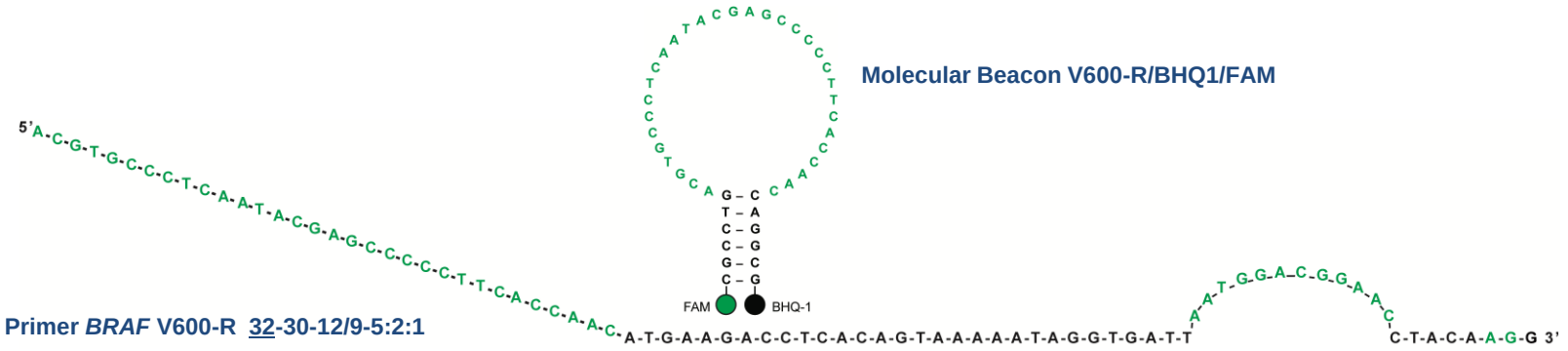

Selection
5′
3′
Exponential Amplification
5′
3′
5′
3′
3′
Linear Amplification After the
SuperSelective Primer is Used Up
5′

Detection of *BRAF* V600E Mutant DNA Fragments in the Presence of 40,000 *BRAF* Wild-type DNA Fragments

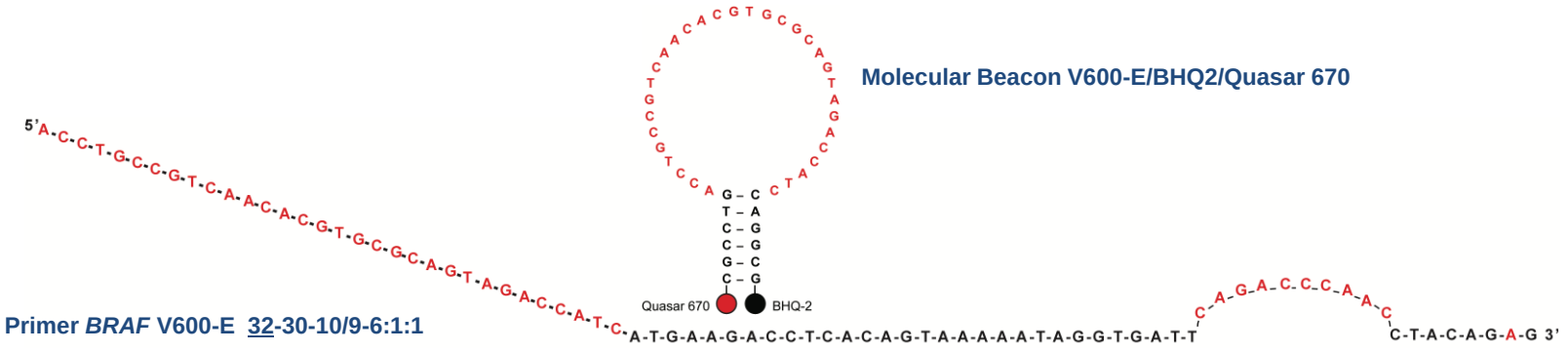
SuperSelective Primer 32-28-12/9-7:1:1 and Fluorescein-labeled Molecular Beacons



Primer Design for Multiplex Quantitation of Rare Mutant Target Sequences



Primer *BRAF* V600-R 32-30-12/9-5:2:1



Primer *BRAF* V600-E 32-30-10/9-6:1:1

BRAF Reverse primer 5' A-T-C-A-G-T-G-G-A-A-A-A-A-T-A-G-C-C-T-C-A-A-T-T-C-T-T-A-C-C-A-T-C-C 3'

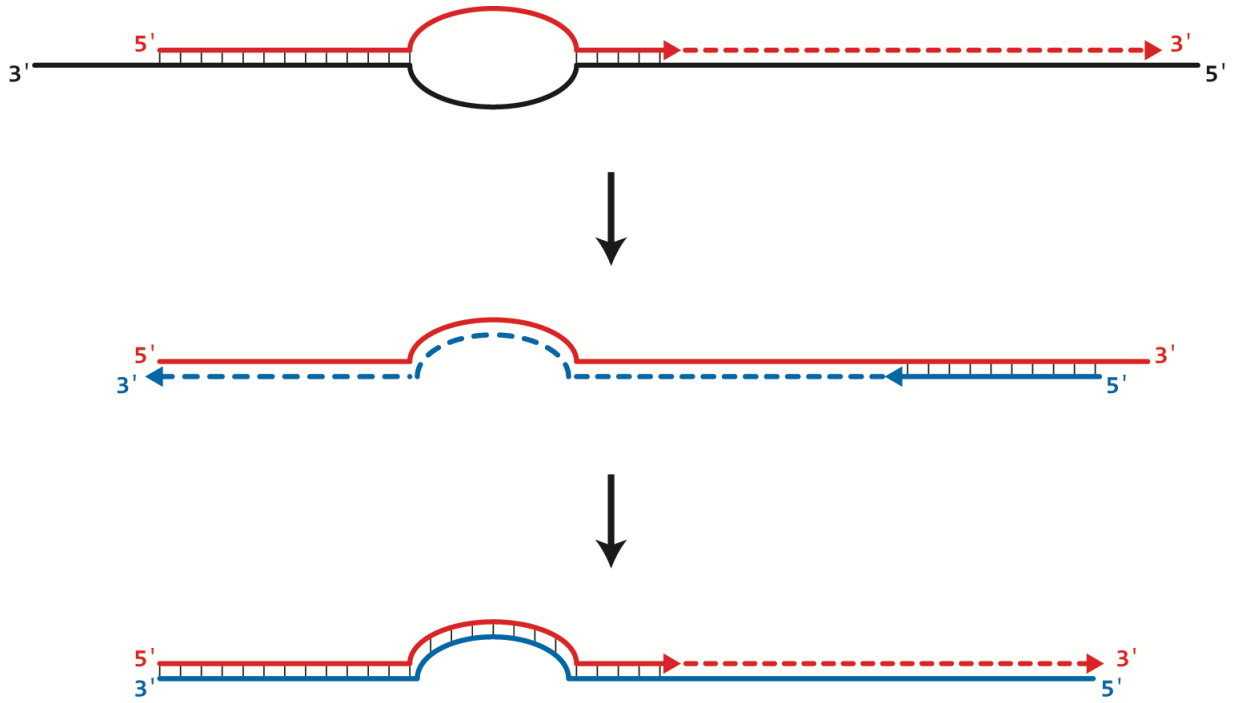
Reaction Conditions

10	mM	Tris-HCl (pH 8.0)
50	mM	KCl
2.5	mM	$MgCl_2$
1.5	Units	Platinum Taq DNA polymerase
250	µM	Each deoxyribonucleoside triphosphate
60	nM	SuperSelective primer *BRAF* V600-R
60	nM	SuperSelective primer *BRAF* V600-E
1,000	nM	*BRAF* Reverse primer
300	nM	Molecular beacon *BRAF* V600-R/BHQ1/FAM
300	nM	Molecular beacon *BRAF* V600-E/BHQ2/Quasar 670

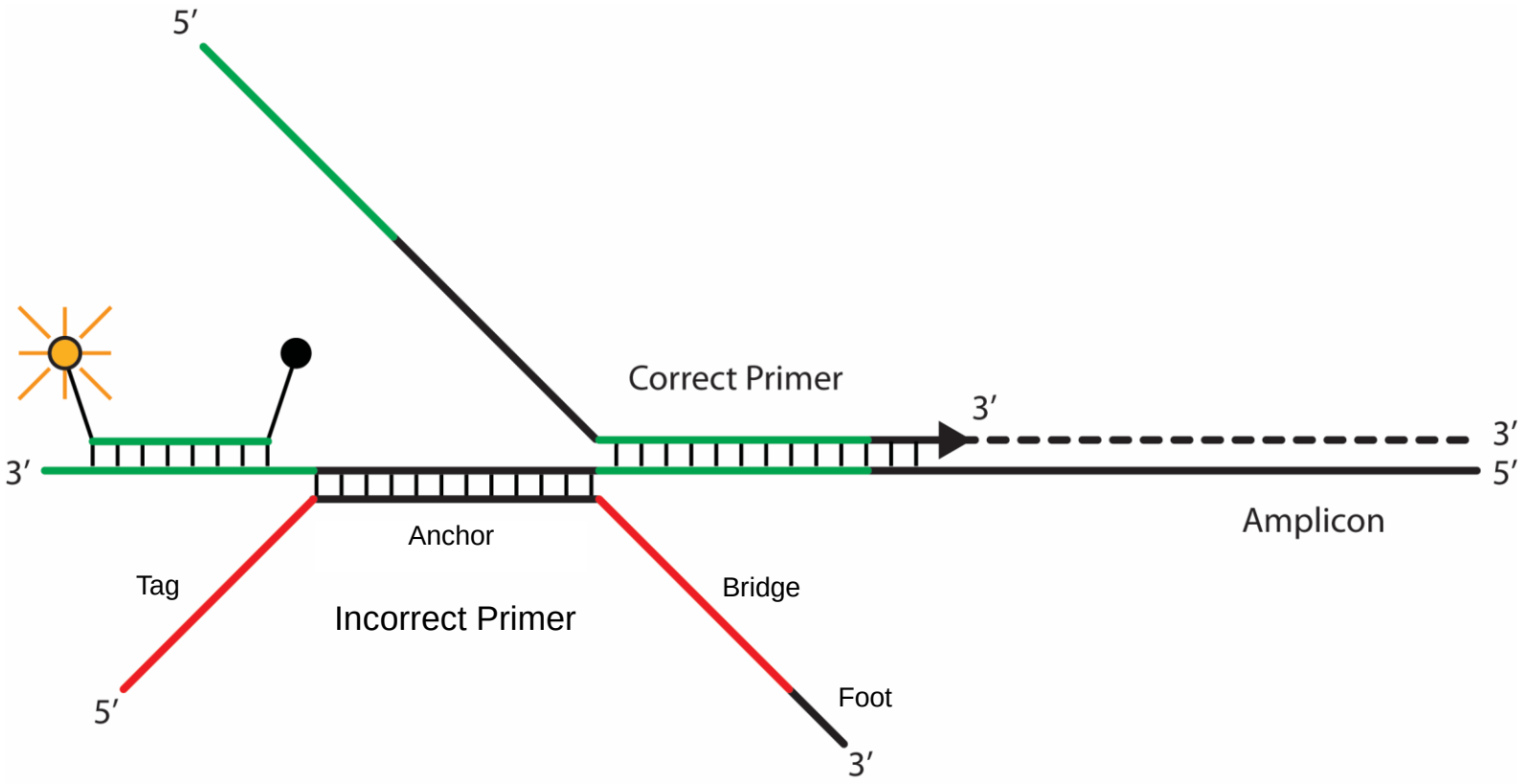
Thermal Cycling Program

1	hold	2	min	95 ˚C
55	cycles	20	sec	95 ˚C
		20	sec	60 ˚C (monitor fluorescence)
		20	sec	72 ˚C

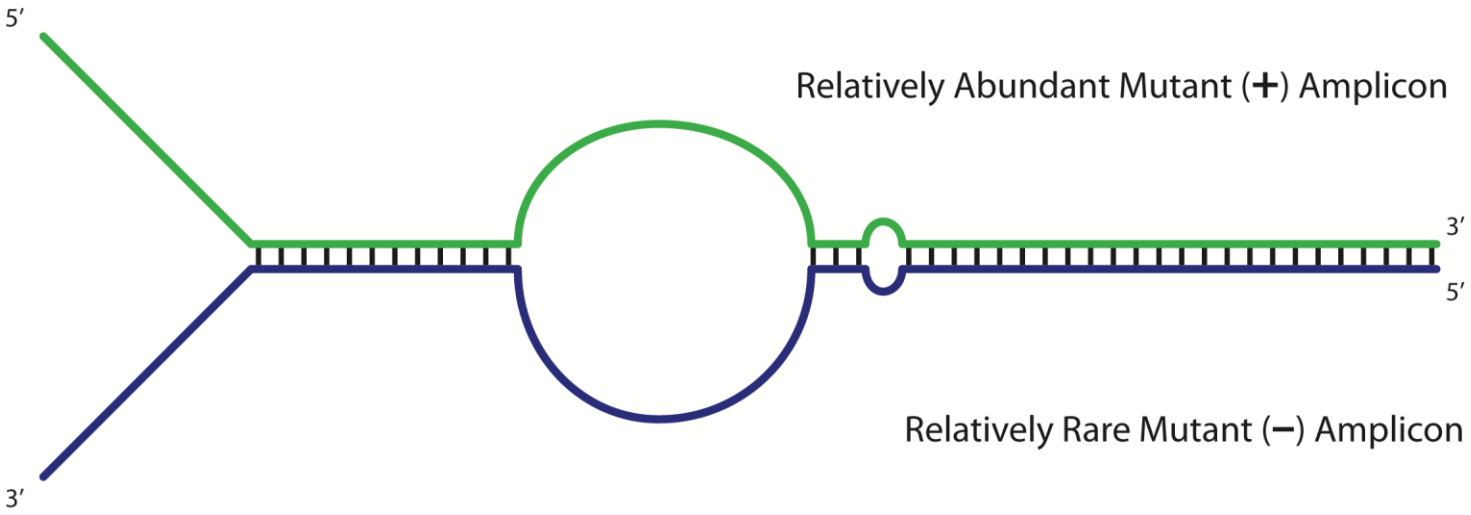
Synthesis of Amplicons



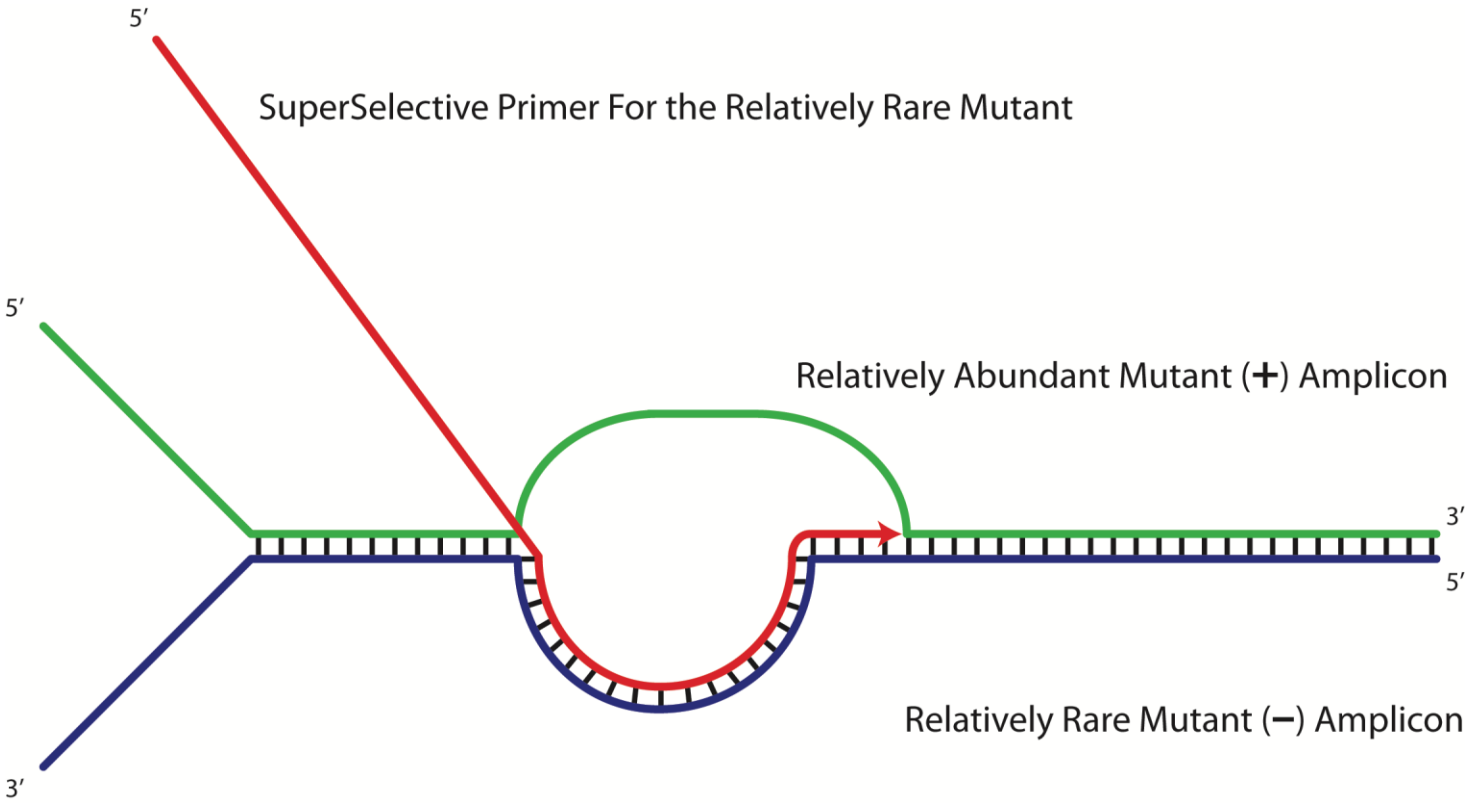
Amplicons are Only Copied by their Correct SuperSelective Primer in Multiplex Assays



Amplicon Heteroduplex is Not Entirely Double Stranded



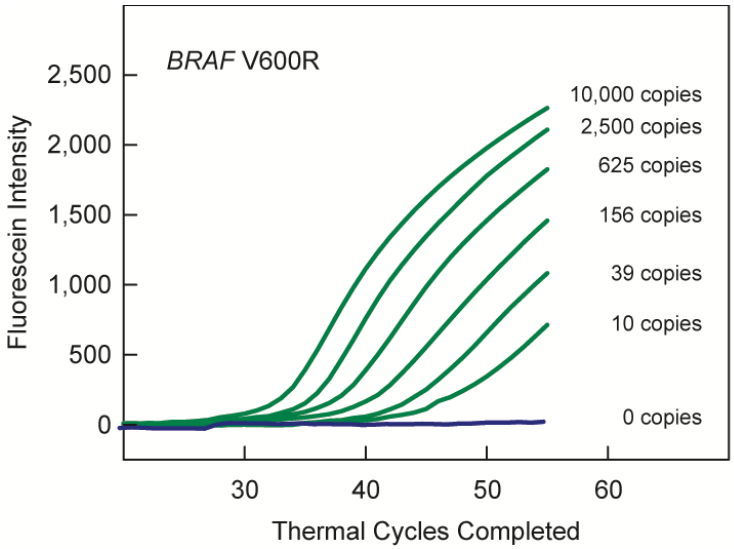
Amplicon Heteroduplex Does Not Prevent Exponential Amplification of the Relatively Rare Mutant

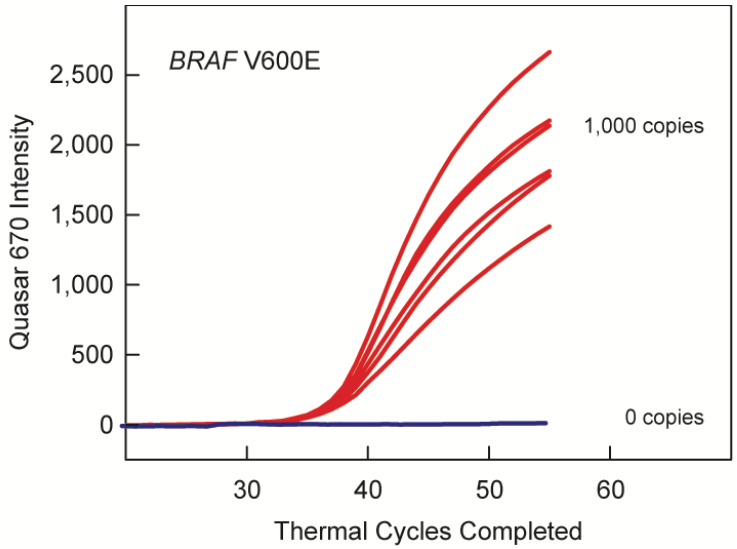


Non-Symmetric Duplex Assay for Different Numbers of *BRAF* Mutant V600R Templates

60 nM primers for *BRAF* V600E; 60 nM primers for *BRAF* V600R; 1,000 nM reverse primers
300 nM *BRAF* V600E molecular beacon; 300 nM *BRAF* V600R molecular beacon

1,000 *BRAF* V600E templates; 10,000 *BRAF* wild-type templates

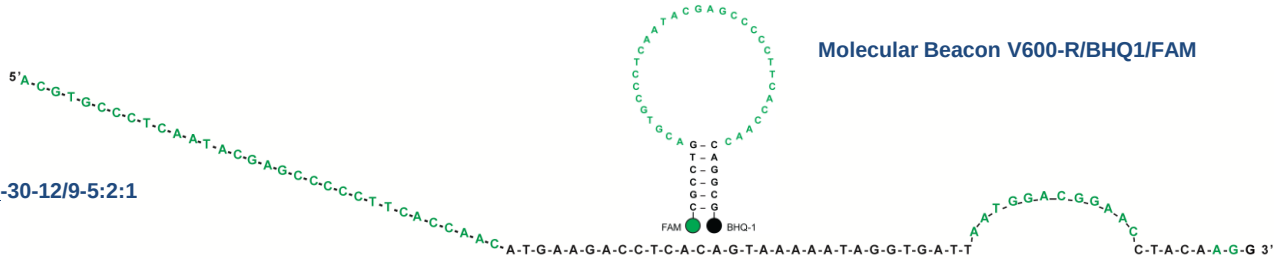




Inclusion of a Primer for a Reference Gene to Assess Relative Abundance

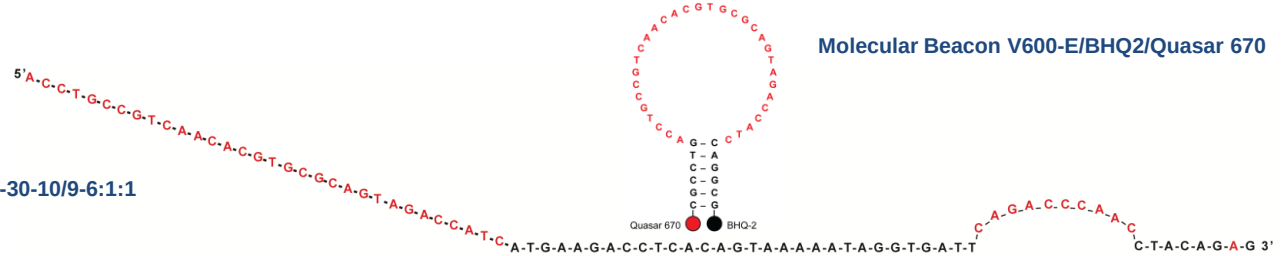
Molecular Beacon V600-R/BHQ1/FAM

Primer *BRAF* V600-R 32-30-12/9-5:2:1



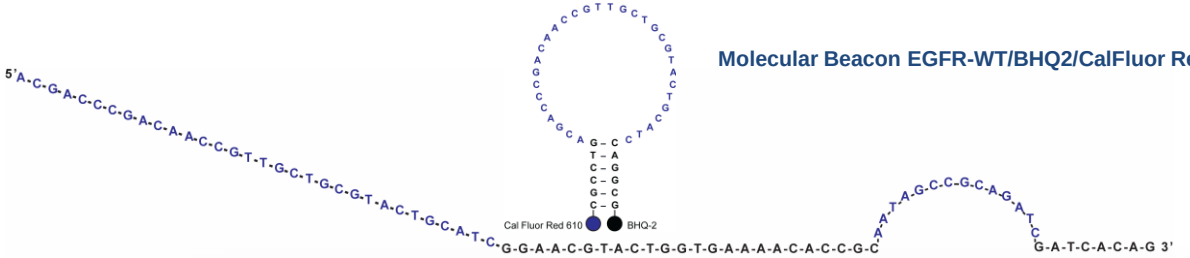
Molecular Beacon V600-E/BHQ2/Quasar 670

Primer *BRAF* V600-E 32-30-10/9-6:1:1



Molecular Beacon EGFR-WT/BHQ2/CalFluor Red 610

Primer *EGFR*-WT 32-25-14/9-8:0:0



***BRAF* Reverse primer**

5' A-T-C-A-G-T-G-G-A-A-A-A-A-T-A-G-C-C-T-C-A-A-T-T-C-T-T-A-C-C-A-T-C-C 3'

***EGFR* Reverse primer**

5' C-A-T-G-G-T-A-T-T-C-T-T-T-C-T-C-T-T-C-C-G-C-A-C-C-C-A 3'

Reaction Conditions

10	mM	Tris-HCl (pH 8.0)
50	mM	KCl
2.5	mM	$MgCl_2$
1.5	Units	Platinum Taq DNA polymerase
250	µM	Each deoxyribonucleoside triphosphate
60	nM	SuperSelective primer *BRAF* V600-R
60	nM	SuperSelective primer *BRAF* V600-E
1,000	nM	*BRAF* Reverse primer
60	nM	SuperSelective primer *EGFR*-WT
500	nM	*EGFR* Reverse primer
300	nM	Molecular beacon *BRAF* V600-R/BHQ1/FAM
300	nM	Molecular beacon *BRAF* V600-E/BHQ2/Quasar 670
300	nM	Molecular beacon *EGFR*-WT/BHQ2/CalFluorRed 610

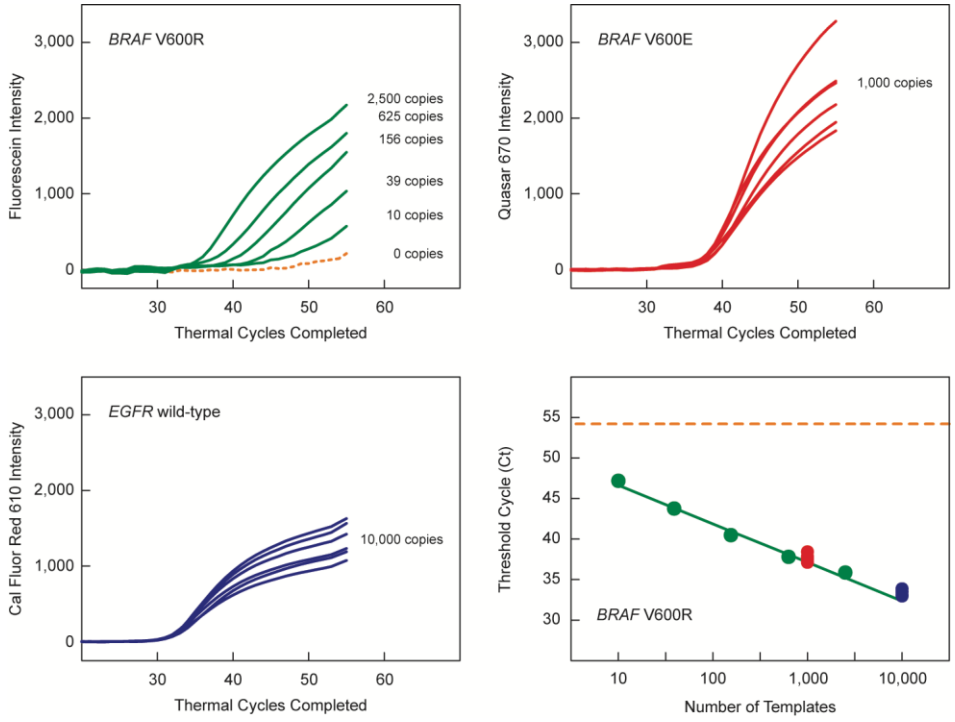
Thermal Cycling Program

1	hold	2	min	95 ˚C
55	cycles	20	sec	95 ˚C
		20	sec	60 ˚C (monitor fluorescence)
		20	sec	72 ˚C

Non-Symmetric Triplex Assay (Three Differently Colored Molecular Beacons) for Different Numbers of *BRAF* Mutant V600R Templates

60 nM primers for *BRAF* V600E; 60 nM primers for *BRAF* V600R; 60 nM primers for *EGFR* WT
1,000 nM common *BRAF* reverse primers; 500 nM *EGFR* reverse primer;
300 nM of each molecular beacon

Dilutions of *BRAF* V600R templates; 1,000 *BRAF* V600E templates;
10,000 *BRAF* wild-type templates; 10,000 *EGFR* wild-type templates



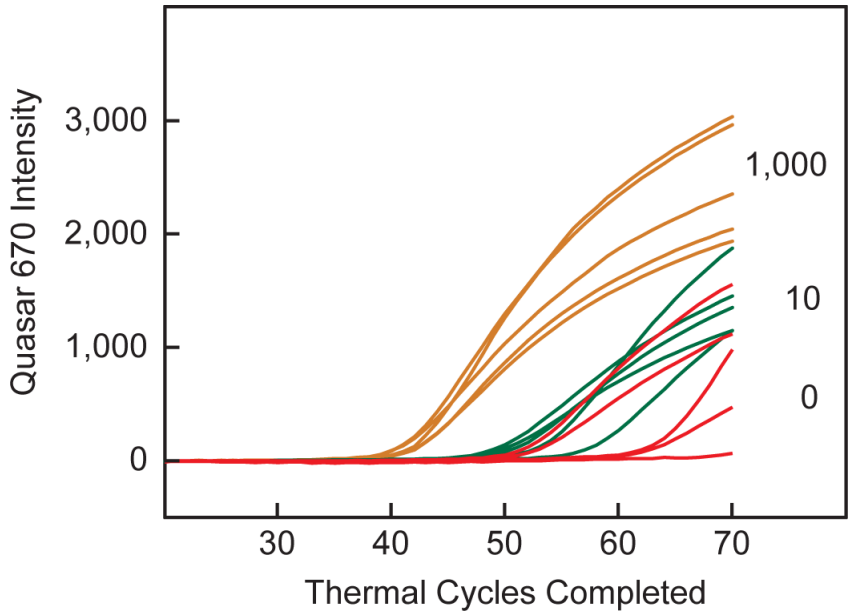
Vargas, Kramer, Tyagi, and Marras (2016)

Multiplex Real-Time PCR Assays that Measure the Abundance of Extremely Rare Mutations Associated with Cancer

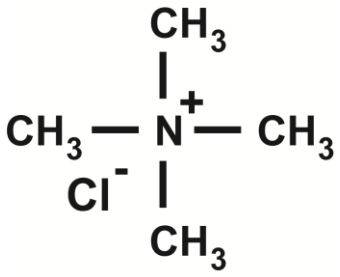
PLoS ONE 11, e156546

Insufficient Suppression of Signals from 40,000 Closely Related Wild-type Fragments Can Cause False-negative or False-positive Conclusions When Mutants are Rare

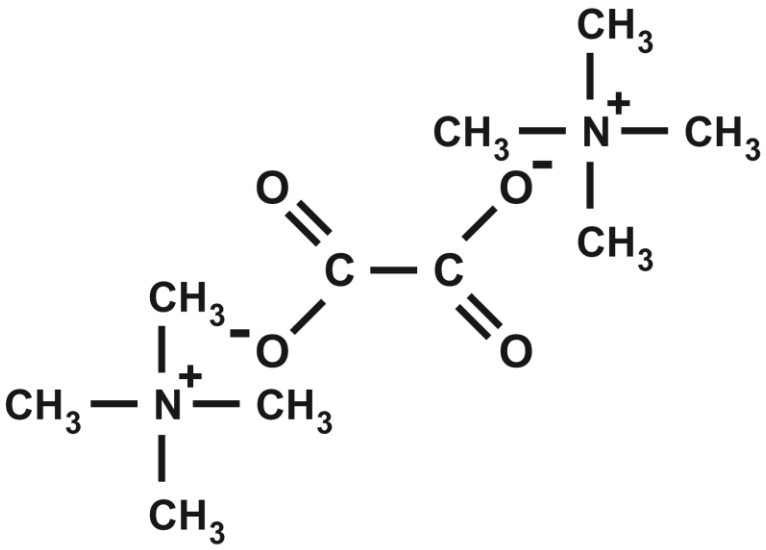
SuperSelective Primer *BRAF* V600E 32-30-10/9-6:1:1



Selectivity Enhancing Agents



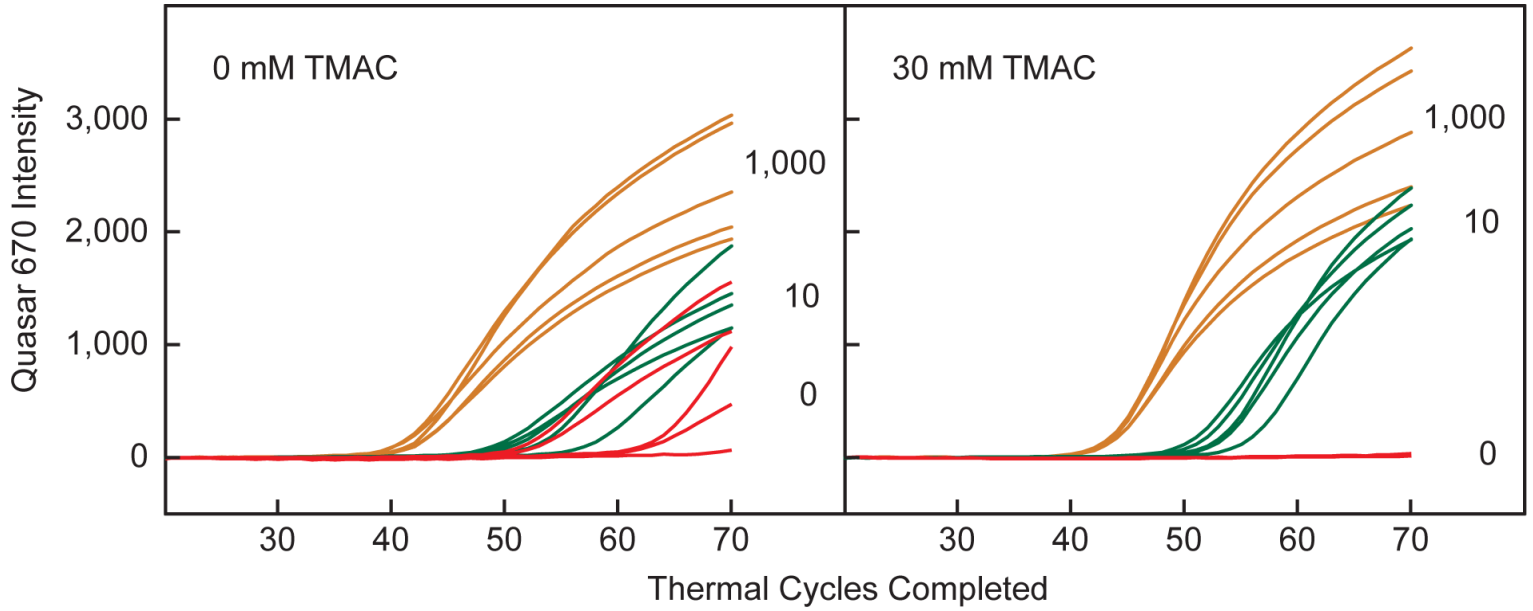
Tetramethylammonium Chloride



bis-Tetramethylammonium Oxalate

Suppression of Signals from 40,000 Closely Related Wild-type Templates by Tetramethylammonium Chloride

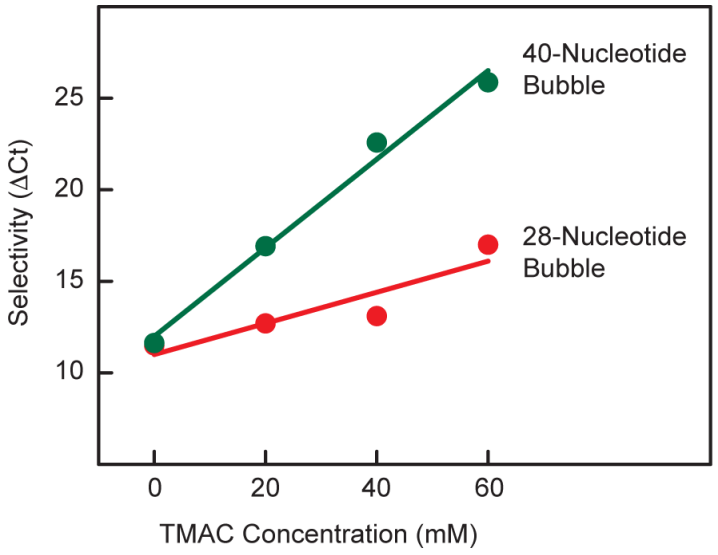
SuperSelective Primer *BRAF* V600E 32-30-10/9-6:1:1



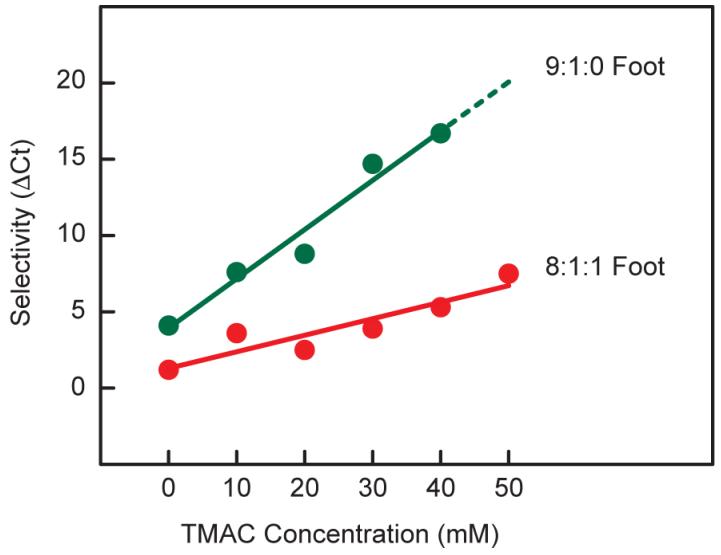
Selectivity Enhancement Depends on Bubble Circumference and on Location of the Interrogating Nucleotide

(Bubble Effect on 100,000 Mutants Compared to 100,000 Wild Types)
(Interrogating Nucleotide Effect on 4,000 Mutants Compared to 400,000 Wild Types)

***BRAF* V600E 32-24-18/18-8:1:1**
***BRAF* V600E 32-24-10/14-8:1:1**

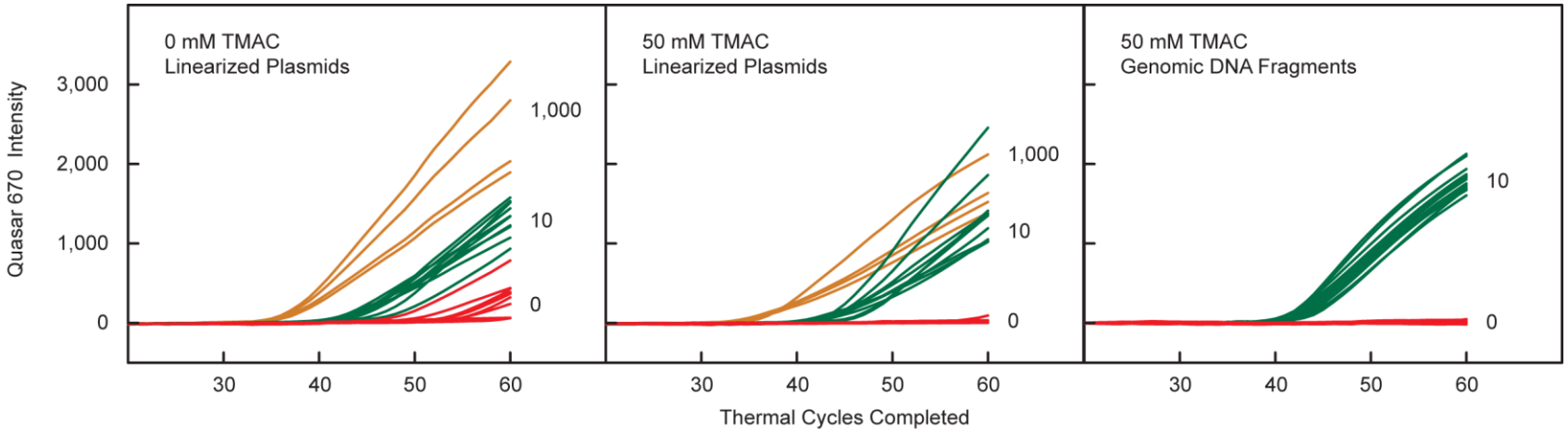


***BRAF* V600E 32-24-14/14-9:1:0**
***BRAF* V600E 32-24-14/14-8:1:1**



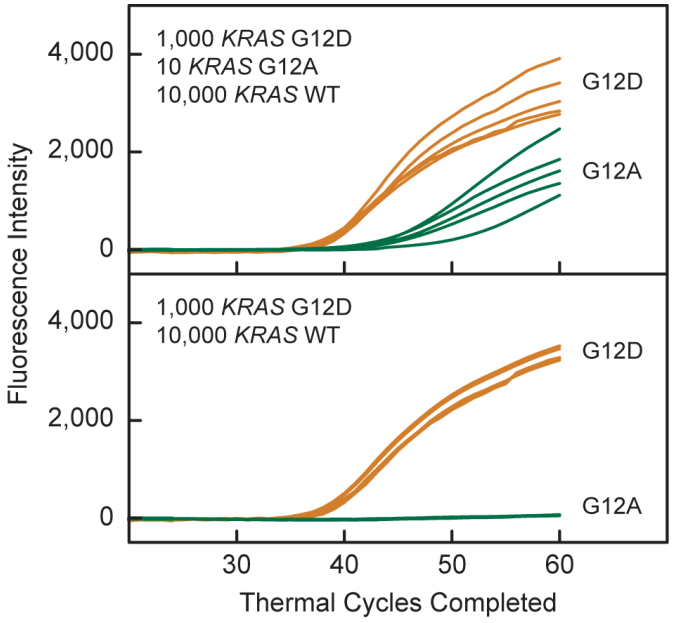
Combination of 50 mM TMAC and SuperSelective Primers with a Longer Foot Sequence and a 3'-Terminal Interrogating Nucleotide Completely Suppresses Synthesis from 40,000 Closely Related Wild-type Fragments

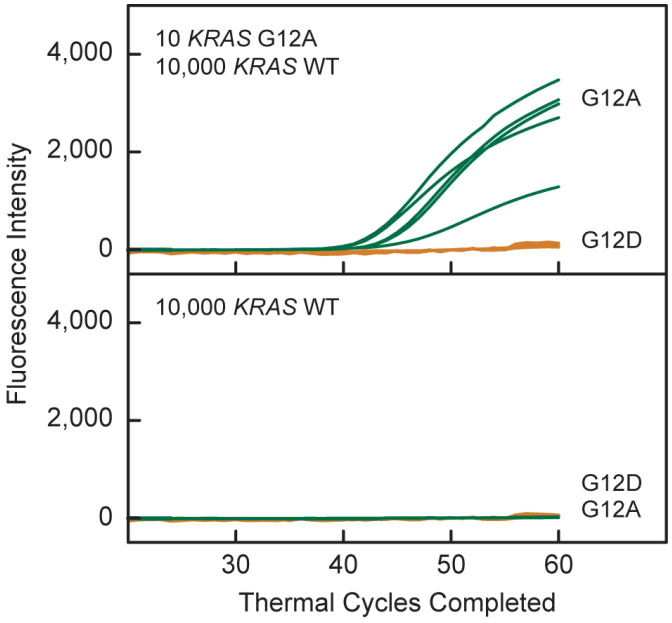
SuperSelective Primer *BRAF* V600E 32-24-14/14-9:1:0



Multiplex PCR Assay for Closely Related Mutant Alleles Carried Out in the Presence of 60 mM Tetramethylammonium Chloride

Primer *KRAS* G12A 32-28-14/12-8:1:0 and Molecular Beacon for KRAS G12A
Primer *KRAS* G12D 32-28-19/10-8:1:0 and Molecular Beacon for KRAS G12D



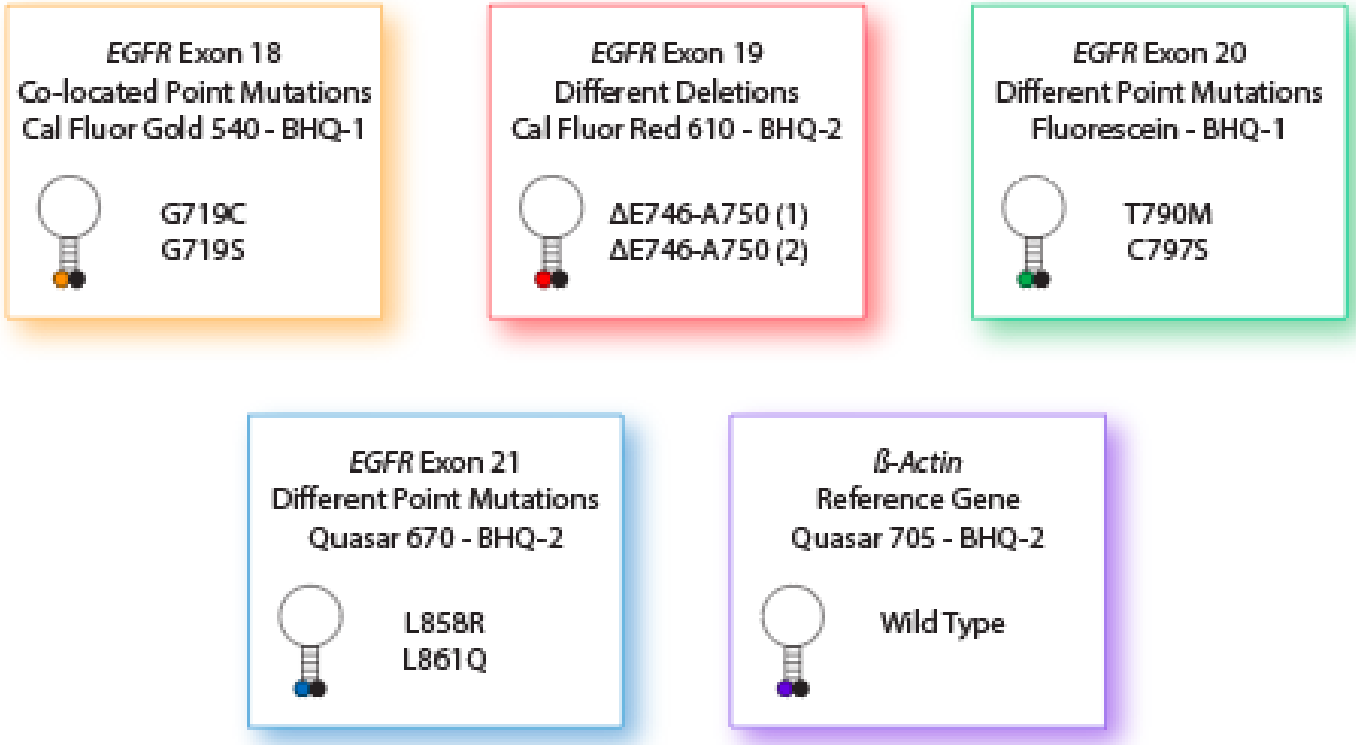


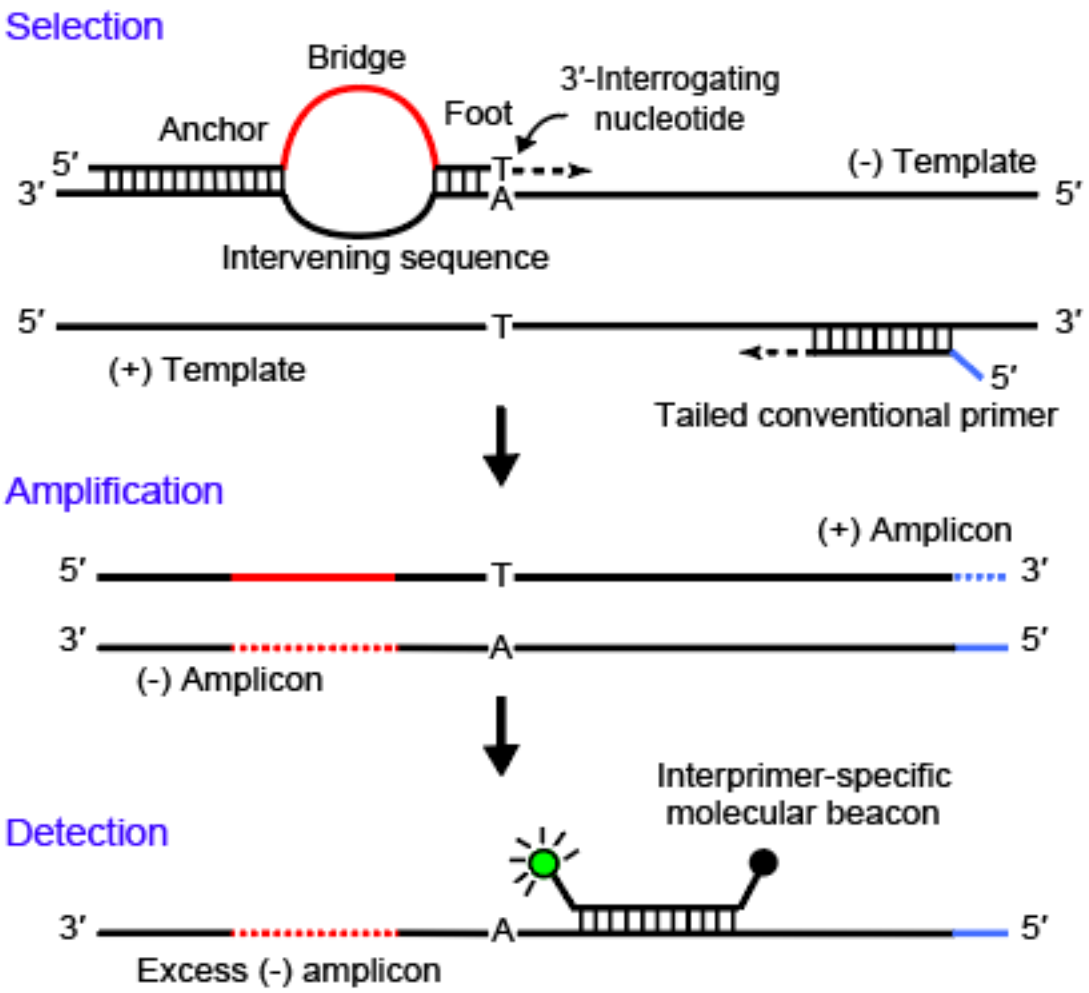
Vargas, Marras, Tyagi, and Kramer (2018)

Suppression of Wild-Type Amplification by Selectivity Enhancing Agents in PCR Assays that Utilize SuperSelective Primers for the Detection of Rare Somatic Mutations

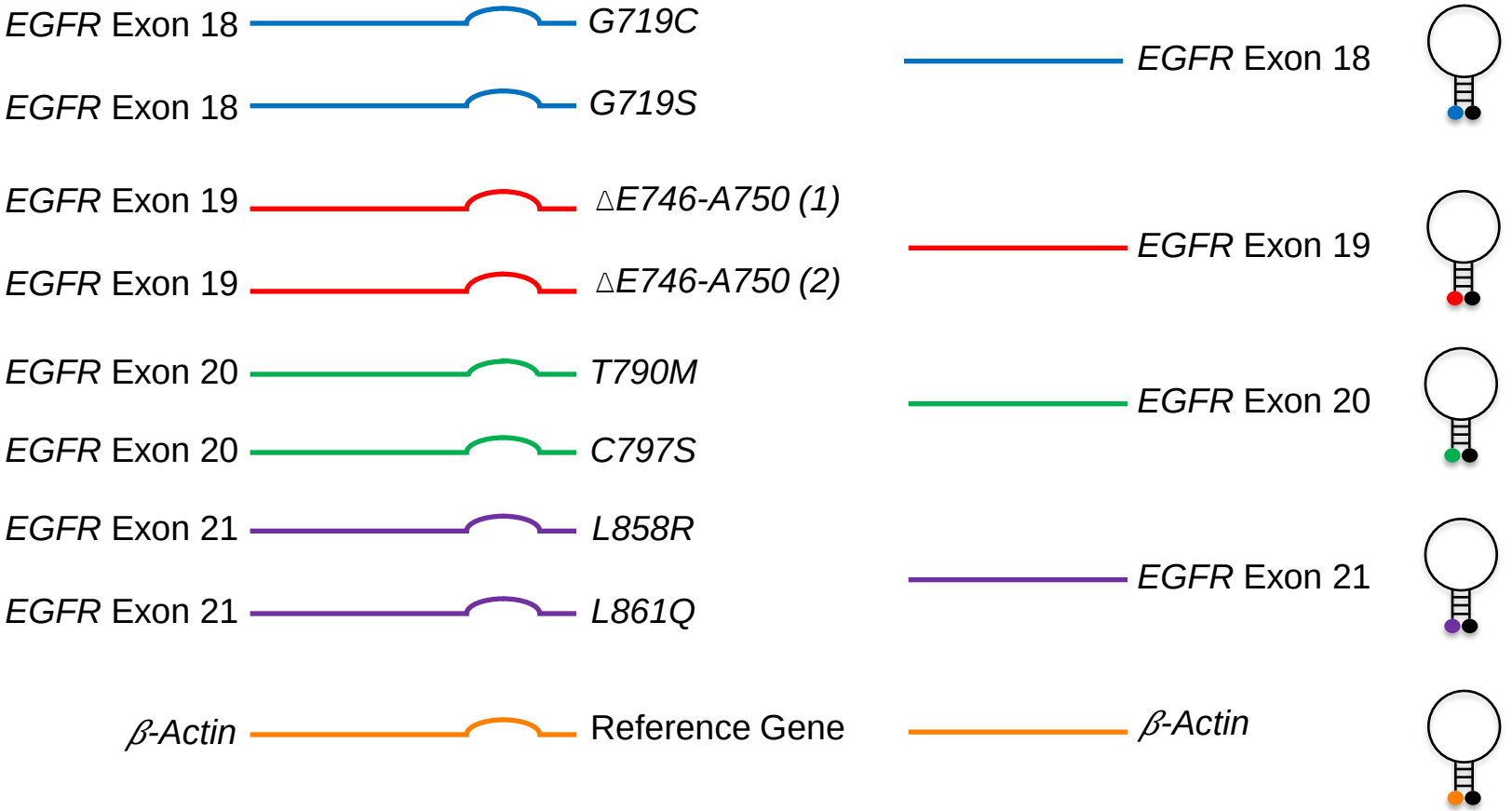
Journal of Molecular Diagnostics 20, 415-427

Multiplex SuperSelective PCR Assay For Groups of Mutations

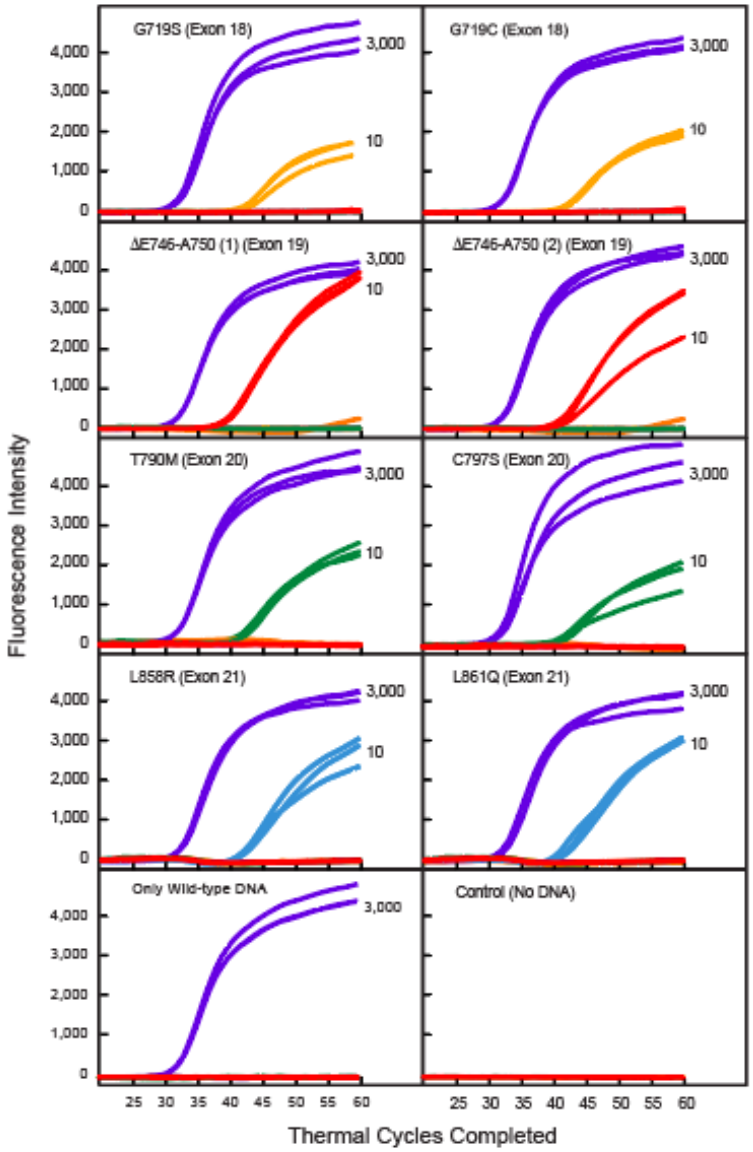



Selection
Bridge
3′-Interrogating
nucleotide
Foot
Anchor
5′
3′
T
A
(-) Template
5′
Intervening sequence
5′
T
3′
(+) Template
5′
Tailed conventional primer
Amplification
(+) Amplicon
5′
T
3′
3′
A
5′
(-) Amplicon
Interprimer-specific
molecular beacon
Detection
3′
A
5′
Excess (-) amplicon

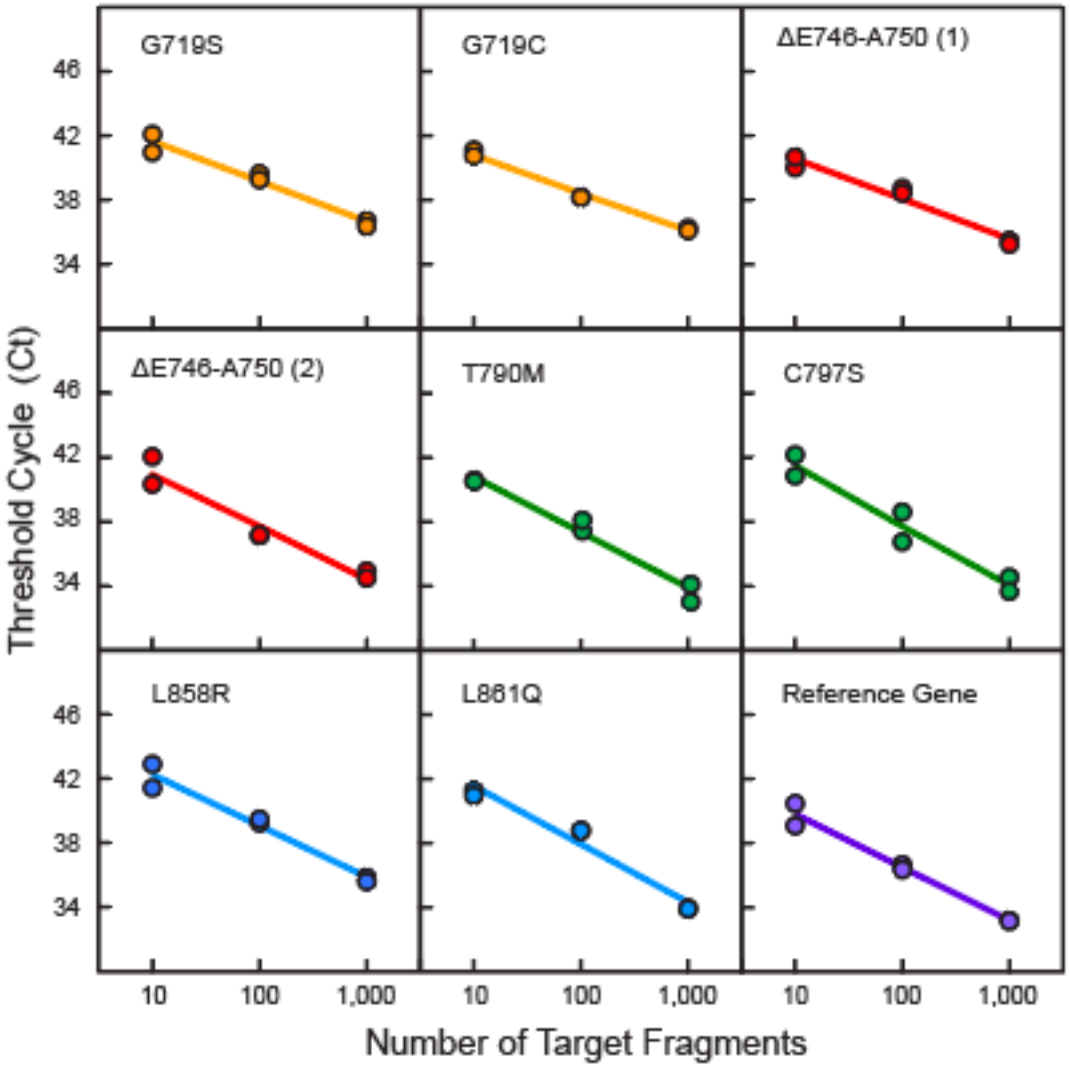
EGFR Mutations Assay Elements



Confirmation of Assay Sensitivity



Quantitative Response of the Multiplex Assay to Different *EGFR* Mutations



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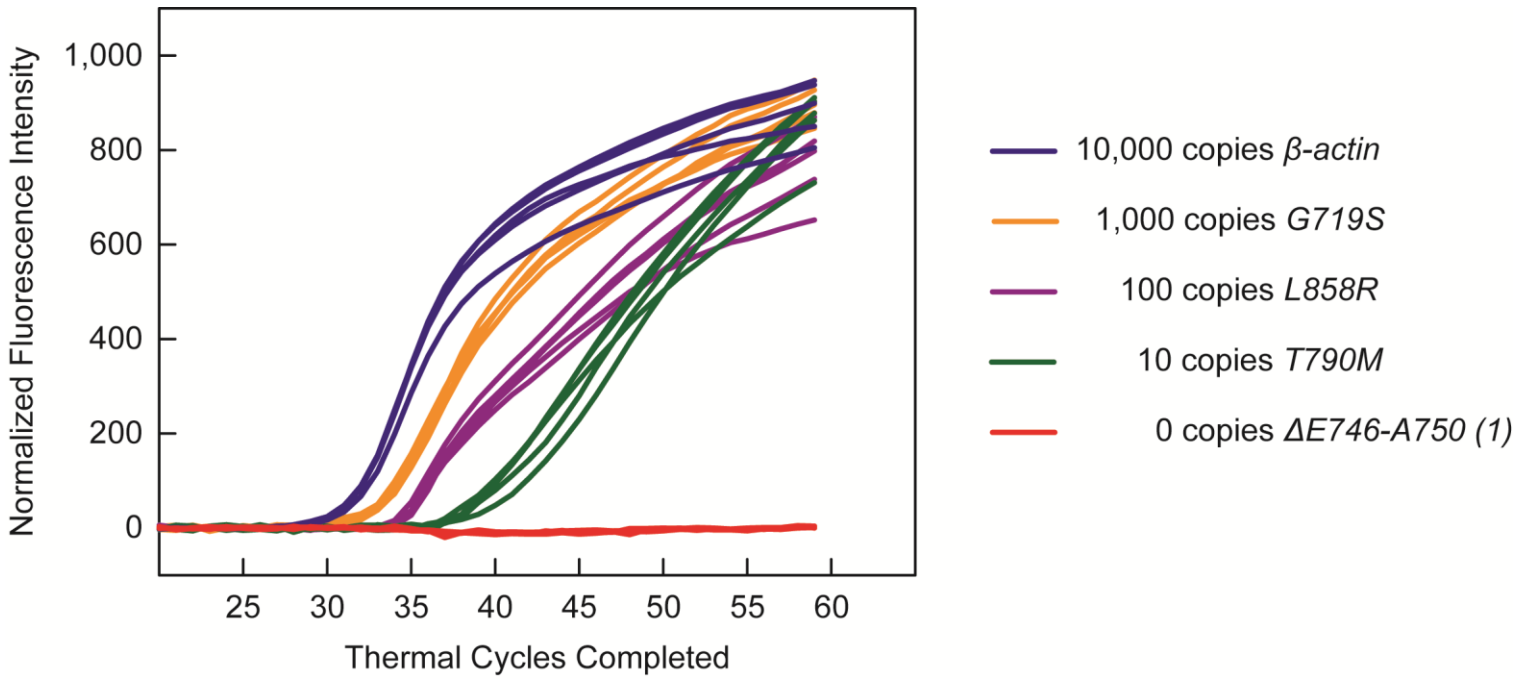
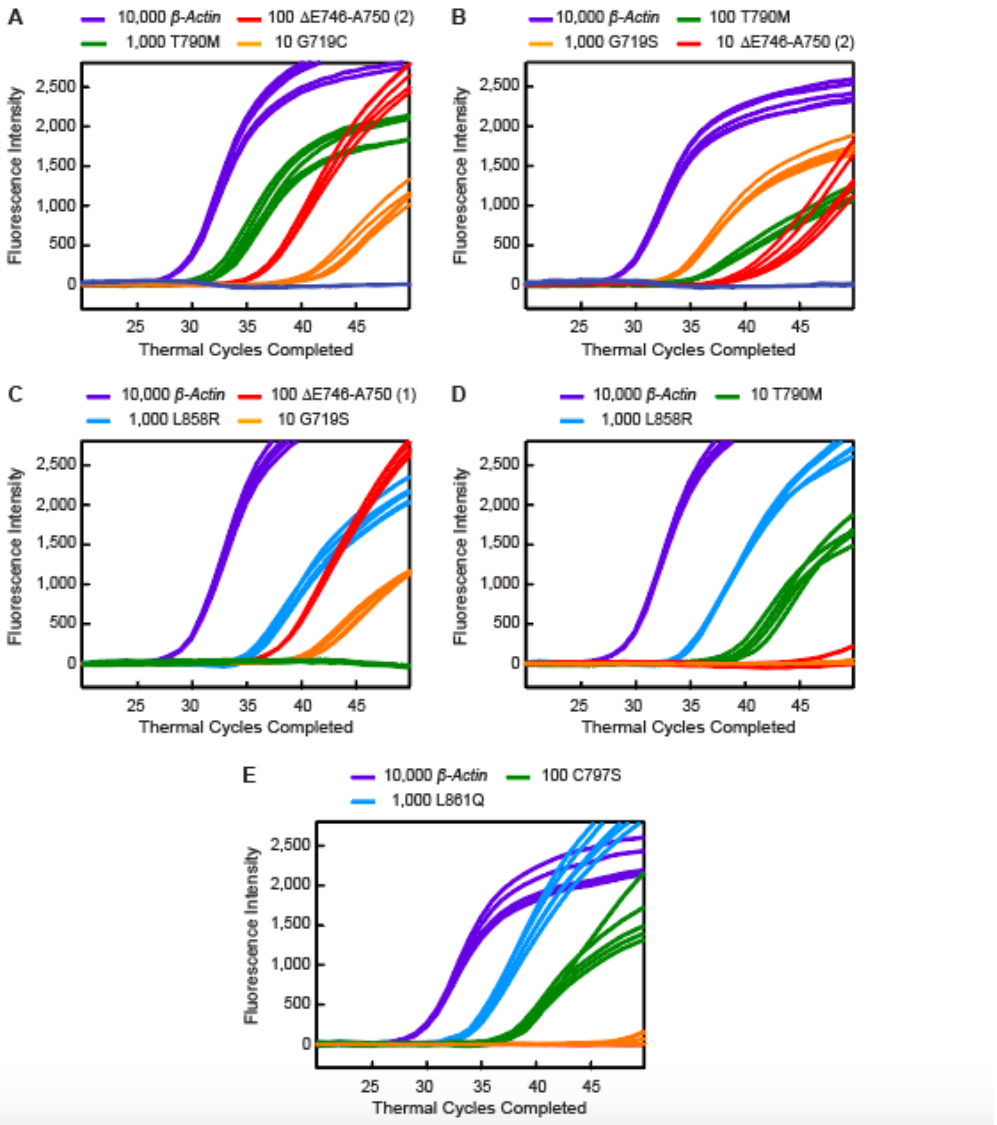
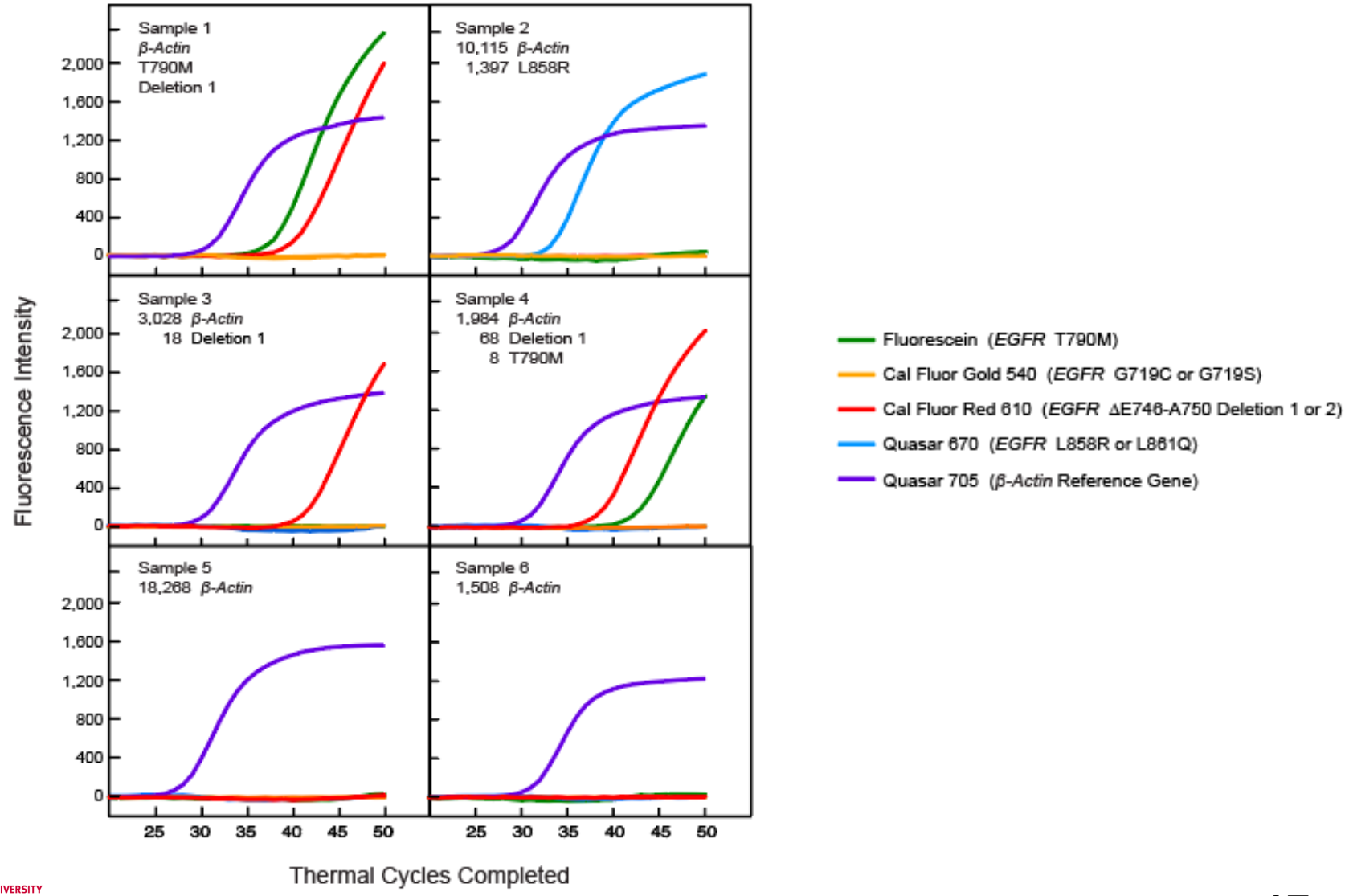
Five Repetitions of a Multiplex Real-time SuperSelective PCR Assay of a Sample Containing Different *EGFR* Mutations



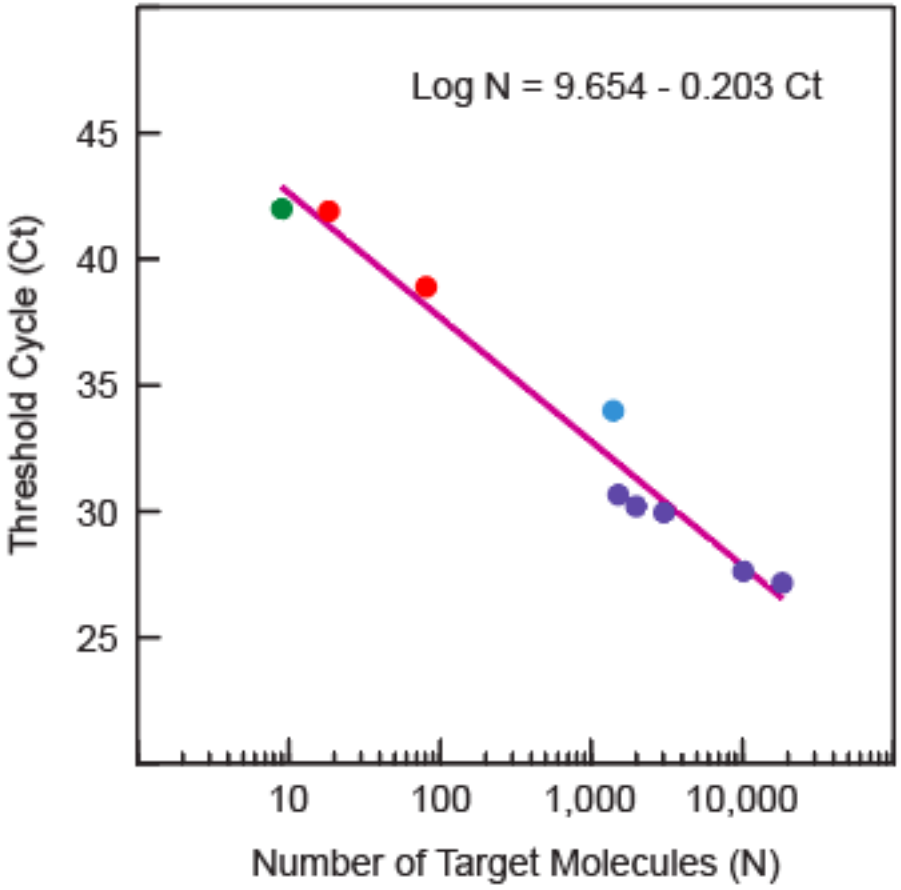
Examples of Multiplex Real-time SuperSelective PCR Assays for the Detection and Quantification of Somatic Mutations in the Human *EGFR* gene



Multiplex real-time PCR assays utilizing cell-free DNA fragments isolated from the plasma in liquid biopsy samples obtained from patients with non-small cell lung cancer



Plot of the threshold cycle obtained for each detected target sequence verses the logarithm of the number template fragments present in the sample added to initiate each multiplex PCR assay

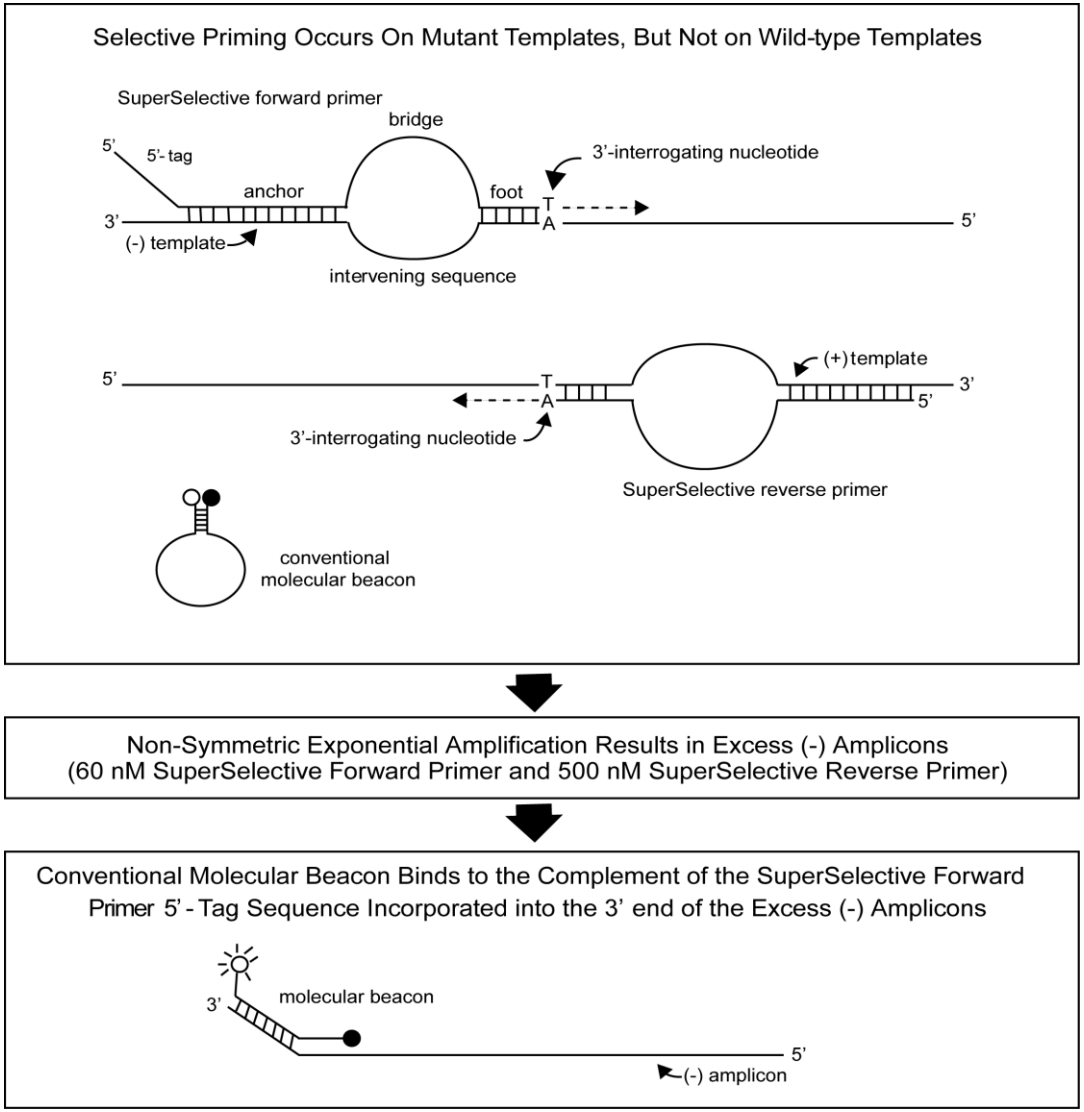


Vargas, Tyagi, Marras, Moerzinger, Abin-Carriquiry, Cuello, Rodriguez, Martinez, Makhnin, Farina, Patel, Chuang, Li, and Kramer (2022)

Multiplex SuperSelective PCR Assays for the Detection and Quantitation of Rare Somatic Mutations in Liquid Biopsies

Journal of Molecular Diagnostics 24, 189-204

SuperSelective Primer Pair for the Selective Amplification of *EGFR* G719C Mutant DNA Fragments in the Presence of Abundant Normal Human Genomic DNA Fragments



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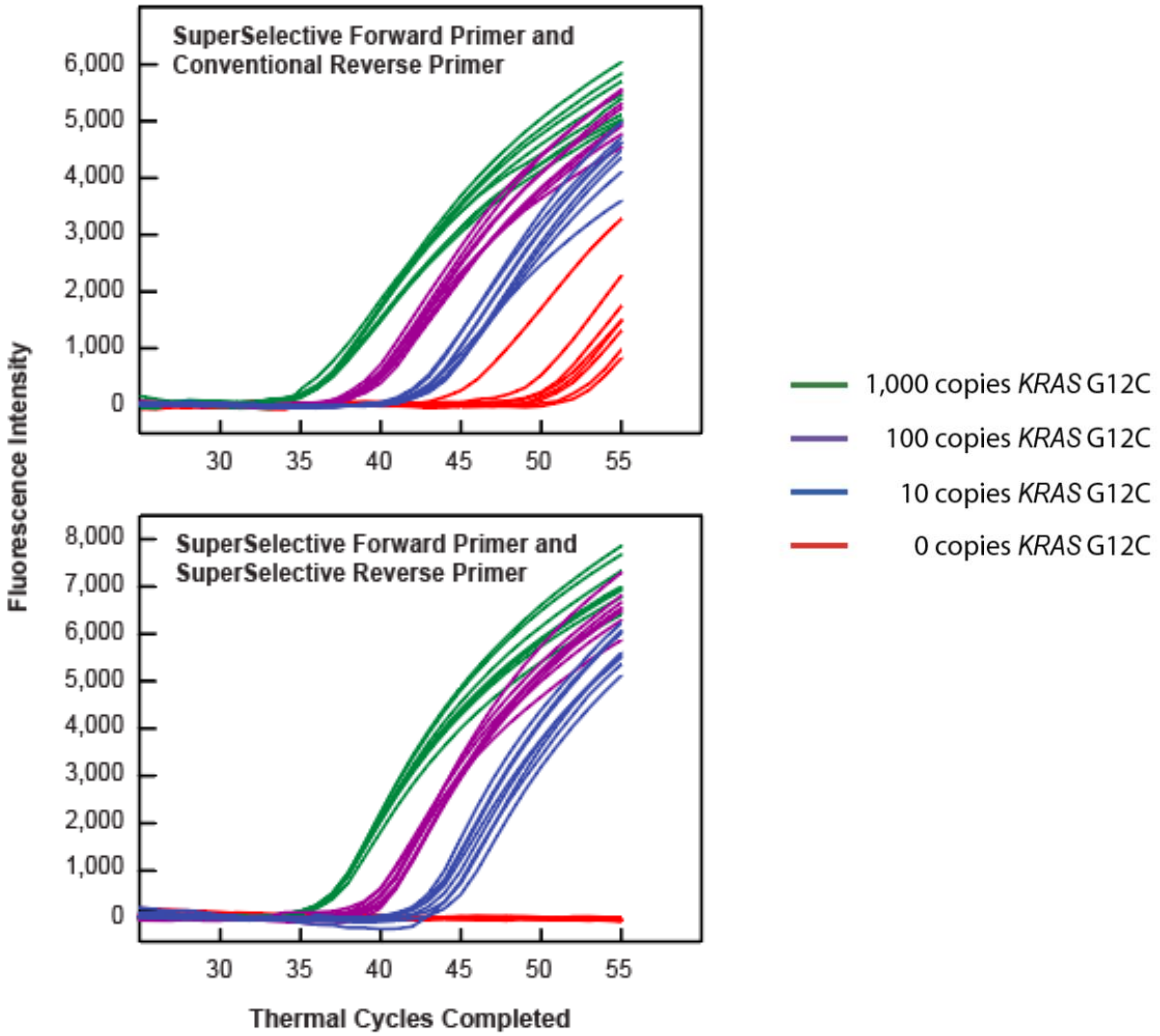
Demonstration of the Selectivity and Sensitivity of an Assay that Utilizes a Pair of SuperSelective PCR Primers for the Amplification of Rare *KRAS* G12C Mutant DNA Fragments in the Presence of DNA Fragments from 10,000 Normal Human Genomes



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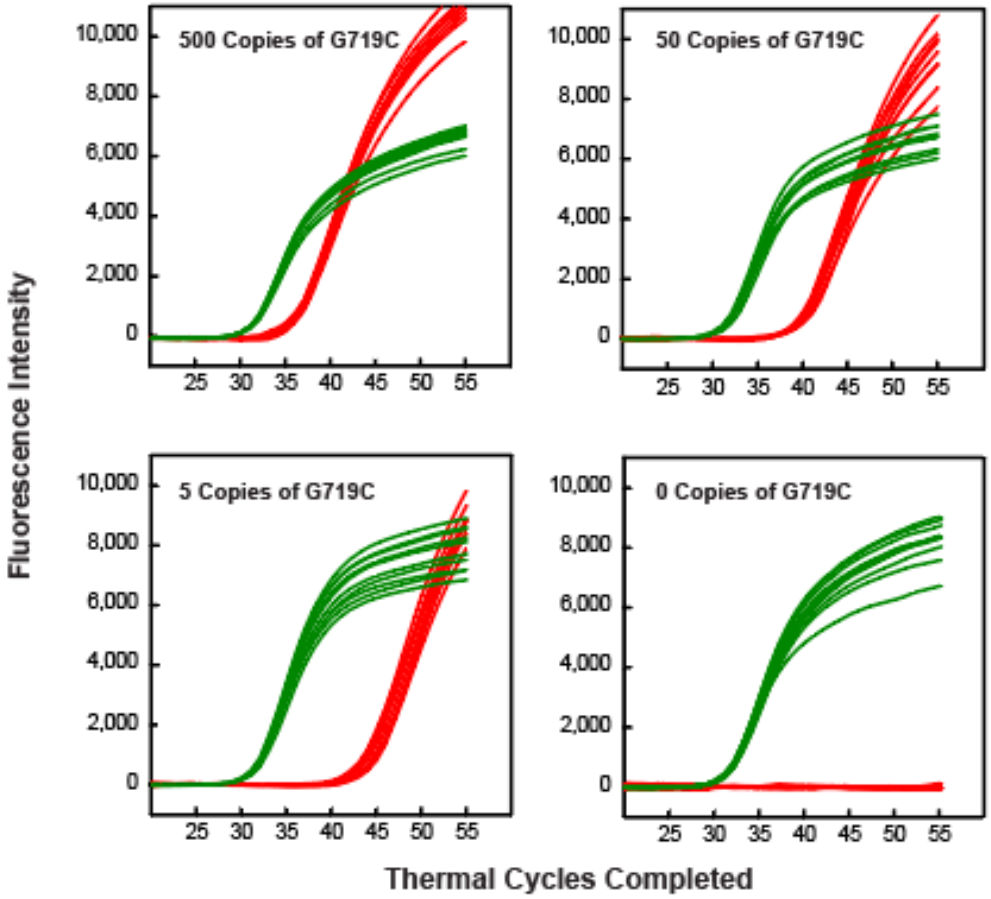
Demonstration of the Selectivity and Sensitivity of an Assay that Utilizes a Pair of SuperSelective PCR Primers for the Amplification of Rare Mutant DNA Fragments in the Presence of Abundant Human Genome Fragments

All Reactions Contained DNA Fragments from the Entire Human Genome, Including 10,000 Copies of the *β-actin* Reference Gene (Green Lines), 10,000 copies of the Wild-type *EGFR* Gene, and Different Quantities of a Plasmid Containing the *EGFR* G719C Mutant Sequence (Red Lines)



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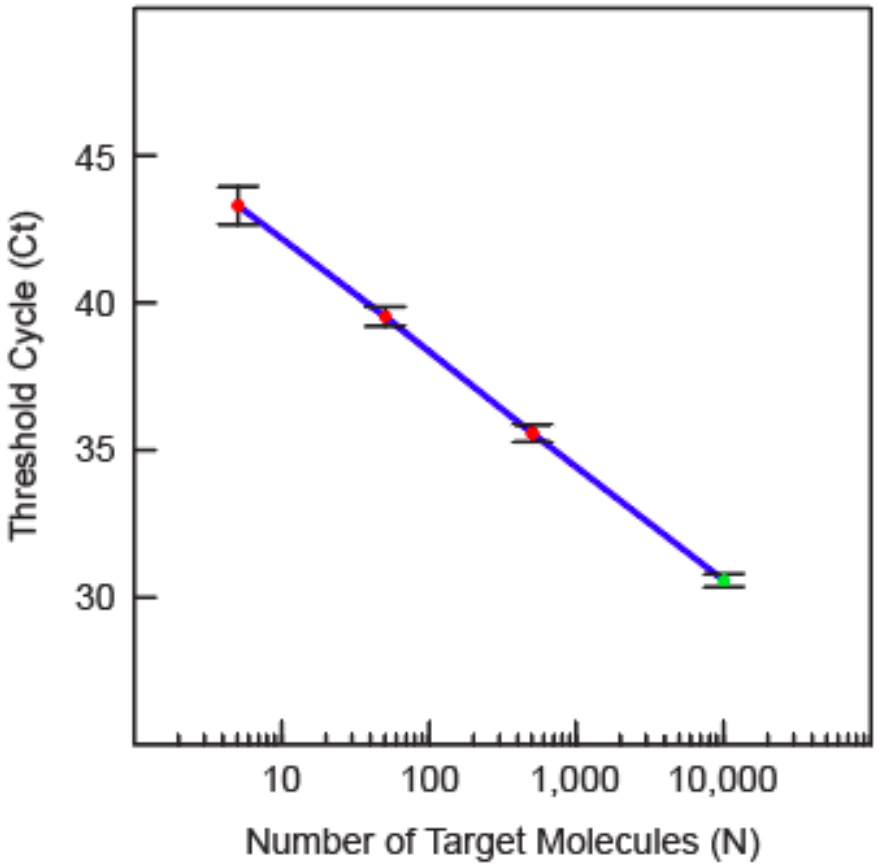
Inverse linear relationship between the mean Ct value of each set of *EGFR* G719C mutant DNA fragments (red dots) and the logarithm of the amount of those target DNA molecules present in each sample, including the mean Ct value of the *β-actin* reference gene fragments (green dot) contained in the 10,000 copies of the entire human genome



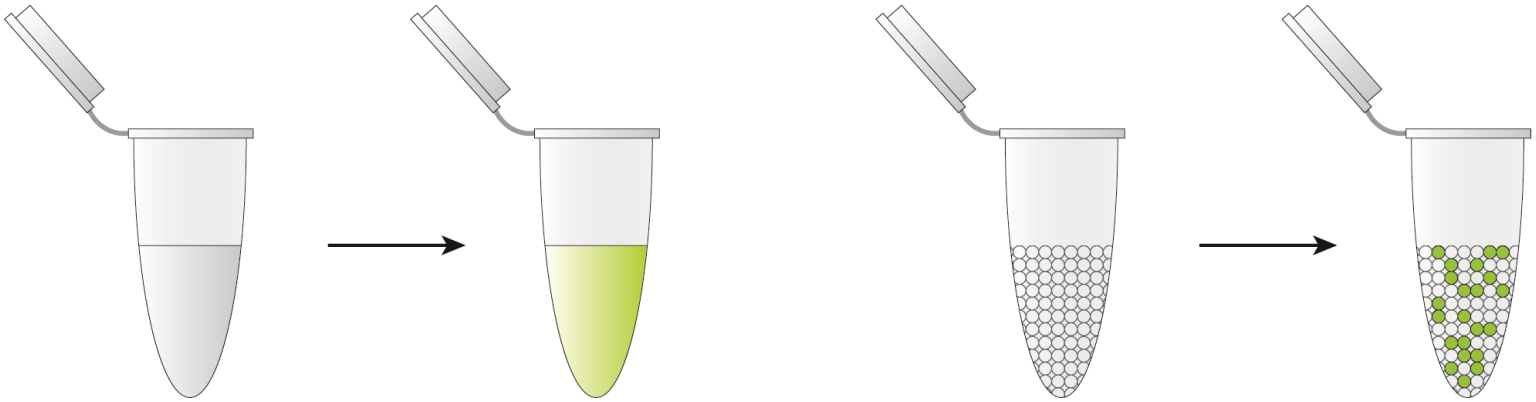
Kramer and Vargas (2021)

SuperSelective Primer Pairs for Sensitive Detection of Rare Somatic Mutations

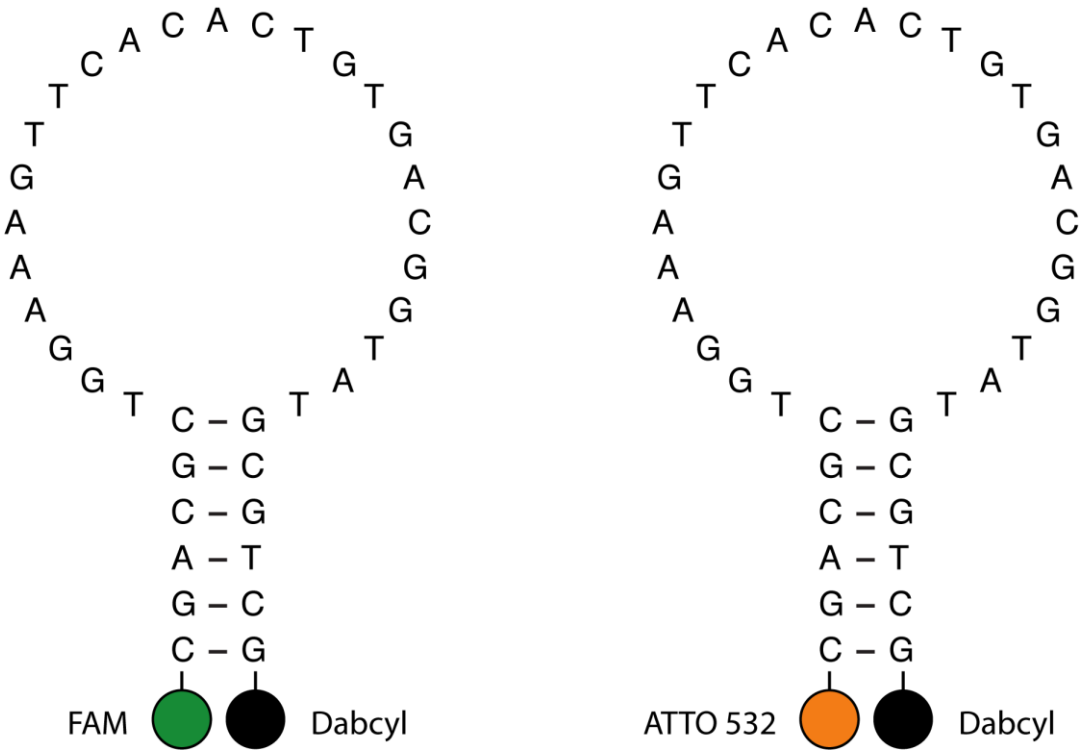
Nature Scientific Reports 2021, 11:22384

Conventional PCR

Digital PCR



Color-coded Molecular Beacons for the 16S Ribosomal RNA Gene of *Streptococcus pneumonia*

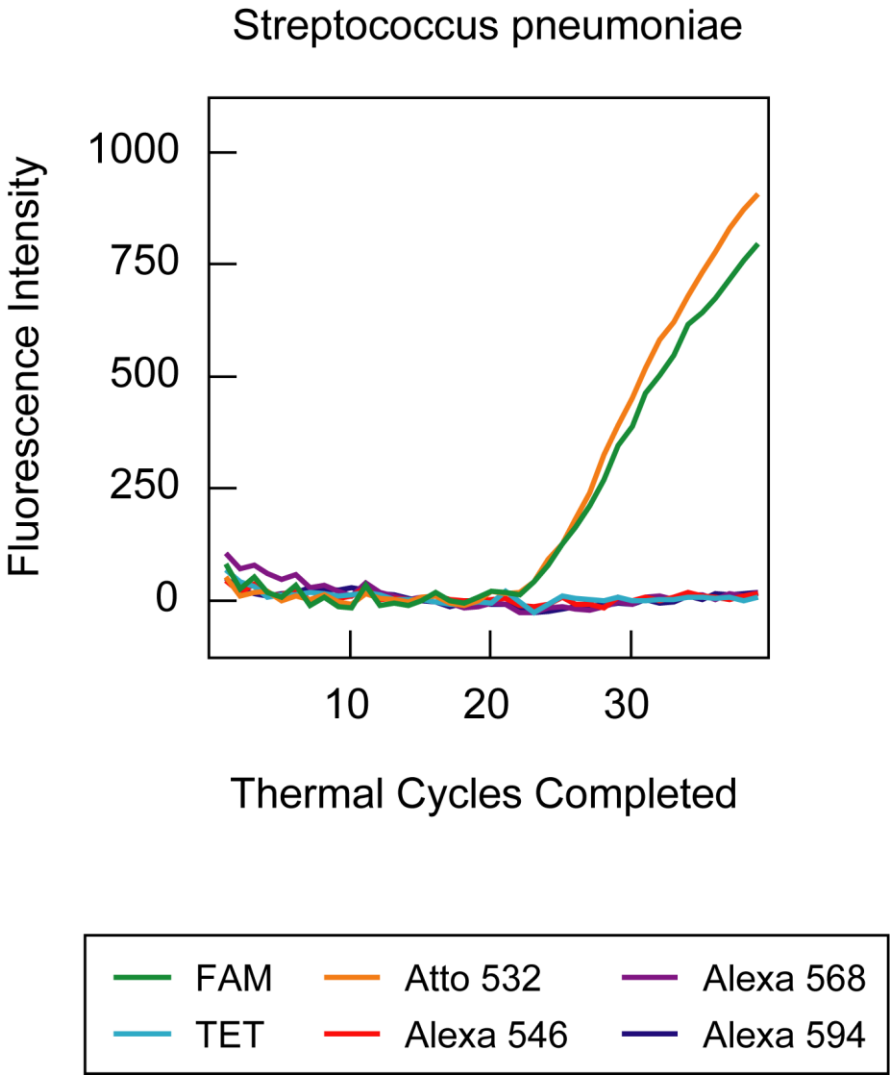


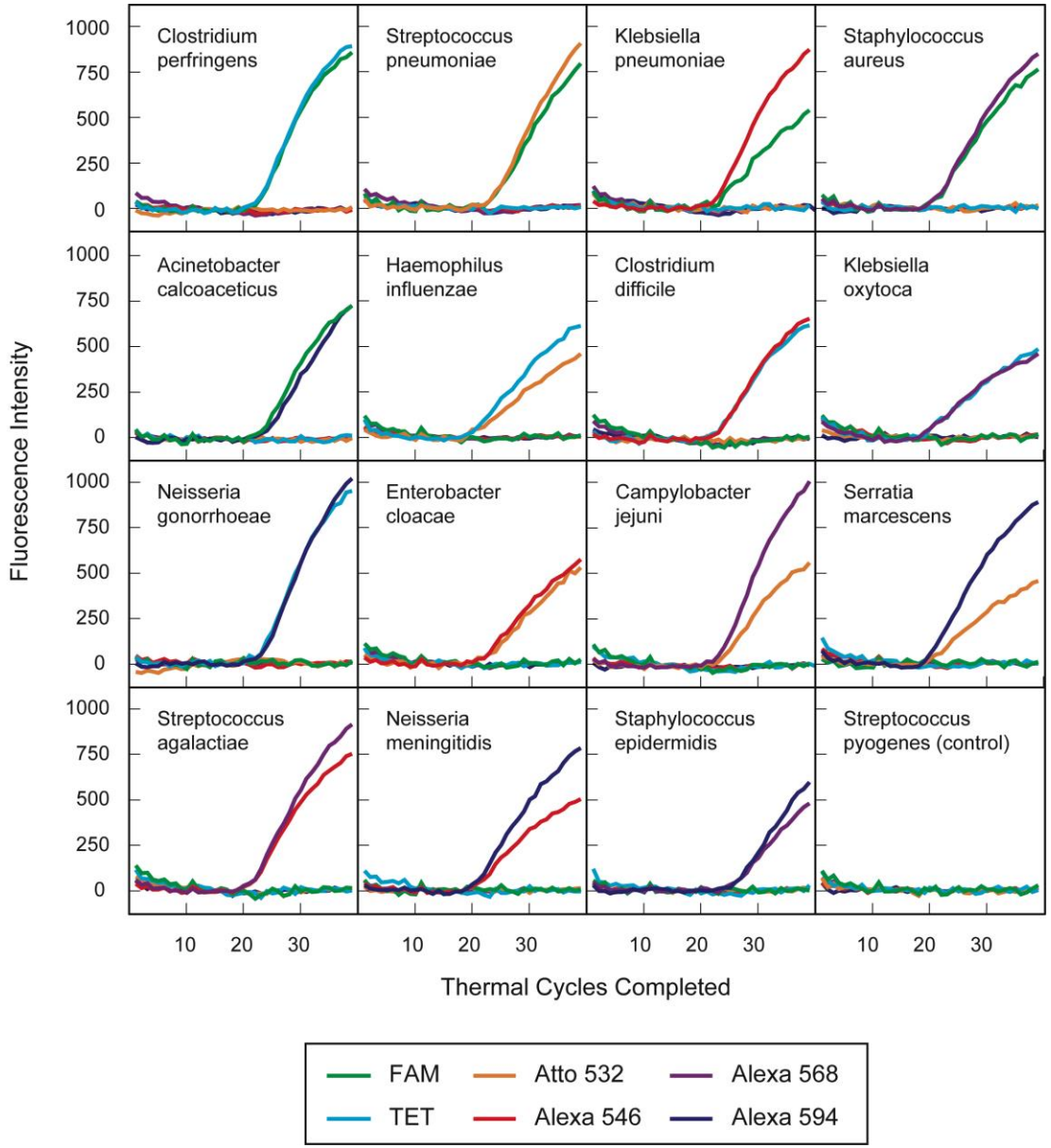
Color Duplex Coding

Bacterial Species	FAM	TET	Atto 532	Alexa 546	Alexa 568	Alexa 594
Clostridium perfringens	●	●				
Streptococcus pneumoniae	●		●			
Klebsiella pneumoniae	●			●		
Staphylococcus aureus	●				●	
Acinetobacter calcoaceticus	●					●
Haemophilus influenzae		●	●			
Clostridium difficile		●		●		
Klebsiella oxytoca		●			●	
Neisseria gonorrhoeae		●				●
Enterobacter cloacae			●	●		
Campylobacter jejuni			●		●	
Serratia marcescens			●			●
Streptococcus agalactiae				●	●	
Neisseria meningitidis				●		●
Staphylococcus epidermidis					●	●

Duplex-coded molecular beacons

Species	Sequence (5' → 3')
Clostridium perfringens	FAM–CGACGC–TCTTTGGGGAAGATAATGACGGT–GCGTCG–Dabcyl TET–CGACGC–TCTTTGGGGAAGATAATGACGGT–GCGTCG–Dabcyl
Streptococcus pneumoniae	FAM–CGACGC–TGGAAAGTTCACACTGTGACGGTAT–GCGTCG–Dabcyl Atto-532–CGACGC–TGGAAAGTTCACACTGTGACGGTAT–GCGTCG–Dabcyl
Klebsiella pneumoniae	FAM–CGCAGC–AGGAAGGCGGTGAGGTTAATA–GCTGCG–Dabcyl Alexa-546–CGCAG--AGGAAGGCGGTGAGGTTAATA--CTGCG–BHQ-2
Staphylococcus aureus	FAM–CGCAGC–AGTAACTGTGCACATCTTGACG–GCTGCG–Dabcyl Alexa-568–CGCAG--AGTAACTGTGCACATCTTGACG--CTGCG–BHQ-2
Acinetobacter calcoaceticus	FAM–CGACGC–GAGGAGGAGGCTACTGAAGTTAATA–GCGTCG–Dabcyl Alexa-594–CGCTG----GGAGGAGGCTACTGAAGTTAATA--CAGCG–BHQ-2
Haemophilus influenzae	TET–CGACGC–AGGAAGGTTGATGTGTTAATAGTA–GCGTCG–Dabcyl Atto-532–CGACGC–AGGAAGGTTGATGTGTTAATAGTA–GCGTCG–Dabcyl
Clostridium difficile	TET–CGCACG–ACTCTGTCCTCAAGGAAGATAATG–CGTGCG–Dabcyl Alexa-546–CGCAC--ACTCTGTCCTCAAGGAAGATAATG--GTGCG–BHQ-2
Klebsiella oxytoca	TET–CGCTGC–AGGTTAATAACCTCAGCAATTG–GCAGCG–Dabcyl Alexa-568–CCGCG--AGGTTAATAACCTCAGCAATTG--CGCGG–BHQ-2
Neisseria gonorrhoeae	TET–CGACGC–GAAGAAAAGGCCGTTGCCAATATCG–GCGTCG–Dabcyl Alexa-594–CGCAG---AAGAAAAGGCCGTTGCCAATATCG--CTGCG–BHQ-2
Enterobacter cloacae	Atto-532–CGCAGC–GGAGGAAGGTGTTGTGG–GCTGCG–Dabcyl Alexa-546–CGACG---GAGGAAGGTGTTGTGG--CGTCG–BHQ-2
Campylobacter jejuni	Atto-532–CGCAGC–GCGTGGAGGATGACACTTTTCGGAG–GCTGCG–Dabcyl Alexa-568–CGCAG--GCGTGGAGGATGACACTTTTCGGAG--CTGCG–BHQ-2
Serratia marcescens	Atto-532–CGCAGC–CTTAATACGTTCATCAATTGACGTT–GCTGCG–Dabcyl Alexa-594–CGACG--CTTAATACGTTCATCAATTGACGTT--CGTCG–BHQ-2
Streptococcus agalactiae	Alexa-546–CGCAG–CGTTGGTAGGAGTGGAAAATCTA–CTGCG–BHQ-2 Alexa-568–CGCAG–CGTTGGTAGGAGTGGAAAATCTA–CTGCG–BHQ-2
Neisseria meningitidis	Alexa-546–CGACG–AAGAAAAGGCTGTTGCTAATATCA–CGTCG–BHQ-2 Alexa-594–CGACG–AAGAAAAGGCTGTTGCTAATATCA–CGTCG–BHQ-2
Staphylococcus epidermidis	Alexa-568–CGCAG–AGAACAAATGTGTAAGTAACTATG–CTGCG–BHQ-2 Alexa-594–CGCAG–AGAACAAATGTGTAAGTAACTATG–CTGCG–BHQ-2


Streptococcus pneumoniae
Fluorescence Intensity
1000
750
500
250
0
10
20
30
Thermal Cycles Completed
FAM
TET
Atto 532
Alexa 546
Alexa 568
Alexa 594



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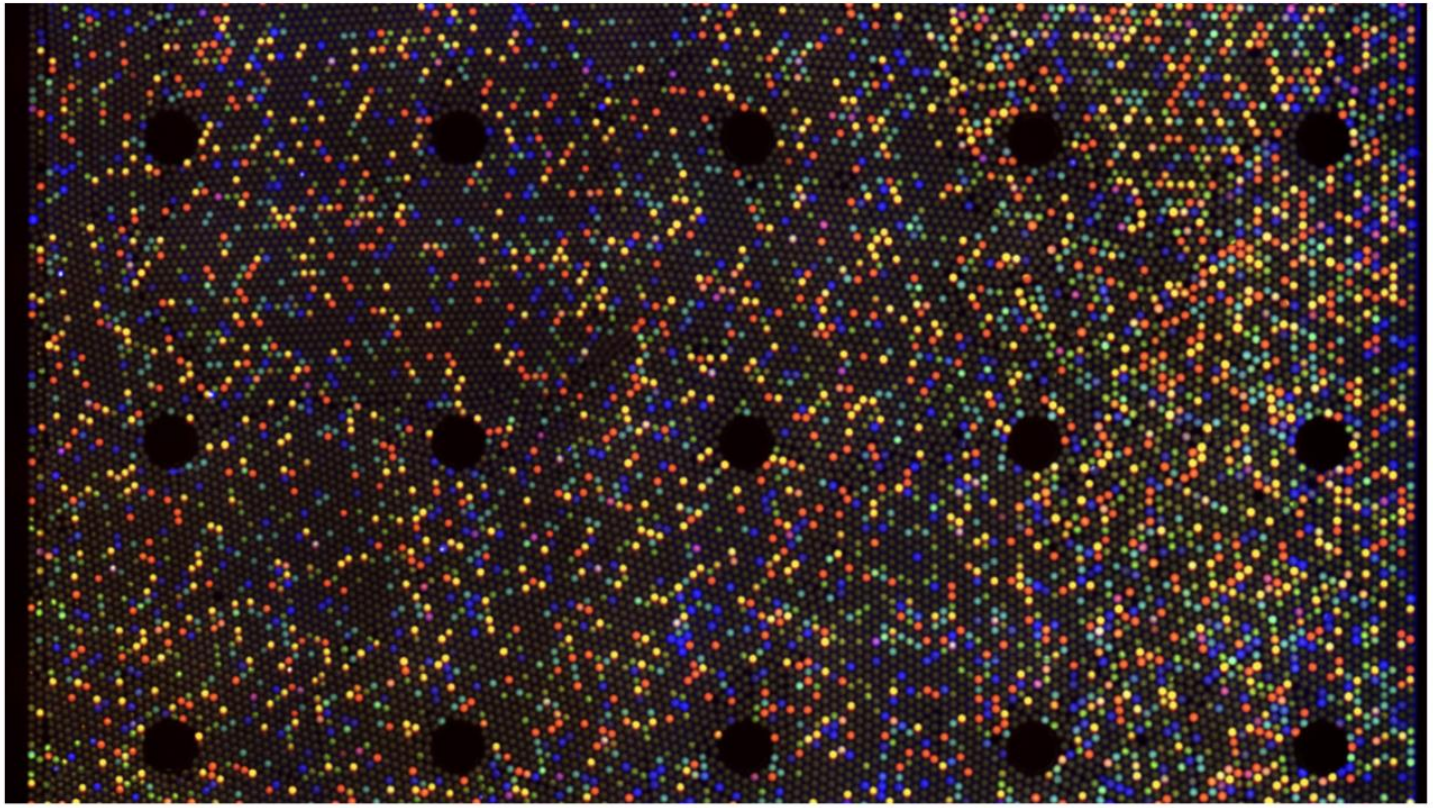
Marras, Tyagi, Antson, and Kramer (2019)

Color-Coded Molecular Beacons for Multiplex PCR Screening Assays

PLoS ONE 14, e0213906

Multicolor Digital PCR



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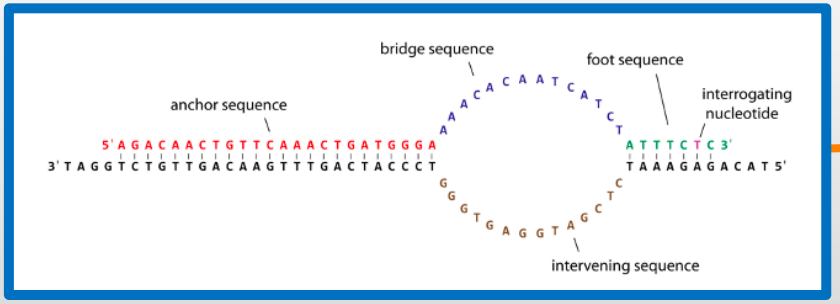
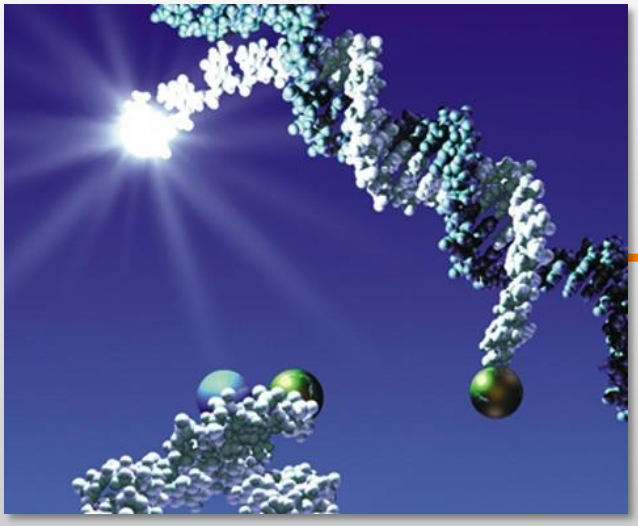


Diana Vargas

Acknowledgements



Salvatore Marras

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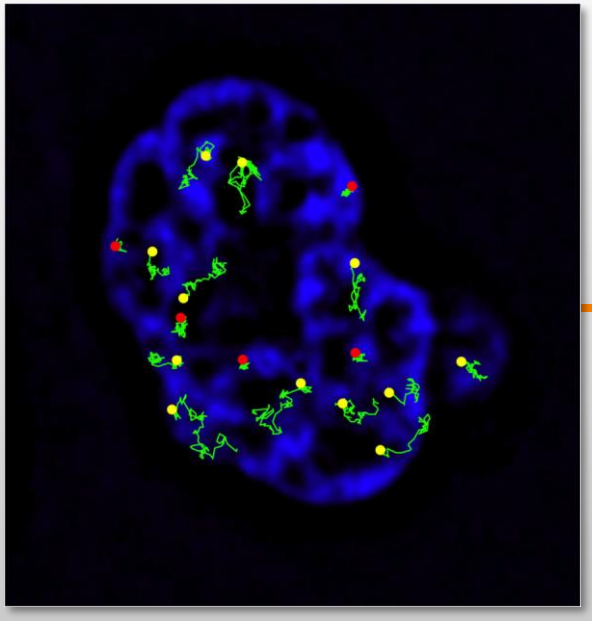

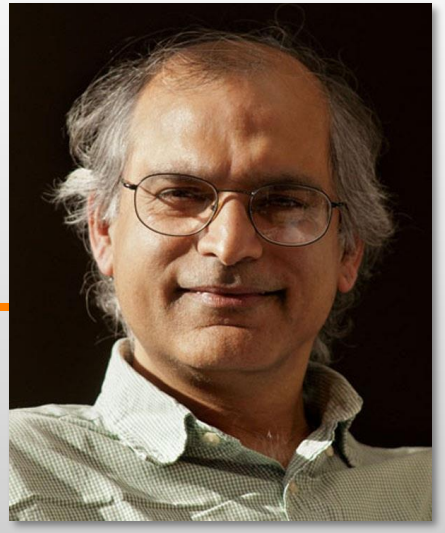

Sanjay Tyagi