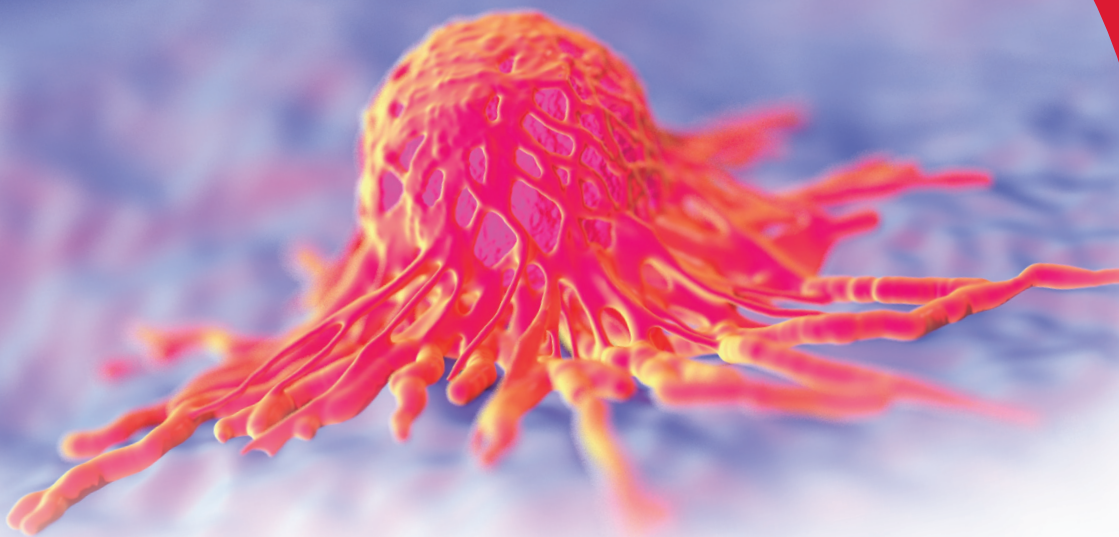


PointMan



# PointMan™ NRAS DNA Enrichment Kit

Instructions for use of PointMan™ NRAS  
DNA enrichment kit

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## Section 1 Kit Contents

### Kit Contents: PointMan NRAS Enrichment Kit

The PointMan NRAS kit contains 3 separate primer mixes and control primers. Please read tube contents carefully before preparing individual reactions.

- PointMan NRAS codon 12 primer mix (24 reactions BLACK)
- PointMan NRAS codon 13 primer mix (24 reactions BLACK)
- PointMan NRAS codon 61 primer mix (24 reactions BLACK)
- Control primer mix NRAS codon 12 (24 reactions WHITE)
- Control primer mix NRAS codon 13 (24 reactions WHITE)
- Control primer mix NRAS codon 61 (24 reactions WHITE)
- PointMan NRAS Mastermix with SYBRgreen (225 reactions GREEN)
- Sequencing primer forward NRAS codon 12 (100 reactions YELLOW)
- Sequencing primer reverse NRAS codon 12 (100 reactions YELLOW)
- Sequencing primer forward NRAS codon 13 (100 reactions YELLOW)
- Sequencing primer reverse NRAS codon 13 (100 reactions YELLOW)
- Sequencing primer forward NRAS codon 61 (100 reactions YELLOW)
- Sequencing primer reverse NRAS codon 61 (100 reactions YELLOW)
- 1% Positive control NRAS codon 12 (8 reactions RED)
- 1% Positive control NRAS codon 13 (8 reactions RED)
- 1% Positive control NRAS codon 61 (8 reactions RED)
- RNase/DNase free water (CLEAR)

## Section 2 Shipping and Storage

The PointMan NRAS kit is shipped frozen and must still be frozen upon arrival. If the PointMan NRAS kit is not frozen upon arrival, the outer packing has been opened during transit, the shipment does not contain a packing note, instruction booklet or the reagents please contact the EKF Molecular technical services department ([info@ekfmolecular.com](mailto:info@ekfmolecular.com)). The PointMan NRAS kit should be stored at -15°C to -25°C and protected from sunlight.

When stored under the recommended storage conditions in the original packaging, the PointMan NRAS kit is stable for 6 months from the date of purchase. Repeated thawing and freezing should be avoided. We recommend a maximum of 7 freeze-thaw cycles.

### **Section 3** Product Use Limitations

EKF Molecular Diagnostics sell products solely for use in laboratory research to gain information for use by the purchaser (the “Permitted Use”). By purchasing a PointMan enrichment kit, purchasers undertake that they are purchasing for the Permitted Use only and that purchasers will not use products for any other use, including without limitation: diagnostics for medical purposes or commercial purposes and will not resell any products. Products are covered by patent applications owned by or licenced to EKF Molecular Diagnostics (please see [www.ekfmolecular.com](http://www.ekfmolecular.com)). By purchasing any PointMan enrichment kit, purchasers acquire a non-exclusive licence to use the product for the Permitted Use only. EKF Molecular Diagnostics do not warrant that any use of a product will not infringe any patent or any other intellectual property rights whatsoever of any third party. Polymerase Chain Reaction (PCR) is covered by several patents owned by Hoffman-Roche Inc and Hoffman-LaRoche, Ltd. Purchase of EKF Molecular Diagnostics kits does not include or provide any licence with respect to any patents owned by Hoffman-La Roche or others.

### **Section 4** Product Warranty and Guarantee

We warrant to you that any product purchased from us will on delivery: conform in all material respects to its specification; be of satisfactory quality (within the meaning of the Sale of Goods Act 1979, as amended) and fit for the purpose held out by us; be free from material defects in design, material and workmanship and; comply with all applicable statutory and regulatory requirements.

You may reject any product which does not comply with the above; in the case of a defect that is apparent on normal visual inspection, within five business days of delivery and in the case of a latent defect, within a reasonable time of the latent defect having become apparent. If you reject a product described as above you may either require us to replace the rejected product or require us to repay the price of the rejected goods in full, following return of the goods.

## Section 5 Quality Control

As part of our routine quality assurance program, all EKF Molecular Diagnostics products are manufactured to ISO9001 standards and monitored to ensure the highest levels of performance and reliability. products are manufactured to ISO9001 standards and monitored to ensure the highest levels of performance and reliability.

## Section 6 Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles. All chemicals and biological material must be considered as potentially hazardous. Specimens are potentially infectious and must be treated accordingly.

Discard sample and assay waste in accordance with your local safety regulations.

## Section 7 Introduction to PointMan

There is an unmet clinical need for the accurate detection and genotyping of rare somatic mutations. The PointMan system is a novel method for selective amplification of genotype specific sequences. Unlike allele specific priming, PointMan does not, in the first instance, achieve results by attempting to selectively prime and amplify mutant sequences. Such methods are 'leaky', need extensive optimisation and have limited resolution. By contrast, PointMan uses allele specific primers for priming on the wild-type (WT) sequence and this then drives those WT templates into terminally extended products that are not available for exponential amplification. The variant sequences are uninhibited and therefore free to undergo exponential amplification and enrichment.

The system uses 4 primers in total (fig.1). The internal primers confer the selective power of the assay and are called the enriching primers. The external primers are unmodified and are simply used to PCR amplify across the region of interest (amplifying primers).

## Section 7 Introduction to PointMan

The enriching primers have the 3' terminal residue situated over the SNP and this residue is homologous with the WT sequence. A second important feature of the enriching primers is that they also contain a PCR blocking residue. Whilst this does not prevent binding of the primer and extension on the genomic DNA template, it does prevent the enriching primers from participating in the second and all subsequent rounds of PCR amplification (fig.1). The single stranded template made by the enriching primer on the WT sequences blocks the extension of the full length amplicon from the amplifying primer. In this way, exponential amplification on WT sequences is terminated.

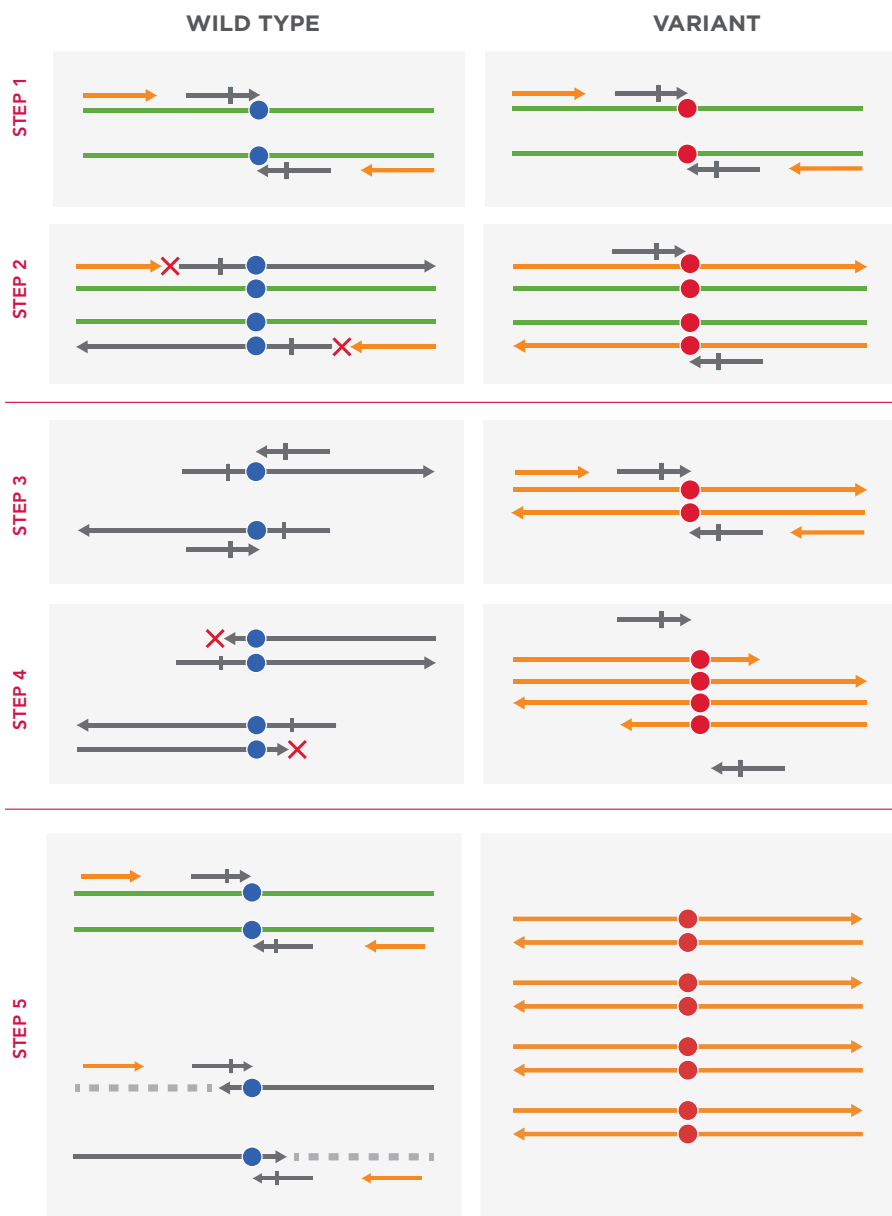
On variant sequences, the enriching primer does not extend as it is displaced by the enzyme during the extension step. This allows for full exponential amplification across the variant region (fig.1).

The PointMan NRAS kit will exponentially amplify deletions and alteration in the sequences highlighted in red below. For a full list of the mutations enriched please visit: [www.ekfmolecular.com](http://www.ekfmolecular.com)

Codon 12      3' GTT GGA GCT GGT **GGC** GTA GGC AAG AGT 5'

Codon 13      3 GTT GGA GCT GGT GGC **GTA** GGC AAG AGT 5'

Codon 61      3'GAC ACA GCA GGT **CAA** GAG GAG TAC AGT 5'



**Figure 1: Figure 1: PointMan amplifies the SNP sequence but blocks amplification of the WT sequence**



**Step 1** Enriching primers (—→) with blocking moiety (✗) complimentary to the wild-type sequence and a mismatch at the 3' end to the variant (mutant) sequence (the base of interest is shown as: wild-type – blue oval; variant - red oval). Amplifying primers (—→) complimentary to sequences flanking the region of interest.

**Steps 2 Wild-Type** Enriching primers anneal to wild-type with high avidity due to match at 3' end; Extension from enriching primers occurs, further increasing avidity. Amplifying primers anneal and extend, but extension is terminated by presence of the extending enriching primer.

**Variant.** Enriching primers do not efficiently anneal and extend due to mismatch at the 3' end and stringency of PCR conditions. Amplifying primers therefore extend right through the region of interest, without hindrance.

**Step 3 & 4 Wild Type** In the subsequent cycle, only the product of extension from the enriching primers provides priming sites for further replication, but extension is terminated by the blocking moieties, and therefore the product does not itself contain any priming sites. Therefore no further replication is possible in subsequent cycles, and the reaction is driven into linear amplification based only on the original sample material.

**Variant.** In subsequent cycles, stemming from the full replicons unencumbered by blocking moieties, exponential amplification proceeds in the usual way.

Note that there is no risk of false positives from mispriming: no extraneous source of variant sequence is introduced in the form of primer-dimers (unlike allele specific PCR and its variants)

## Section 8 Equipment and Reagents to be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

- DNA sample (see below)
- Dedicated pipettes (adjustable) for PCR master mix preparation\*
- Dedicated pipettes (adjustable) for dispensing of DNA\* sample
- Sterile pipette tips with filters
- Benchtop centrifuge with rotor for 1.5 ml reaction tubes
- Real-time PCR instrument
- Sterile microcentrifuge tubes for preparing master mixes

## Section 9 Suitable Sample Material

PointMan will work with any high quality source of extracted human DNA and is therefore compatible with a wide range of extraction protocols. DNA samples should be quality checked and quantified using a UV spectrophotometer or similar approach.

## General precautions

The user should always pay attention to the following:

Use sterile pipette tips with filters and make sure that pipettes have been calibrated according to the manufacturer's instructions.

Store and extract positive materials (samples and positive controls) separately from all other reagents and add them to the reaction mix in a spatially separated facility or within a laminar flow cabinet.

Thaw all components thoroughly on ice before starting an assay.

When thawed, mix the components by inverting each tube 10 times and centrifuge briefly. Do not vortex the mastermix as this may inactivate the Taq DNA polymerase.

Use extreme caution to prevent contamination of PCRs with synthetic control material. We recommend using separate, dedicated pipettes for setting up reaction mixes and adding DNA template. Preparation and dispensing of reaction mixes must be carried out in a separate area to the addition of sample.

Reagents for the PointMan EGFR kit have been optimally diluted. EKF Molecular Diagnostics do not recommend further dilution of reagents as this may result in a loss of performance. We do not recommend using reaction volumes of less than 15 µl as this may increase the risk of false negatives.

All reagents in the PointMan EGFR Kit are formulated specifically for use with the stated tests. All reagents supplied in the PointMan EGFR Kit are intended to be used solely with the other reagents in the same Kit. Substitutions to the reagents in the kit must not be made if optimal performance is to be maintained.

Only use the Taq DNA polymerase that is provided in the kit. Do not substitute with Taq DNA polymerase from other kits of the same or any other type, or with Taq DNA polymerase from another supplier.

\* Ensure that all instruments have been checked and calibrated according to the manufacturer's recommendations.

## Section 10 Principles of the Test

### PointMan Primer Mix (Black)

This primer mix contains the 4 primers required for a PointMan reaction. When used in conjunction with the PointMan™ Mastermix and user supplied DNA sample, this reaction will selectively enrich the PCR reaction for variant sequences, if present. Following PointMan reaction completion, the enriched product can be sequenced to confirm if the variant sequence is present in the tissue sample.

### Control Primer Mix (White)

The PointMan reaction blocks amplification of the wide type (WT) sequence, thus it follows that clinical samples which contain only the WT sequence may not produce any amplification product. Experimentally, it has been demonstrated that if the sample contains less than 1000 copies of WT sequence, no PCR product will accumulate. For this reason it is essential to confirm that the sample has been extracted correctly and is competent to support PointMan PCR amplification.

The control primer mix contains only the amplifying primers without the enriching primers. This mix will therefore produce a post reaction product on both WT and variant sequences. The control primer mix is used to confirm that a biological sample is competent for PCR and therefore valid for PointMan analysis. A positive signal from the control assay primers is essential to enable validation of negative results.

### PointMan Mastermix with SYBRgreen (Green)

The PointMan Mastermix is specifically formulated to provide optimal blocking of amplification on WT sequences whilst allowing efficient amplification of variant sequences. Consistent with the PointMan process outlined above, the enzyme is exonuclease deficient. The mastermix also contains SYBRgreen. This allows the monitoring of product accumulation in real time and also permits melting curve analysis to ensure that a single amplicon, corresponding to EGFR, has been amplified. Analysis of the SYBRgreen data following PointMan enrichment enables the user to validate that PointMan™ has produced a PCR product prior to sample sequencing. The PointMan Mastermix cannot be substituted for another enzyme and buffer system as this will greatly affect the sensitivity of the enrichment.

### PointMan Sequencing Primers (Yellow)

To confirm the presence of variant sequences following PointMan™ enrichment, samples can be sequenced using the sequence primers provided to prime the reaction. The sequencing primer is unlabelled and is supplied lyophilized.

## Section 11 Bench-side Protocol

To minimise the risk of contamination with foreign DNA, EKF Molecular Diagnostics recommend that all pipetting be performed in a PCR clean environment. Ideally this would be a designated PCR cabinet. Filter tips are recommended for all pipetting steps.

### 1. Pulse-spin each tube in a centrifuge before opening.

This ensures all products are situated in the base of the tube and prevents reagent loss upon opening the tube.

### 2. Resuspend all components according to the table below using RNase/DNase free water supplied.

COMPONENT	VOLUME
PointMan™ primer mix	72 µl
Control primer mix	72 µl
Forward sequencing primer*	110 µl
Reverse sequencing primer*	110 µl
1% positive control **	100 µl

\* 3.2 pmol per µl when resuspended

\*\* Contamination risk - contains high copy number of positive control template

Preparation of a PointMan™ reaction

Please note that the PointMan EGFR enrichment kit contains 3 separate primer mixes and control primers. Please read tube contents carefully before preparing individual reactions.

1. Make up reaction mixes

For each sample make up a reaction mix for both the PointMan™ test and the control well. Remember to include additional reactions for the positive and negative controls.

COMPONENT	1 REACTION
PointMan™ primer mix (BLACK)	2 µl
PointMan™ Mastermix with SYBRgreen (GREEN)	10 µl
RNAse/DNAse free water (CLEAR)	3 µl
Final volume	15 µl
Control primer mix (WHITE)	2 µl
PointMan™ Mastermix with SYBRgreen (GREEN)	10 µl
RNAse/DNAse free water (WHITE)	3 µl
Final volume	15 µl

2. Pipette 15 µl of relevant reaction mix into each well according to your PointMan™ experimental plate set up. e.g.

	SAMPLE 1	SAMPLE 2	SAMPLE 3	SAMPLE 4	SAMPLE 5	POSITIVE CTRL	NTC
PointMan™	●	●	●	●	●	●	●
Control primer	●	●	●	●	●	●	●

3. Add 5 µl of DNA sample to each reaction

Ideally 1-10 ng of high quality DNA should be used. For no template controls (NTC)/ negative control wells replace sample with RNAse/DNAse free water. For the 1% positive control well, use 5 µl of control template (Red).

## Section 12 Amplification Protocol

PointMan™ uses a carefully optimised 4 step cycling parameter. Please follow this protocol exactly using a block based real time PCR machine.

COMPONENT	STEP	TIME	TEMP
	Enzyme Activation (if required)	2 mins	95 °C
50 CYCLES	Denaturation	10 s	95 °C
	Primer Annealing	20 s	50 °C
	Enriching Primer Displacement	1 s	70 °C
	Extension*	30 s	60 °C
MELT CURVE	60-90 °C in 1 °C intervals *		

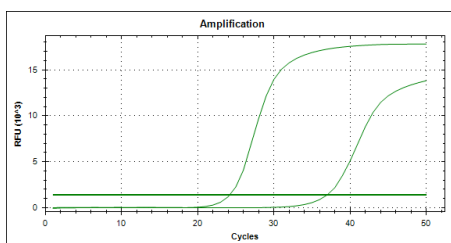
\* Flourogenic data should be collected during these steps through the SYBRgreen/FAM channel

## Section 13 Interpretation of Results

### Real time PCR traces

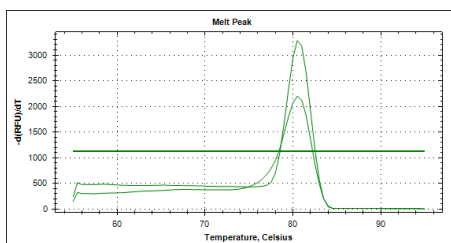
Analysis of the real time PCR data should give amplification plots in the region of CT=25-40. Please note that variant traces will appear before the WT traces due to exponential replication of variant sequence.

### rtPCR Plots for NRAS codon 12



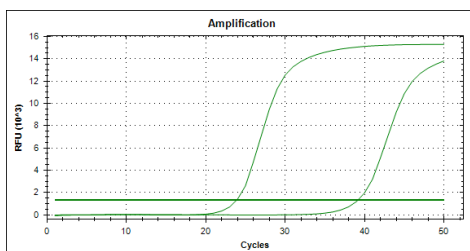
### Melting curve analysis

The melting curve for NRAS codon 12 should give a single peak with a melting temperature of 80°C as shown below.



### rtPCR Plots for NRAS codon 13

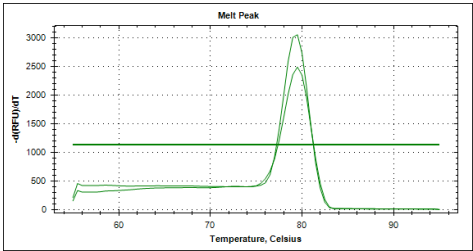
The melting curve should give a single peak with a melting temperature of 88°C as shown above.



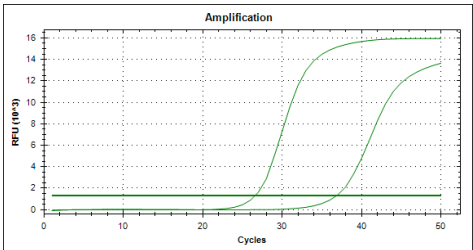


**Melting curve analysis**

The melting curve for NRAS codon 13 should give a single peak with a melting temperature of 80°C as shown below 79°C as shown above.

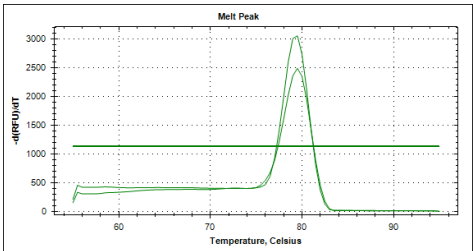


**rtPCR Plots for NRAS codon 61**



**Melting curve analysis**

The melting curve for NRAS codon 61 should give a single peak with a melting temperature of 79°C as shown below



## Section 14 Table 1: Data Interpretation

CONTROL PRIMERS	POINTMAN PRIMER MIX	INTERPRETATION
Amplicon produced	Amplicon produced with melting Tms of 80°C (codon 12 13), 79°C, (codon 61).	Positive for a variant sequence. Confirm by sequence analysis
Amplicon produced	NO Amplicon produced	Reaction successful but no variant sequences amplified. The sample is WT
NO Amplicon produced	NO Amplicon produced	The sample has failed. Either there was insufficient amplifiable or PCR inhibitors have prevented a successful test

### Mutation Details

Numerous mutations and deletions in NRAS exons 12, 13 and 61 are reported, the most common are listed below. For a full list of the possible mutations enriched by this component of the kit, please visit [www.ekfmolecular.com](http://www.ekfmolecular.com)

Cosmic IDs are taken from the catalogue of Somatic Mutations in Cancer ([www.sanger.ac.uk/genetics/CGP/cosmic](http://www.sanger.ac.uk/genetics/CGP/cosmic))

Table 2: List of NRAS mutations and COSMIC IDs.

Mutation	Sequence	Base Change	Cosmic ID
Gly12Ser 3'	GTG GTT GGA GCA <b>AGT</b> GGT GTT GGG AAA 5'	34 G>A	563
Gly12Arg 3'	GTG GTT GGA GCA <b>CGT</b> GGT GTT GGG AAA 5'	34 G>C	561
Gly12Cys 3'	GTG GTT GGA GCA <b>TGT</b> GGT GTT GGG AAA 5'	34 G>T	562
Gly12Asn 3'	GTG GTT GGA GCA <b>AAT</b> GGT GTT GGG AAA 5'	34 35 GG>AA	12723
Gly12Pro 3'	GTG GTT GGA GCA <b>CCT</b> GGT GTT GGG AAA 5'	34 35 GG>CC	559
Gly12Tyr 3'	GTG GTT GGA GCA <b>TAT</b> GGT GTT GGG AAA 5'	34 35 GG>TA	560
Gly12Asp 3'	GTG GTT GGA GCA <b>GAT</b> GGT GTT GGG AAA 5'	35 G>A	564
Gly12Ala 3'	GTG GTT GGA GCA <b>GCT</b> GGT GTT GGG AAA 5'	35 G>C	565
Gly12Cys 3'	GTG GTT GGA GCA <b>GTT</b> GGT GTT GGG AAA 5'	35 G>T	566
Gly12Glu 3'	GTG GTT GGA GCA <b>AAG</b> GGT GTT GGG AAA 5'	35 36 GT > AG	144577
Gly12Gly 3'	GTG GTT GGA GCA <b>AGC</b> GGT GTT GGG AAA 5'	36 T>C	567
Gly13Ser 3'	GTT GGA GCA GGT <b>AGT</b> GTT GGG AAA AGC 5'	37 G>A	571
Gly13Cys 3'	GTT GGA GCA GGT <b>CGT</b> GTT GGG AAA AGC 5'	37 G>C	569
Gly13Thr 3'	GTT GGA GCA GGT <b>TGT</b> GTT GGG AAA AGC 5'	37 G>T	570
Gly13Asn 3'	GTT GGA GCA GGT <b>AAT</b> GTT GGG AAA AGC 5'	37 38 GG>AA	24668
Gly13Tyr 3'	GTT GGA GCA GGT <b>TAT</b> GTT GGG AAA AGC 5'	37 38 GG>TA	568
Gly13Asp 3'	GTT GGA GCA GGT <b>GAT</b> GTT GGG AAA AGC 5'	38 G>A	573
Gly13Ala 3'	GTT GGA GCA GGT <b>GCT</b> GTT GGG AAA AGC 5'	38 G>C	575
Gly13Val 3'	GTT GGA GCA GGT <b>GTT</b> GTT GGG AAA AGC 5'	38 G>T	574
Gly13Val 3'	GTT GGA GCA GGT <b>GTC</b> GTT GGG AAA AGC 5'	38 39 GT>TC	572
Gly13Gly 3'	GTT GGA GCA GGT <b>GGC</b> GTT GGG AAA AGC 5'	39 T>C	576
Gln61Lys 3'	CTG GAT ACA GCT GGT <b>AAA</b> GAA GAG TAC 5'	180 181 AC>TA	12730
Gln61Lys 3'	CTG GAT ACA GCT GGA <b>AAA</b> GAA GAG TAC 5'	181 C>A	580
Gln61Glu 3'	CTG GAT ACA GCT GGA <b>GAA</b> GAA GAG TAC 5'	181 C>G	581
Gln61Arg 3'	CTG GAT ACA GCT GGA <b>AGA</b> GAA GAG TAC 5'	181 182 CA>AG	579
Gln61Leu 3'	CTG GAT ACA GCT GGA <b>TTA</b> GAA GAG TAC 5'	181 182 CA>TT	12725
Gln61Lys 3'	CTG GAT ACA GCT GGA <b>AAG</b> GAA GAG TAC 5'	181 183 CAA>AAG	53223
Gln61Pro 3'	CTG GAT ACA GCT GGA <b>CCA</b> GAA GAG TAC 5'	182 A>C	582
Gln61Arg 3'	CTG GAT ACA GCT GGA <b>CGA</b> GAA GAG TAC 5'	182 A>G	584
Gln61Leu 3'	CTG GAT ACA GCT GGA <b>CTA</b> GAA GAG TAC 5'	182 A>T	583
Gln61Arg 3'	CTG GAT ACA GCT GGA <b>CGG</b> GAA GAG TAC 5'	182 183 AA>GG	33693
Gln61Leu 3'	CTG GAT ACA GCT GGA <b>CTG</b> GAA GAG TAC 5'	182 183 AA>TG	30646
Gln61His 3'	CTG GAT ACA GCT GGA <b>CAC</b> GAA GAG TAC 5'	183 A>C	586
Gln61Gln 3'	CTG GAT ACA GCT GGA <b>CAG</b> GAA GAG TAC 5'	183 A>G	587
Gln61His 3'	CTG GAT ACA GCT GGA <b>CAT</b> GAA GAG TAC 5'	183 A>T	585

## Section 15 Notices and Disclaimers

These products are sold exclusively for research and development (R&D) use by the purchaser. PointMan enrichment kits may not be used for human or veterinary in vitro diagnostic (IVD) applications and they may not be re-sold, distributed or re-packaged without express written authorisation from EKF Molecular Diagnostics.

**License statement:** Enzymes designed and sold for use in Amplification Patent Rights and/or Sequencing Patent Rights. A license under US Patents 4,683,202, 4,683,195, 4,965,188, and 5,075,216 or their foreign counterparts, owned by Hoffmann-La Roche Inc. and F. Hoffmann-La Roche Ltd. ("Roche"), has an up-front fee component and a running-royalty component. The purchase price of this product includes limited, nontransferable rights under the running-royalty component to use only this amount of the product to practice the Polymerase Chain Reaction ("PCR") and related processes described in said patents solely for the research and development activities of the purchaser when this product is used in conjunction with a thermal cycler whose use is covered by the up-front fee component. Rights to the up-front fee component must be obtained by the end user in order to have a complete license to use this product in the PCR process. These rights under the up-front fee component may be purchased from Perkin-Elmer or obtained by purchasing an authorized Thermal Cycler. No right to perform or offer commercial services of any kind using PCR, including without limitation reporting the results of purchaser's activities for a fee or other commercial consideration, is hereby granted by implication or estoppel. Further information on purchasing licenses to practice the PCR process may be obtained by contacting the Director of Licensing at The Perkin-Elmer Corporation, 850 Lincoln Center Drive, Foster City, California 94404 or at Roche Molecular Systems, Inc., 1145 Atlantic Avenue, Alameda, California 94501.

The purchase of EKF Molecular Diagnostics reagents cannot be construed as an authorization or implicit license to practice PCR under any patents held by Hoffmann-LaRoche Inc or others.

PointMan Mastermix containing GoTaq® Hot Start Polymerase manufactured by Promega Corporation for distribution by EKF Molecular Diagnostics. Licensed to Promega under U.S. Patent Nos. 5,338,671 and 5,587,287 and their corresponding foreign patents.



# Section 16 Notes

# Section 16 Notes

