



Precipio, Inc.

Instructions for Use

**EGFR Exon 20 Mutation Analysis using ICE COLD-PCR for
Detection with Sanger Sequencing**

Table of Contents

Manufacturer	2
Intended Use	2
Reagent Preparation	2
Kit Components and Storage Conditions	2
Reagents Required but not Supplied	3
Primary Sample Collection, Handling and Storage	4
Notes for this Assay	5
ICE COLD-PCR Protocol	5
Thermal Cycler Program for ICE COLD-PCR Enrichment	8
Quality Control of ICE COLD-PCR Products (OPTIONAL)	8
Sanger Sequencing of the ICE COLD-PCR products using the ABI 3730xl DNA Analyzer	9
Technical Support/Questions:	9

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Manufacturer

This kit was manufactured by Precipio, Inc. at 8813 F Street, Omaha, NE 68127, USA.
USA Telephone 1-203-787-7888

Intended Use

For professional use only. Precipio's ICEme Kit offerings are *in vitro* diagnostic assays that enrich clinically actionable somatic mutations in a target of interest. This kit is specifically designed to enrich for mutations in Exon 20 of the EGFR gene that are of clinical relevance. This kit is designed to be used in a clinical diagnostic laboratory by suitably trained personnel testing DNA extracted from formalin-fixed paraffin embedded tissues or from plasma using a PCR-based amplification/enrichment method and Sanger Sequencing.

















Reagent Preparation

All reagents supplied with this kit are ready to use. Some components will need to be thawed, vortexed and/or spun in a microcentrifuge before use; check details in Assay Procedure below. Reagents do need to be combined to produce Master Mixes and reaction mixtures; full details are given in the procedure below.

Kit Components and Storage Conditions

The EGFR Exon 20 Kit contains the components to perform ICE COLD-PCR amplification with mutation enrichment as well as Sanger sequence detection for 24 total samples with controls. Enough reagents, including controls, are present to accommodate 4 batches of 6 samples. Recommended storage conditions are listed in **Table 1**.

Table 1: Kit components with recommended storage conditions.

Reagent, ICE COLD-PCR	Lid Color	Color of Label	Number of Tubes	Volume/Tube (μL)	Storage (°C)
10X Primer/RS-Oligo Mix	Clear 	Blue 	1	290	-20
2X Polymerase Master Mix	Clear 	Green 	4	360	-20
Wild-Type Control	Clear 	White 	1	66	-20
1% T790M Control	Clear 	Red 	1	66	-20
1% C797S TA Control	Clear 	Red 	1	66	-20
1% C797S GC Control	Clear 	Red 	1	66	-20
Reagent, Sequencing	Lid Color	Color of Label	Number of Tubes	Volume Total (μL)	Storage (°C)
Forward Sanger Sequencing Primer, 10 μM	Clear 	Orange 	1	60	-20
Reverse Sanger Sequencing Primer, 10 μM	Clear 	Orange 	1	60	-20

This kit was designed specifically to enrich the following mutations:

1. c.2369C>T; p.T790M
2. c.2389T>A, p.C797S
3. c.2390G>C, p.C797S

Reagents Required but not Supplied

1. Molecular Biology Grade Water: Thermo Fisher Catalog # AM9937

Primary Sample Collection, Handling and Storage

This kit can be used with the following:

- DNA extracted from formalin-fixed paraffin-embedded tumor samples (FFPE slides & blocks) or fine needle aspirations (FNAs)
 - For optimal DNA extraction from FFPE, the tissue should be fixed in neutral buffered formalin for 14 – 24 hours, placed in ethanol and then embedded in paraffin following standard histological practices.
 - Tumor biopsies are a heterogeneous mixture of tumor cells and non-tumor cells. In addition, the tumor itself is a heterogeneous mixture of tumor cells with mutations and tumor cells without mutations. Because these somatic mutations may not be evenly distributed throughout the tumor, the resultant mutational analysis of different sections from the same tumor may be different.
 - To increase the probability of detecting a mutation, DNA from the tumor region of the tissue should be isolated by scraping only the tumor area from the glass slide using a fresh, sterile scalpel for each new slide. It is recommended that at least two independent analyses are performed for each sample.
 - The formalin-fixation process used in preparing FFPE tumor biopsy samples may result in deamination of cytosines. Deamination converts cytosine to uracil. The polymerase will recognize this uracil as a thymine and incorporate an adenine in the copied strands. The correct allele G is then replaced with an A causing an artifact mutation due to the fixation process and not a true somatic mutation. It is **highly** suggested that an extraction protocol which incorporates **Uracil-N-Glycosylase** be used for FFPE Extractions.
- Circulating free DNA (cfDNA) from plasma or serum
 - The following collection tubes can be used to collect whole blood for subsequent extraction of cfDNA from plasma:
 - Lavender top EDTA tube
 - Streck Cell Free DNA BCT® Tube

NOTE: Extraction of cfDNA should be performed within 24 hrs of collection with the lavender top EDTA tube. See manufacturer’s guidance for each tube type.

NOTE: Upon separation via centrifugation, if the sample is red or orange in color, this denotes the sample is hemolyzed and therefore not suitable for downstream plasma analysis. A redraw is required. Plasma should have a yellow or light yellow color and show distinct separation on top of the blood.

NOTE: Storage and shipping conditions of the whole blood may affect quantity and quality of cfDNA in relation to full length DNA.

 - The preferred extraction kit for ICE COLD-PCR applications is the Bioo Scientific NextPrep-Mag™ cfDNA Isolation Kit (Refer to protocol PRO1005 Plasma Extraction Protocol_MagBind 20Sep2018.docx). The Qiagen QIAamp® Circulating Nucleic Acid Kit (PRO1001 Plasma Extraction Protocol_QIAgen 20Sep2018.docx) was also tested.
- DNA isolated from other body fluids.

Other considerations:

1. It is recommended that all samples and controls are extracted with the same protocol to avoid any variations due to reagents and/or buffers.
2. All DNA samples should be diluted to the same starting concentration with the same dilution buffer.
3. Ensure sample to sample uniformity.
4. Any additional control DNA to be analyzed should be of the same quality and quantity as the sample DNA.

Notes for this Assay

- The ICE COLD-PCR assay has been optimized using the **Bio-Rad C1000** thermal cycler; however, the ICE COLD-PCR assay can be optimized/run on most other thermal cyclers.
- A single sequencing primer is provided for uni-directional sequencing.
- Due to heterogeneity associated with tumors, biopsy samples may contain normal cells as well as Wild-Type and mutant tumor cells.
- The Limit of Detection (LOD) of any mutations present in the sample DNA following ICE COLD-PCR is dependent on the sensitivity of the downstream sequence detection platform used.
- Only the DNA polymerase(s) supplied with this kit should be used as indicated for the specific assay or downstream application.
- This is a mutation enrichment assay. Any mutation or mismatch covered by the RS-Oligo will be enriched during the ICE COLD-PCR process. There are two possible sources of false positive results related to this assay:
 - o Polymerase errors. While the polymerase included in the kit is a high-fidelity enzyme, there is the possibility of a polymerase-induced error.
 - o The formalin-fixation process used in preparing FFPE tumor biopsy samples

NOTE: Variants caused by polymerase errors or deamination of cytosine do not repeat upon re-analysis. Therefore, it is recommended that any such mutation be confirmed by duplicate analysis using the same extracted genomic DNA..

ICE COLD-PCR Protocol

IMPORTANT! Use dedicated hood/room for ICE COLD-PCR reaction setup to avoid contamination from post-PCR products.

IMPORTANT! The following procedures are optional but highly recommended prior to PCR setup:

- Turn on UV light inside hood or a UV crosslinker.
 - Prior to preparing Master Mixes, UV crosslink all empty Master Mix tubes. Also UV crosslink 1.7 mL tubes containing appropriate volume of Molecular Biology Grade Water needed for Master Mix preparation. These tubes should be UV irradiated for 10 min.
 - Make sure all work areas are prepared for analysis of low level mutations. This includes correct use of the PCR Workstation, dedicated pipettes, tips, 10% bleach solution and/or DNA Away™ solutions.
1. Remove controls, 10X Primer/RS-Oligo Mix and 2X Polymerase Master Mix from freezer and thaw on ice.
 2. Once thawed, vortex all kit components ~3 - 5 sec to mix thoroughly. Briefly centrifuge 5 sec to ensure no liquid remains on tube lids and place on ice.
 3. **Example experiment layout:** Follow **Table 2** for layout to simplify later steps of the procedure. The

following 5 controls are required for each setup: **NTC** (No Template Control) and the control DNAs provided by the kits, including **WT** (EGFR Wild-Type), **T790M_1per** (1% EGFR-T790M), **C797S-TA_1per** (1% EGFR_C797S-TA) and **C797S-GC_1per** (1% EGFR_C797S-GC).

NOTE: Additional controls may be added in place of test samples.

Table 2A: Proposed experimental layout for the ICE COLD-PCR for **24** samples in one batch.

	1	2	3	4
A	SMP01	SMP09	SMP17	WT
B	SMP02	SMP10	SMP18	T790M_1per
C	SMP03	SMP11	SMP19	C797S-TA_1per
D	SMP04	SMP12	SMP20	C797S-GC_1per
E	SMP05	SMP13	SMP21	
F	SMP06	SMP14	SMP22	
G	SMP07	SMP15	SMP23	NTC
H	SMP08	SMP16	SMP24	

Table 2B: Proposed experimental layout for the ICE COLD-PCR for **6** samples in one batch.

	1	2
A	SMP01	WT
B	SMP02	T790M_1per
C	SMP03	C797S-TA_1per
D	SMP04	C797S-GC_1per
E	SMP05	
F	SMP06	
G	NTC	
H		

4. Use **Table 3** as a guide for calculating and preparing the ICE COLD-PCR Master Mix assuming a DNA volume of 15 μL at 3.3 $\text{ng}/\mu\text{L}$. Please note that less total DNA can be added to the reaction.
 - a. If the extracted DNA concentration is $> 3.3 \text{ ng}/\mu\text{L}$, dilute to a working concentration of 3.3 $\text{ng}/\mu\text{L}$ and use 15 μL of the diluted DNA for analysis.
 - b. If the extracted DNA concentration is $\leq 3.3 \text{ ng}/\mu\text{L}$, add 15 μL of extracted DNA for analysis.

Table 3: Calculations for Master Mix preparation

Reagent:	1X reaction*
UV-treated Molecular Biology Grade Water (μL)	5
2X Polymerase Master Mix (μL)	25
10X Primer/RS-Oligo Mix (μL)	5
Total Volume PCR Master Mix for 1 reaction (μL)	35
Volume DNA added to reaction (μL)	15

**Multiply volumes in this table by the number of samples being tested.*

NOTE: A Master Mix volume slightly greater than this calculation will be required to allow for losses during pipetting.

NOTE: Amount of UV-treated Molecular Biology Grade Water and volume of DNA can be adjusted accordingly.

NOTE: We suggest **50** ng of DNA should be used for each PCR reaction in order to potentially detect $\geq 0.1\%$ mutation present in the starting material (minimum 18 ng). Up to 20 μL DNA can be used in each PCR reaction with adjustment of volume of water used.

NOTE: If DNA contains contaminants (ethanol carryover, EDTA etc.), increasing DNA volume may decrease PCR efficiency.

5. Label a 1.7 mL-centrifuge tube for PCR Master Mix preparation.
6. Add required volume of molecular biology grade water to Master Mix tube.
7. Add required volume of 2X Polymerase Master Mix to Master Mix tube.
8. Add required volume of 10X Primer/RS-Oligo Mix to Master Mix tube.
9. Cap Master Mix tube, vortex for $\sim 3 - 5$ sec, briefly centrifuge and store on ice until use.
10. Label a 96-well plate with appropriate sample information.
11. Pipette appropriate volume of ICE COLD-PCR Master Mix into applicable wells. If using a repeat pipettor, ensure that there is no spillage or splashing from well to well.
12. To appropriate wells, add required volume of each DNA sample, control, or water (NTC). Use separate pipette tips for each sample and avoid cross-contamination of the samples by splashing. Securely cap all wells containing DNA samples, controls, and NTC with 8-cap strips.

NOTE: Addition of the kit controls last lessens the chance of contaminating any test sample wells.

13. Vortex (1/2 speed) for $\sim 3 - 5$ sec. Centrifuge for ~ 5 sec to ensure all solutions are collected at the bottom of wells or tubes. If not, repeat centrifugation.

Thermal Cycler Program for ICE COLD-PCR Enrichment

Use the thermal cycler protocol in **Table 4** for ICE COLD-PCR Enrichment.

IMPORTANT! Ensure that the following ramp rates are used depending on the thermal cycler:

- BioRad C1000 Touch: 1.5°C/sec
- BioRad Tetrad II: default (3.0°C/sec)
- Applied Biosystems Veriti: 100%

Table 4: Thermal cycler conditions for ICE COLD-PCR enrichment.

Step	Temperature	Time	Cycles
Initial Denaturation	98 °C	30 sec	1
Main ICE COLD-PCR Amplification	98 °C	10 sec	45
	69 °C	30 sec	
	74 °C	30 sec	
	63 °C	30 sec	
	72 °C	20 sec	
Amplification	98 °C	10 sec	5
	63 °C	10 sec	
	72 °C	20 sec	
Final Extension	72 °C	5 min	1
Hold	12 °C	∞	

NOTE: Samples can be stored at -20 °C post amplification.

Quality Control of ICE COLD-PCR Products (OPTIONAL)

1. Analyze an aliquot of ICE COLD-PCR product alongside an aliquot of 100-bp DNA mass ladder on a 2% agarose gel to verify amplification of DNA.
2. Only a single band corresponding to the main ICE COLD-PCR product should be observed.
3. If multiple bands are present check quality of input DNA.
4. If no product is observed, ensure quality of input template DNA was sufficient.
5. No ICE COLD-PCR products should be visible in No Template Control sample.
 - a. If DNA products of the expected product size are visible in No Template Control sample, contamination is likely and sample should not be taken to sequence analysis.

Sanger Sequencing of the ICE COLD-PCR products using the ABI 3730xl DNA Analyzer

1. Sequencing primers are supplied for use for DNA sequencing of all samples tested. The primers are supplied at a 10 μ M working solution.
2. Please follow your standard laboratory protocol for DNA sequencing. If your laboratory does not have a DNA sequencing protocol, you can find a full protocol for Sanger Sequencing from ICE COLD-PCR products on the Precipio website (PRO1007 Sanger Sequencing Protocol 10Oct2018.docx).
3. For analysis on the ABI 3730xl, the following Run Module parameters should be used to run ICE COLD-PCR products:

Name	Value	Range
Oven_Temperature	60	18...70 DegC
PreRun_Voltage	15.0	0...15 kV
PreRun_Time	180	1...1800 sec
Injection_Voltage	1.5	0...15 kV
Injection_Time	15	1...90 sec
First_ReadOut_Time	200	100...16000 ms
Second_ReadOut_Time	200	100...16000 ms
Run_Voltage	13.4	0...15 kV
Voltage_Number_Of_Steps	30	0...100 Steps
Voltage_Step_Interval	15	0...180 secs
Voltage_Tolerance	0.6	0...6.0 kV
Current_Stability	30.0	0...2000 uA
Ramp_Delay	450	1...1800 sec
Data_Delay	250	1...1800 sec
Run_Time	1000	300...14000 sec

Technical Support/Questions:

For any questions regarding the kit or IFU, please contact us at Techsupport@precipiodx.com or call 1-203-787-7888 ext. 509.