**COLD-PCR Preliminary Report 041612**

**Ref:**

1. **Replacing PCR with COLD-PCR enriches variant DNA sequences and redefines the sensitivity of genetic testing,** Nat. Med. 2008 May; 149(5): 579- 584
2. **COLD-PCR–Enhanced High-Resolution Melting Enables Rapid and Selective Identification of Low-Level Unknown Mutations,** Clin Chem. 2009 December; 55(12): 2130–2143

|  |  |  |  |
| --- | --- | --- | --- |
| **Reaction Component** | | **Ours** | **Makrigiorgos Papers** |
| Template DNA | WT ref DNA | from **Clonetech**\*\* | No mention of source (Nat. Med)  Male gDNA from **Promega** (Clin. Chem.) |
|  | Mutant DNA | gDNA of **HTB38** cell line, purchased from ATCC  [HTB38 mutation: p53 exon 8 codon 273 G>A] | **SW480** cell line purchased from ATCC (gDNA from the cell line isolated by lab)  [SW480 mutation: p53 exon 8 codon 273 G>A] |
| Primers | 210bp and 87bp amplicons | Primers were ordered based on sequences published in the Nat. Med paper |  |
| Enzyme | COLD-PCR | **Jump Start Taq DNA Polymerase** (from Sigma) | **Jump Start Taq DNA Polymerase** (from Sigma) (Nat. Med)  **Phusion DNA Polymerase** (from Finnzymes) (Clin. Chem.) |
| Dye | COLD-PCR | **0.1X LC Green** | **0.1X LC Green** (Nat.Med and Clin. Chem) |

\*\*: WT ref gDNA obtained from Clonetech. This genomic DNA is isolated from the whole blood of disease free sources. The sources of whole blood for the Human Genomic DNA have tested negative for HIV antibodies and Hepatitis B surface antigen.

Protocol:

1. Amplification of a 210bp product from WT and mutant DNA (separately) using primer pair A5-A6 (Ref 1). This amplification was done using the HF2-High Fidelity PCR kit from Clonetech (Ref 1).
2. This PCR product was used as a template for the COLD-PCR reactions.

* The paper mentions that the template for the COLD-PCR was a **247bp** product. However, no primer information is available for a 247bp product.

1. Using primer pair A1-A2 (Ref 1), the 87bp product was amplified from the WT and mutant 210bp template (in separate reactions) in the presence of 1X LC Green to determine Tm of the 87bp amplicon. Cycling conditions (Ref1):

95oC, 120secs

95oC,15secs;   
55oC, 30secs, plate read;

72oC, 1min

GOTO 2 30 times

1. WT amplicon had a Tm of 86oC

Mutant amplicon had a Tm of 85.4oC.

* Nat. Med Paper does not mention how much lower the Tc is from the Tm of the WT amplicon. It simply states that the Tc for the 87bp amplicon is 83.5oC.

1. To determine Tc, COLD-PCR reactions have been performed 85.5oC and 85oC (Ref 1)
   1. primer pair A1-A2
   2. template DNA 210bp product from Mutant and WT gDNAs at ratios of 1:10, 1: 25, 1:50 (at 1X, 0.1X and 0.01X template DNA amt)
   3. cycling conditions (Ref 1):

95oC; 120secs

95oC; 15secs

55oC; 30secs, plate read

72oC; 1min

GOTO step 2 10\* times

95oC; 15secs

70oC; 8mins

85.5oC; 3secs   
55oC; 30secs, plate read

72oC; 1min

GOTO step 5 30\* times

\*: These cycle numbers were increased to 15 and 40 when using very small amounts of template DNA (0.1X and 0.01X)

1. The original Nat. Med paper did not use HRMA for downstream processing and uses 0.1X LC Green for the COLD-PCR. HRMA requires 1X LC Green (manufacturer’s recommendation and also stated in Ref 2. Since we are performing HRMA of the COLD-PCR products, the methodology from Ref 2 was followed. According to this,
   1. First step: COLD-PCR with 0.1X LC Green
   2. Second step: Regular PCR at a denaturing temp >90oC. with 1X LC Green. Use this reaction for HRMA directly.

* Ref 2 uses gDNA for the COLD-PCR to generate a 167bp amplicon. This amplicon is used as template for the second round of nested regular PCR (which generates a 135bp product).

1. Our experiment:
   1. First step: COLD-PCR with 0.1X LC Green as described above (point 5).
   2. Second step: Regular PCR with 1X LC Green, using

Primer pair: A1-A2

Template DNA: 1:1000 dilution of the product from first step

Cycling conditions:

95oC; 120secs

95oC; 15secs

55oC; 30secs, plate read

72oC; 1min

GOTO step 2 30 times

Melt Curve@ 65oC to 98oC at 0.2oC/sec with a 10sec hold.

@: melt curve conditions used as recommended by BioRad.

Observations:

1. When checked on a gel the COLD-PCR products as well as the Rd2 Reg PCR products only show smears. According to Ref 1, Tc is the lowest denaturing temperature that reproducibly yields a substantial product. The authors state that the temperature selected as Tc is usually below the Tm of WT, mutant or mismatched sequences.
2. The use of MeltSim (and/or Poland Algorithm) to determine Tc has been recommended in another paper (ie, **Application of COLD-PCR for improved detection of KRAS mutations in clinical samples**, Zuo *et al*, Modern Pathology, 2009, 22: 1023- 1031). In fact, in this paper, the algorithm is unsupported by experimental determination of Tm. For an amplicon Tm of 70.9oC, the authors used a denaturing temp of 80oC for the COLD-PCR.
3. At 85.5oC for which extensive HRMA has been done so far, we cannot talk of reproducibility, since the gel shows only smears and we do not have any other method to check the PCR products.
   * Ref 2 supports all HRM data with sequencing information.
   * Also, we cannot determine the enrichment obtained if we do not have a method to detect what products we have.

The papers do not show any gel pictures and do not state what should be expected to be seen on them.

1. Mixed template reactions seem to have a higher Tm than WT reactions. The PCR products from each step have not been purified for use as template for subsequent steps. The smears starting from the well, suggest that much larger products are being formed (also indicated be the higher Tm). Is it possible that the gDNA used originally (to generate the 210bp amplicon) is also getting amplified?

Amount of gDNA originally used: 5ng; dilution of this PCR rxn: 1000X).

1. LC Green increases Tm by 1-3oC. Given this perhaps the tm of the WT 87bp amplicon maybe lower ie 83-85oC. Therefore, reactions with much lower denaturing temperatures have to be tried.

Steps forward:

1. Try COLD-PCR at more temperatures (80oC to 90oC in 1oC increments).
2. Change denaturing time (Ref 2 uses a denaturing time of 10secs at Tc).
3. Try the COLD-PCR step directly with 1X LC Green.
4. Check all PCRs on gel.
5. Purify the 210bp amplicon from the PCR rxn and then make template mixes to be used for COLD-PCR.
6. Sequence PCR amplicons when obtained to understand the profile of the products and determine enrichment, if any.