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| Time: 5h  1st round 2h  2nd round 2h  Preparation 30+30  12.5µL  2µL for 25µL | 2. Amplify regions of Bisulfite converted DNA by nested PCR  Mastermix: ZymoTaq PreMix  (or EpiMark HS Taq PreMix, Fermentas HotStart Taq Mix)  1st round (IAP1~IAP4, outer primers) Master mix:  12.5µL 2x preMix  2µL IAP1~IAP4  2µL Bisulfite-treated DNA  8.5µL ddH2O  ---------------------------  25µL  One 25 reaction per sample.  95°C 10’  95°C 30’’ \  59°C 30’’ |=> 32  72°C 60’’ /  72°C 7’  4°C  2nd Run (Primer IAP2~IAP4, inner primers)  12.5µL 2x Fermentas HomeMix  2µL IAP2~IAP4  2µL 1st round product  8.5µL ddH2O  ---------------------------  25µL  2µL for 25µL  Do 2 PCR reactions for each sample, 50µL total per sample.  (Optimal: 5µL for 50µL  5PCR reaction to catch all molecules in the first round, 250 total per sample. )  95°C 10’  95°C 30’’ \  59°C 30’’ |=> 35  72°C 60’’ /  72°C 7’  4°C |