Expression of miR-196b is not exclusively *MLL*-driven but is especially linked to activation of *HOXA* genes in pediatric acute lymphoblastic leukemia

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Online Supplementary Design and Methods

Sample preparation for real-time quantitative polymerase chain reaction

At least 5x10^6 freshly obtained leukemia cells were lysed in Trizol reagent (Gibco, BRL, Life Technologies) and stored at -80 °C until RNA was extracted according to the manufacturer's guidelines with minor modifications, as described previously.[1] RNA pellets were dissolved in TE-buffer [10 mM Tris-HCL,1 mM EDTA (ethylenediaminetetraacetic acid) at pH 8.0] and stored at -80 °C. Prior to cDNA synthesis, RNA was quantified using a NanoDrop 1000 spectrophotometer (Isogen Life Science, De Meern, the Netherlands) and loaded on a 2100 bioanalyzer (Agilent, Amstelveen, the Netherlands) to determine the quality. All RNA samples included had an RNA integrity number of 7.5 or greater.

Real-time quantitative polymerase chain reaction analysis of HOXA genes

RNA was reverse-transcribed into cDNA using random hexamers and oligo dT primers and then diluted to 8 μg/ L as described elsewhere.[1] cDNA was stored at -80 °C before use. The levels of expression of *HOXA3*, *HOXA9* and *HOXA10* transcripts were quantified relative to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). cDNA specific for *HOXA9*, *HOXA10* and *GAPDH* was amplified using dual fluorescence-labeled non-extendable probes; for *HOXA3* SYBR green reagents (Applied Biosystems, Foster City, USA) were used as reported before.[1-3] Probes and primers (sequences listed in *Online Supplementary Table S1*) were purchased from Eurogentec (Seraing, Belgium) after being developed using the OLIGO 6.22 software (Molecular Biology Insights, Cascade, USA) according to standardized guidelines: melting temperatures of 65±1°C for primers and 75±1°C for probes (nearest neighbor method at salt concentration of 303 mM of Na^+ equivalent and 300 nM of primer concentration), specific PCR reactions showing only a single product on the gel and (in the case of SYBR green reactions) a single dissociation curve, an amplification efficiency (calculated according to the equation: E = 10-(1/slope) - 1) of ≥ 95% for all reactions, and a negative non-template control.[1,4] Forty nanograms of cDNA specific for *HOXA9*, *HOXA10* and *GAPDH* were amplified in duplo in a total volume of 50 μL containing 300 nM forward and reverse primers, 50 nM probe, 200 μM each of dNTP, 4 mM $MgCl_2$ and 1.25 U of AmpliTaq gold DNA polymerase in Taqman A buffer (Applied Biosystems). Amplification of cDNA specific for *HOXA3* was performed using SYBR Green reagents in the presence of 1.5 mM $MgCl_2$ as reported by others.[3] After initial denaturation for 10 min at 95°C, samples were amplified for 40 cycles of 15 sec at 95°C and 60 sec at 60°C on an Applied Biosystems 7900HT system with SDS 2.3 analysis software. As all PCR reactions had an efficiency of 95% or more, relative mRNA levels of *HOXA3*, *HOXA9* and *HOXA10* transcripts were normalized to the expression levels of the reference gene *GAPDH* and their expression levels was calculated as a percentage of that of *GAPDH* using the following formula: $2^{-\Delta Ct}$ x 100% where the ΔCt is equal to Ct *HOX* gene minus Ct *GAPDH*.[4]

Real-time quantitative polymerase chain reaction of miR-196b

MiR-196b expression was measured by RT-qPCR using a specific stem-loop reverse transcription primer and probe combination designed by Applied Biosystems (cat. no. 4427975).[5] Endogenous small nucleolar RNA 1 (snoRNA-1 or RNU24, 5'- AUUUGCUAUCUGAGAGAUGGUGAUGACAUUUUAAACCACCAAGAUCGCUGAUGCA-3'cat. no. 4373379) was used as a reference for small RNA-input as snoRNA-1 expression levels do not vary significantly between different subtypes of ALL. As described elsewhere, reverse transcription was performed in a total volume of 7.5 μL containing 5 ng total RNA, 0.25 mM of each dNTP, 3.3 U/μL MultiScribe reverse transcriptase, 1x reverse transcription buffer, 0.25 U/μL RNase inhibitor and 50 nM of specific stem-loop reverse transcription primer (all Applied Biosystems).[6] The 7.5 μL reverse transcription reactions were incubated in duplicate for 30 min at 16 °C, 30 min at 42 °C, 5 min at 85 °C and then kept at 4 °C. cDNA samples were stored at –20 °C until further use.

One microliter of cDNA was amplified in duplicate in a total volume of 10 μL containing 1x TaqMan® Universal PCR Master Mix, 0.2 μM TaqMan® probe, 1.5 μM forward primer and 0.7 μM reverse primer (all Applied Biosystems). Further amplification conditions and the equation used to calculate the level of miR-196b expression relative to the level of snoRNA-1 level are the same as those described above for the *HOXA* and *GAPDH* genes.

References

1. Stam RW, den Boer ML, Meijerink JP, Ebus ME, Peters GJ, Noordhuis P, et al. Differential mRNA expression of Ara-C-metabolizing enzymes explains Ara-C sensitivity in MLL gene-rearranged infant acute lymphoblastic leukemia. Blood. 2003;101(4):1270-6.
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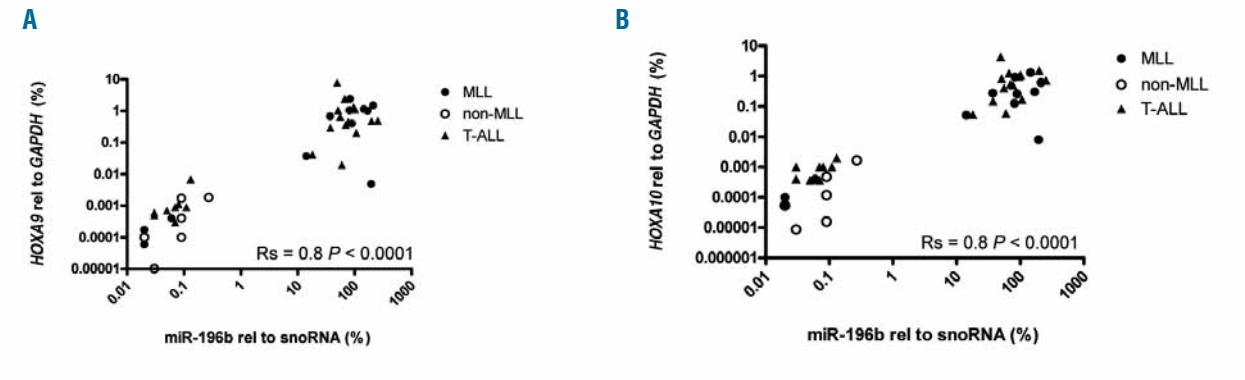
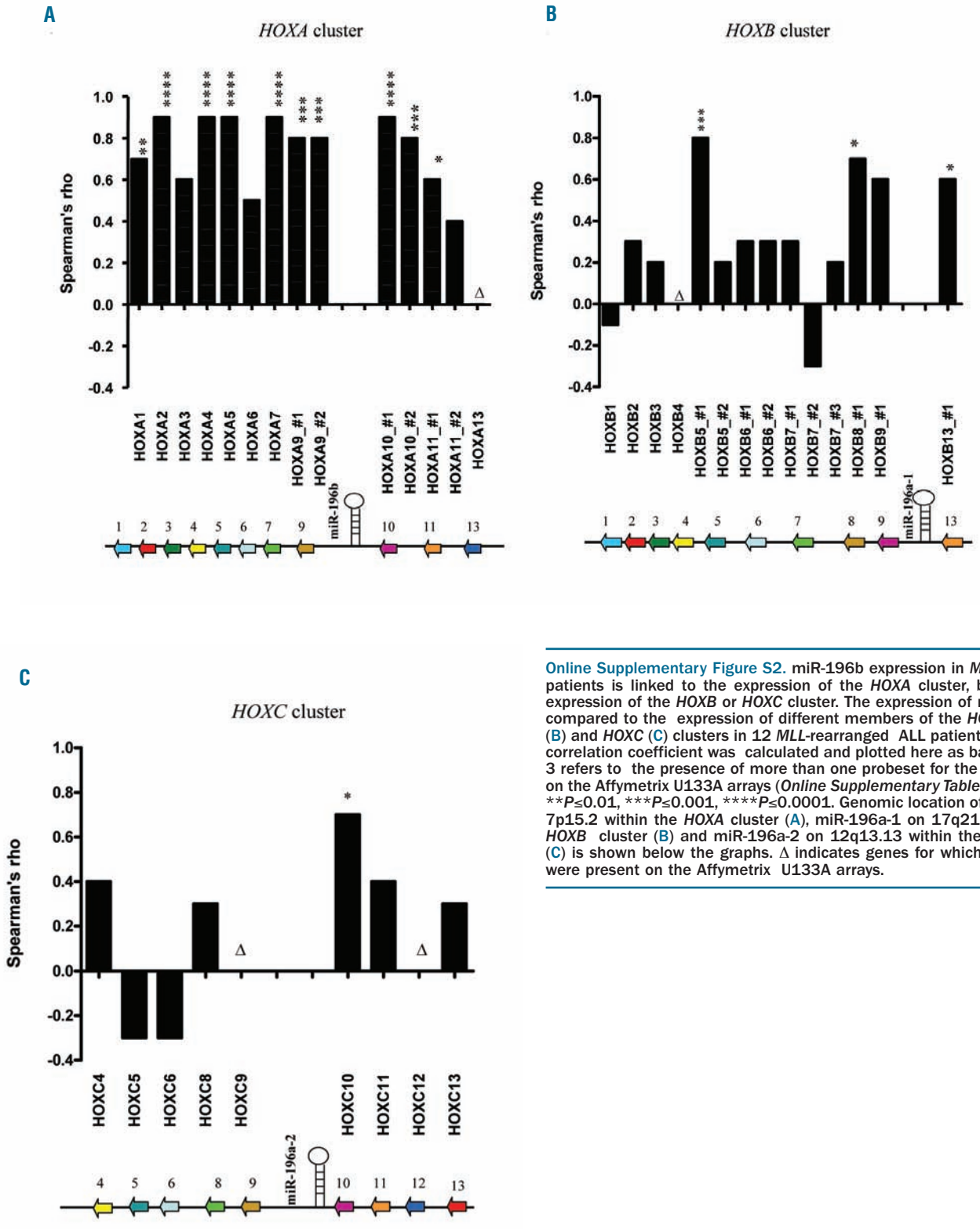
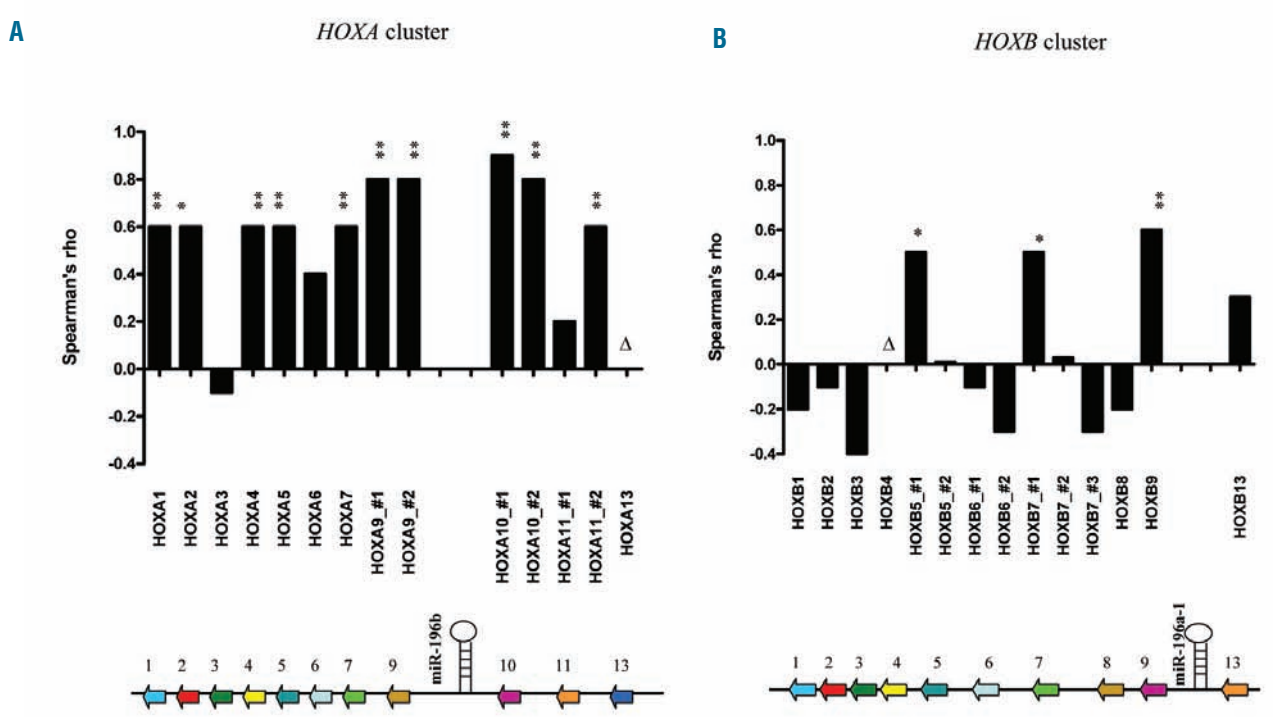


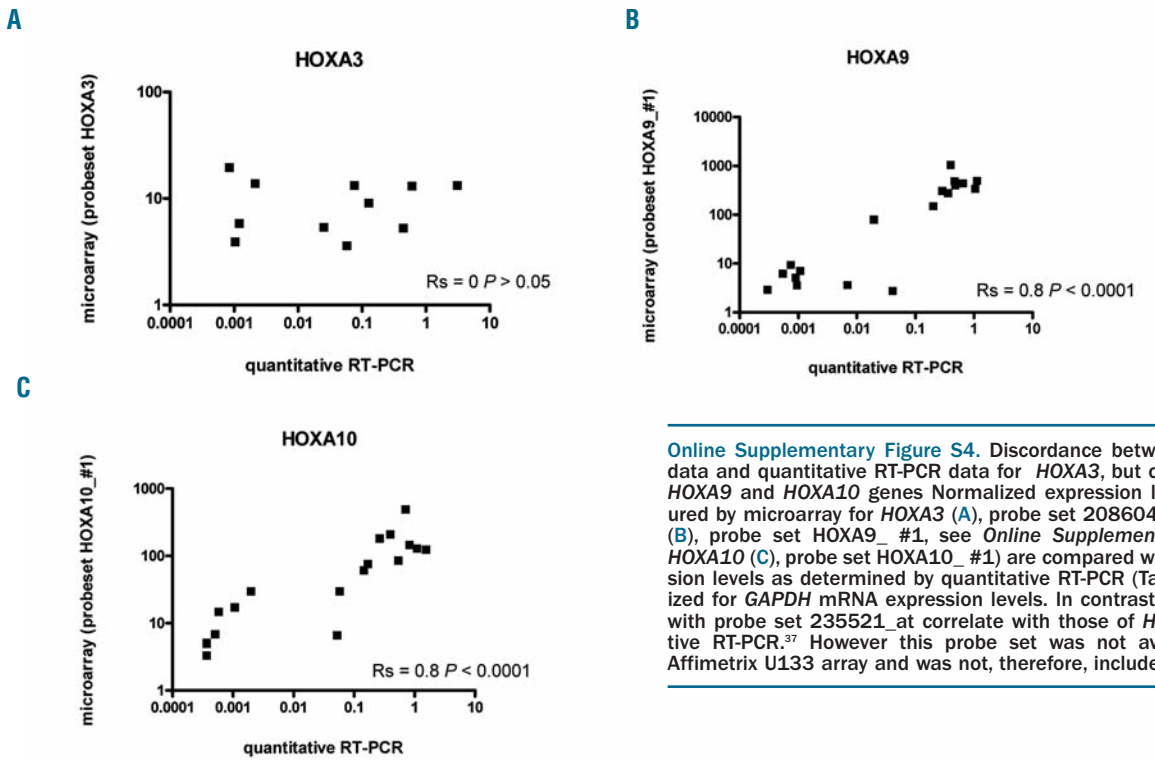
Online Supplementary Figure S1. Correlation between expression levels of miR-196b, *HOXA9* and *HOXA10* irrespective of *MLL*-status. The expression levels are compared between miR-196b and *HOXA9* (A) and between miR-196b and *HOXA10* (B) in 12 *MLL*-rearranged precursor B-ALL (closed circles), 6 other precursor B-ALL patients without *MLL*- translocation (open circles) and 22 T-ALL patients (triangles). The expression level of miR-196b was normalized for the expression level of snoRNA-1 as measured by quantitative stem-loop RT-qPCR whereas the expression of *HOXA9* and *HOXA10* transcripts was normalized for *GAPDH* mRNA expression levels as measured by quantitative RT-qPCR.



Online Supplementary Figure S2. miR-196b expression in *MLL*-rearranged patients is linked to the expression of the *HOXA* cluster, but not to the expression of the *HOXB* or *HOXC* cluster. The expression of miR-196b was compared to the expression of different members of the *HOXA* (A), *HOXB* (B) and *HOXC* (C) clusters in 12 *MLL*-rearranged ALL patients. Spearman's correlation coefficient was calculated and plotted here as bars. # 1, 2 and 3 refers to the presence of more than one probeset for the specific gene on the Affymetrix U133A arrays (*Online Supplementary Table S1*). **P*≤0.05, ***P*≤0.01, ****P*≤0.001, *****P*≤0.0001. Genomic location of miR-196b on 7p15.2 within the *HOXA* cluster (A), miR-196a-1 on 17q21.32 within the *HOXB* cluster (B) and miR-196a-2 on 12q13.13 within the *HOXC* cluster (C) is shown below the graphs. Δ indicates genes for which no probesets were present on the Affymetrix U133A arrays.



Online Supplementary Figure S3. miR-196b expression in T-ALL patients is linked to the expression of the *HOXA* cluster, but not to the expression of the *HOXB* or *HOXC* cluster. The expression of miR-196b was compared to the expression of different members of the *HOXA* (A), *HOXB* (B) and *HOXC* (C) clusters in 18 T-ALL patients. Spearman's correlation coefficient was calculated and plotted here as bars. # 1, 2 and 3 refers to the presence of more than one probe set for the specific gene on the Affymetrix U133A arrays (*Online Supplementary Table S1*). **P*≤0.05, ***P*≤0.01, ****P*≤0.001, *****P*≤0.0001. Genomic location of miR-196b on 7p15.2 within the *HOXA* cluster (A), miR-196a-1on 17q21.32 within the *HOXB* cluster (B) and miR-196a-2 on 12q13.13 within the *HOXC* cluster (C) is shown below the graphs. Δ indicates genes for which no probe sets were present on the Affymetrix U133A arrays.



Online Supplementary Figure S4. Discordance between microarray data and quantitative RT-PCR data for *HOXA3*, but concordance for *HOXA9* and *HOXA10* genes Normalized expression levels as measured by microarray for *HOXA3* (A), probe set 208604_s_at), *HOXA9* (B), probe set HOXA9_ #1, see *Online Supplementary Table S1*), *HOXA10* (C), probe set HOXA10_ #1) are compared with their expression levels as determined by quantitative RT-PCR (Taqman) normalized for *GAPDH* mRNA expression levels. In contrast, data obtained with probe set 235521_at correlate with those of *HOXA3* quantitative RT-PCR.[37] However this probe set was not available on the Affimetrix U133 array and was not, therefore, included in this study.

Online Supplementary Table S1. Specific primer and probe combinations used for RT-qPCR.

HOXA3	Forward sequence	5'-CAA CCC TAC CCC TGC CAA C-3'
	Reverse sequence	5'-TGC TTT GTG TTT TGT CGA GACT C-3'
	Probe sequence	SYBR green
	amplicon length (bp)	224
HOXA9	Forward sequence	5'-ACG CTT GAC ACT CAC ACT TT-3'
	Reverse sequence	5'-CAG GGT CTG GTG TTT TGT AT-3
	Probe sequence	5'-(FAM)-ATG CTT GTG GTT CTC CTC CAG TTG-(TAMRA)-3'
	amplicon length (bp)	402
HOXA10	Forward sequence	5'-TCC GAG AGC AGC AAA G-3'
	Reverse sequence	5'-CCG CTC TCG AGT AAG GTA-3'
	Probe sequence	5'-(FAM)-TGA AAA CGC AGC CAA CTG G-(TAMRA)-3'
	amplicon length (bp)	296
GAPDH	Forward sequence	5'-GTC GGA GTC AAC GGA TT-3'
	Reverse sequence	5'-AAG CTT CCC GTT CTC AG-3'
	Probe sequence	5'-(FAM)-TCA ACT ACA TGG TTT ACA TGT TCC AA-(TAMRA)-3'
	amplicon length (bp)	365
miR-196b	Forward/Reverse/Probe sequence	patented
	amplicon length (bp)	unknown
snoRNA-1	Forward/Reverse/Probe sequence	patented
	amplicon length (bp)	unknown

Legend to Tabe S1: Sequences for primer /probe combinations of miR-196b and its reference gene snoRNA-1 are unknown, since both combinations are patented by Applied Biosystems

Online Supplementary Table S2. Affymetrix U133A probe set description for *HOX* genes.

gene	probeset ID	localization of probeset
HOXA1	214639_s_at	3' UTR
HOXA2	214457_at	CDS / 3 'UTR *
HOXA3	208604_s_at	CDS
HOXA4	206289_at	3' UTR
HOXA5	213844_at	CDS / 3 'UTR *
HOXA6	208557_at	CDS **
HOXA7	206847_s_at	CDS / 3 'UTR *
HOXA9_#1	214651_s_at	3' UTR
HOXA9_#2	209905_at	3' UTR
HOXA10_#1	213147_at	3' UTR
HOXA10_#2	213150_at	3' UTR
HOX A11_#1	208493_at	CDS **
HOX A11#2	213823_at	3' UTR
HOXA13 Δ	-	-
HOXB1	208224_at	CDS **
HOXB2	205453_at	CDS / 3 'UTR *
HOXB3	208414_s_at	CDS / 3 'UTR *
HOXB4 Δ	-	-
HOXB5_#1	205600_x_at	CDS** / 3 'UTR *
HOXB5_#2	205601_s_at	3' UTR
HOXB6_#1	205366_s_at	3' UTR
HOXB6_#2	205365_at	5' UTR
HOXB7_#1	204778_x_at	CDS** / 3 'UTR *
HOXB7_#2	204779_s_at	3' UTR
HOXB7_#3	216973_s_at	CDS / 3 'UTR *
HOXB8	221278_at	CDS **
HOXB9	216417_x_at	CDS
HOXB13	209844_at	CDS **
HOXC4	206194_at	3' UTR
HOXC5	206739_at	3' UTR
HOXC6	206858_s_at	3' UTR
HOXC8	221350_at	CDS **
HOXC9 Δ	-	-
HOXC10	218959_at	3' UTR
HOXC11	206745_at	3' UTR
HOXC12 Δ	-	-
HOXC13	219832_s_at	3' UTR

Localization refers to part of the transcript to which the probe set hybridizes
Coding domain sequence (= CDS) and untranslated region (= UTR)
* some probes cover CDS whereas other probes of the same probe set cover 3' UTR of the transcript
** partly covering CDS on the 5' end of the transcript
Δ indicates genes for which no probe sets are present on the Affymetrix U133A arrays.