Determination of gene signatures to subgroup melanoma patients using novel branched DNA hybridization assays

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Background

Melanoma is the most life-threatening neoplasm of the skin, with increasing incidence and mortality worldwide. The development of melanoma progresses through discrete stages that have well-known clinical and histological features; however, key underlying molecular events have not been clearly elucidated [1]. Identification of prognostic and predictive biomarkers will help to better understand the biological pathways of relevance; genomic studies of melanomas are necessary. Gene expression signatures have been successfully employed to distinguish cancer subtypes in many tumor types including melanoma [2]; however, melanin content of later-stage melanomas and age of stored formalin-fixed, paraffin-embedded (FFPE) samples can make gene expression analysis difficult [3]. Here we describe two gene expression technologies to validate microarray data, that work either directly on melanoma tissue lysates using the Invitrogen™ QuantiGene™ Plex Assay, or by *in situ* hybridization (ISH) using the Invitrogen™ ViewRNA™ ISH Tissue Assay, which uses FFPE tissue sections (Figure 1). The two assays, based on second-generation branched-DNA nanostructures, enable direct, specific, and quantitative detection of mRNAs without RNA isolation, reverse transcription, or PCR amplification.

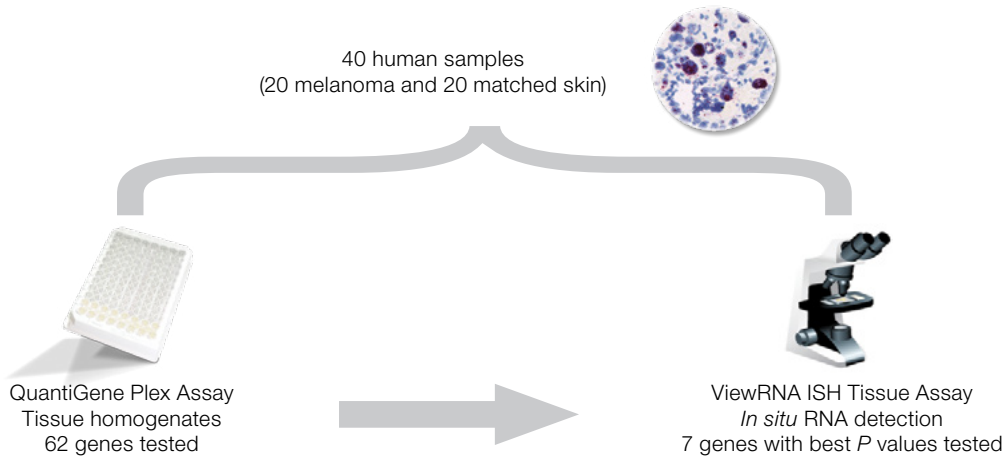

Figure 1. QuantiGene Plex and ViewRNA biomarker assay strategy—melanoma and normal skin.

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QuantiGene Plex Assay overview

Twenty matched-pair melanoma and normal skin samples were prepared for QuantiGene Plex Assays as follows: 5 mg wet-weight, snap-frozen tissue samples were homogenized in 300 µL Invitrogen™ QuantiGene™ Homogenization Solution plus Proteinase K. Homogenates were tested in 40 µL triplicate samples by the QuantiGene Plex Assay using two 36-gene panels as described in the user manual; each panel contained 31 target genes and 5 housekeeping genes. All samples were analyzed using a Luminex® instrument, and all target gene expression was normalized to a geometric mean of the 5 housekeeping genes (PGK1, HPRT, TBP, ACTB, GUSB; see Figure 2).

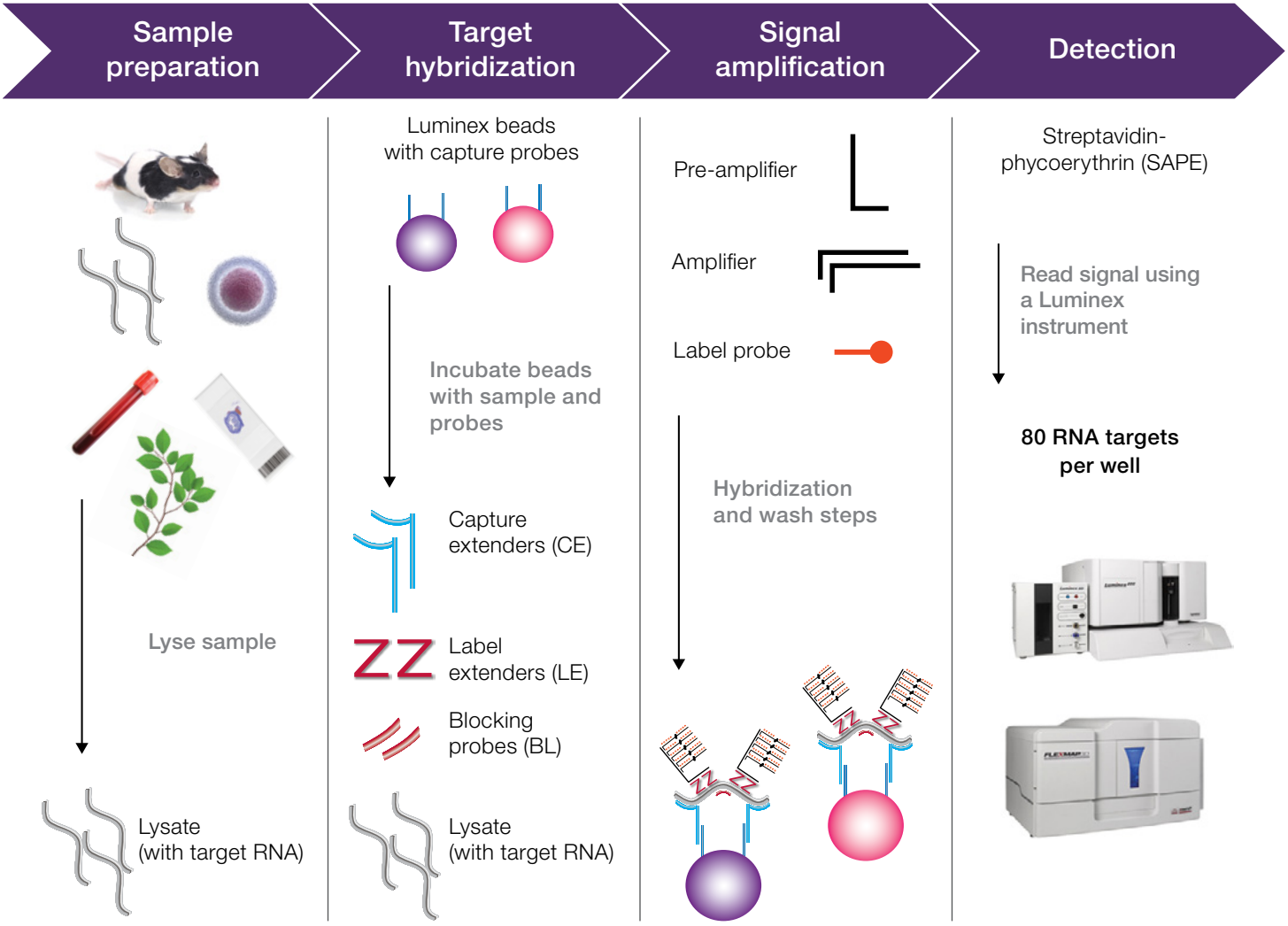


Figure 2. QuantiGene Plex Assay overview. The assay uses Luminex® xMAP® technology to simultaneously measure as many as 80 genes in a single well.

ViewRNA ISH Tissue Assay overview

Snap-frozen tissues were fixed in 10% buffered formalin for 24 hours, rinsed in water, dehydrated, and embedded in paraffin. Tissue sections (5 µm) were deposited on positively charged Fisher Scientific™ SuperFrost™ Plus slides (Cat. No. 12-550-15). All samples were tested in duplicate by the ViewRNA ISH Tissue Assay for the 7 genes with the best P values, including dapB (negative control), ACTB (positive control for all samples), and KRT5 (positive control for normal skin and negative control for metastatic melanoma). Sections were hybridized with probe and labeled with Fast Red, and nuclei were counterstained with DAPI as described in the user manual. 20x/NA 0.75 images were taken with an Olympus™ IX71 microscope and Olympus™ DP71 camera (Figure 3).

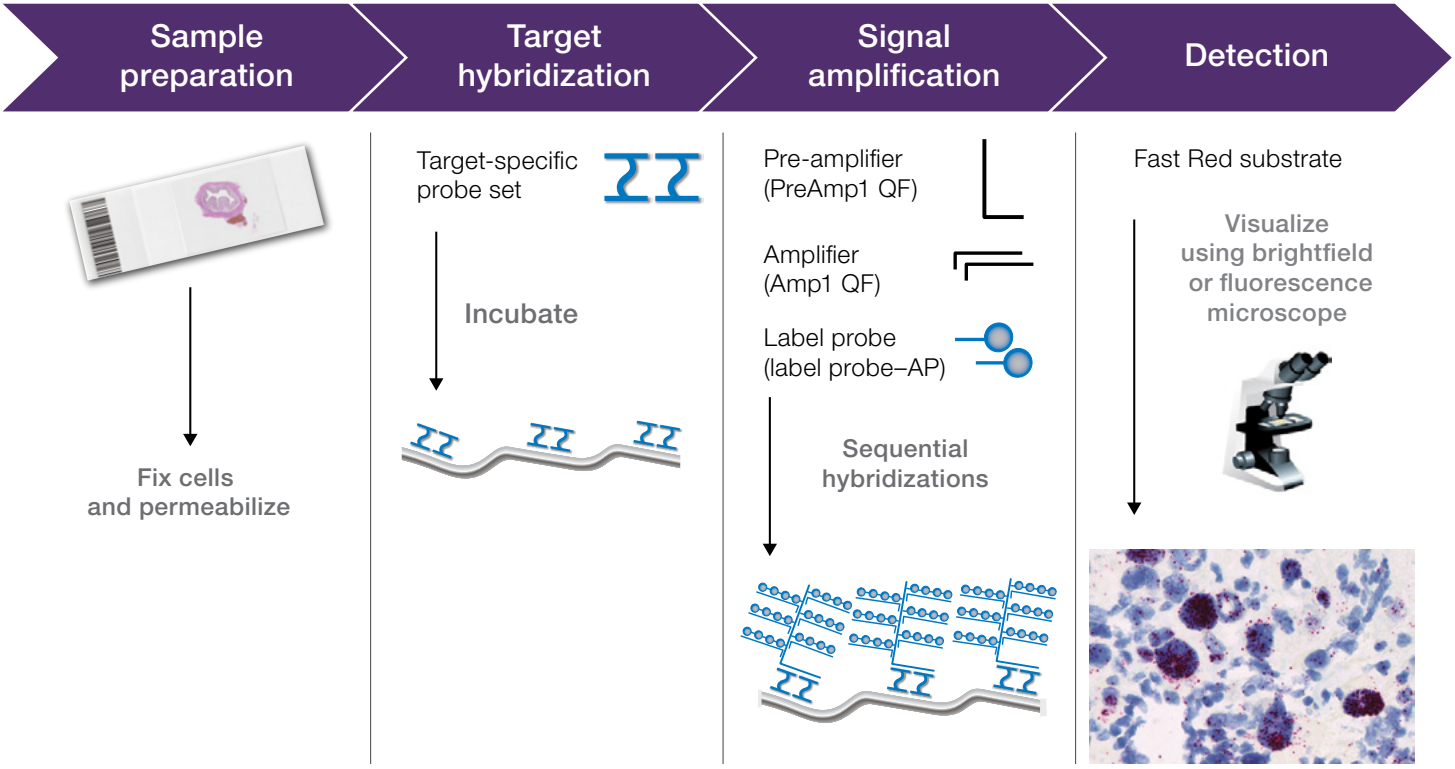


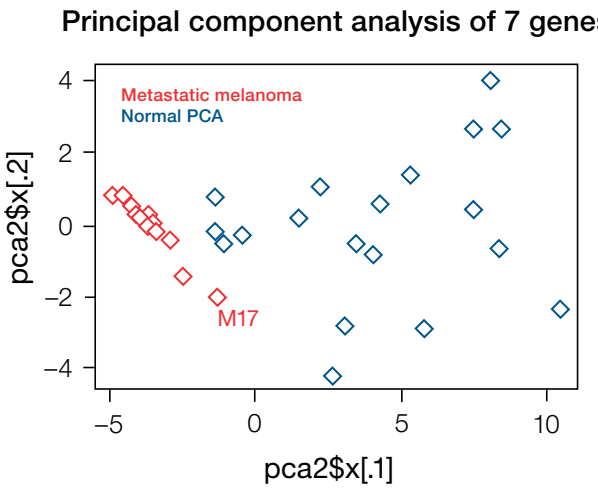
Figure 3. ViewRNA ISH assay. The ViewRNA ISH Tissue Assay, based on branched-DNA signal amplification technology, has the sensitivity and robustness to measure single molecules of mRNA in single cells.

Results

Sixty-two genes related to melanoma have been categorized from the literature using RNA expression to determine the prevalence of these genes in 20 frozen sections of metastatic melanoma tissues, as compared to their corresponding normal skin counterparts. RNA expression was quantified directly from melanoma and normal skin tissue homogenates by the QuantiGene Plex Assay, based on branched DNA technology (Figure 4) [4,5]. Seven genes, demonstrating the most significant difference in expression between tissue types (P values 1.28 x 10–9 to 1.13 x 10–6), were found to be significantly associated with melanoma as compared to normal skin when analyzed by principal component analysis and cluster analysis (Figures 5 and 6). These 7 genes were validated using FFPE tissues from the same donors by the ViewRNA ISH Tissue Assay (Figure 7) [6]. Of the 7 genes, 4 genes were upregulated in the melanoma metastases versus normal skin tissues. These 7 candidate genes gave signal differences in both intensity and spatial recognition between melanoma tumor and normal skin tissue microenvironments. These genes support angiogenesis, immune response and inflammation, DNA replication, cell proliferation and motility, tissue invasion and progression, epidermis development, cell communication, and morphogenesis.

Gene	*P* value	Gene	*P* value
BCL6	**1.28×10^{-9}**	*XRCC6*	1.88×10^{-2}
PTEN	**1.03×10^{-8}**	*DNAJA1*	2.51×10^{-2}
ARPC2	**2.17×10^{-8}**	*CDKN2A*	3.31×10^{-2}
CXCL12	**1.37×10^{-7}**	*NOS2*	4.91×10^{-2}
BRAF	**4.18×10^{-7}**	*CTNNB1*	7.62×10^{-2}
PCNA	**6.33×10^{-7}**	*VEGFC*	1.02×10^{-1}
CLEC3B	**1.13×10^{-6}**	*B3GAT1*	1.19×10^{-1}
MCM6	**1.93×10^{-6}**	*CEACAM1*	1.20×10^{-1}
NME1	**2.39×10^{-6}**	*CCNA1*	1.43×10^{-1}
EMX2	**3.07×10^{-6}**	*GMNN*	1.72×10^{-1}
MKI67	**3.45×10^{-6}**	*TRPM1*	1.96×10^{-1}
TP53	**3.63×10^{-6}**	*IL-8*	2.37×10^{-1}
MMP2	**6.60×10^{-6}**	*APAF1*	2.82×10^{-1}
NCOA3	1.84×10^{-4}	*MCAM*	2.91×10^{-1}
BIRC5	2.21×10^{-4}	*CD8A*	3.22×10^{-1}
PLAT	3.27×10^{-4}	*IL-10*	3.23×10^{-1}
FXYD5	3.67×10^{-4}	*MCM4*	3.47×10^{-1}
RGS1	4.13×10^{-4}	*TFAP2A*	3.75×10^{-1}
SPP1	4.45×10^{-4}	*FGF2*	3.84×10^{-1}
LICAM	1.70×10^{-3}	*BCL2L1*	3.85×10^{-1}
CDKN1A	2.13×10^{-3}	*XRCC5*	3.86×10^{-1}
IL-24	2.40×10^{-3}	*CXCR4*	4.11×10^{-1}
LYVE1	2.46×10^{-3}	*ICAM1*	4.76×10^{-1}
MITF	2.53×10^{-3}	*BCL2*	5.23×10^{-1}
TYR	5.06×10^{-3}	*ATF2*	5.37×10^{-1}
MAP2	7.88×10^{-3}	*WNT2*	5.54×10^{-1}
MLANA	8.26×10^{-3}	*BBC3*	6.51×10^{-1}
TNF	1.11×10^{-2}	*STMN2*	8.32×10^{-1}
CDH3	1.20×10^{-2}	*HSPA4*	8.89×10^{-1}
FN1	1.21×10^{-2}	*CD4*	9.54×10^{-1}
ITGB3	1.43×10^{-2}	*CNN3*	9.98×10^{-1}

Figure 4. Gene expression differences between melanoma and normal skin. Expression of all 62 genes was normalized to the geometric mean of 5 housekeeping genes, and the *P* value for each gene was calculated using a supervised Student's *t*-test. The genes are in the order of their *P* values; the best *P* values are in bold.



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BRAF	4.18×10^{-7}
PCNA	6.33×10^{-7}
CLEC3B	1.13×10^{-6}

M17: Not melanoma; lymph node with pigmented histiocytes

Figure 5. QuantiGene Plex mRNA principal component analysis (PCA). Genes with the best 7 *P* values were analyzed using an unsupervised principal component analysis. Note: Sample 17 was not a melanoma sample (lymph node with pigmented histiocytes).

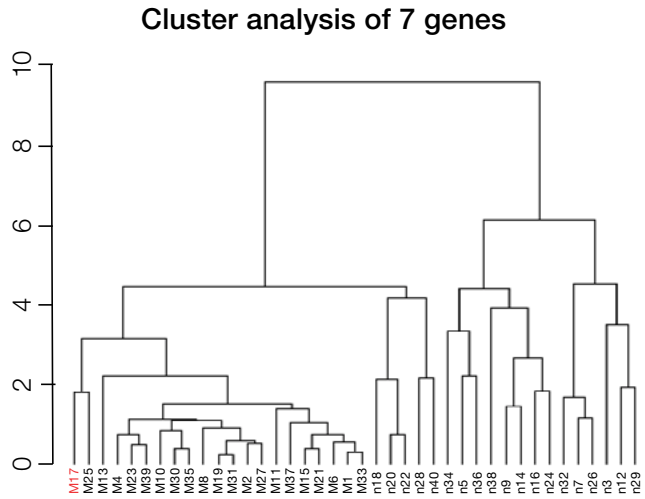

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Figure 6. QuantiGene Plex mRNA cluster analysis. Genes with the best 7 *P* values were analyzed using an unsupervised cluster analysis. Note: Sample 17 was not a melanoma sample (lymph node with pigmented histiocytes).

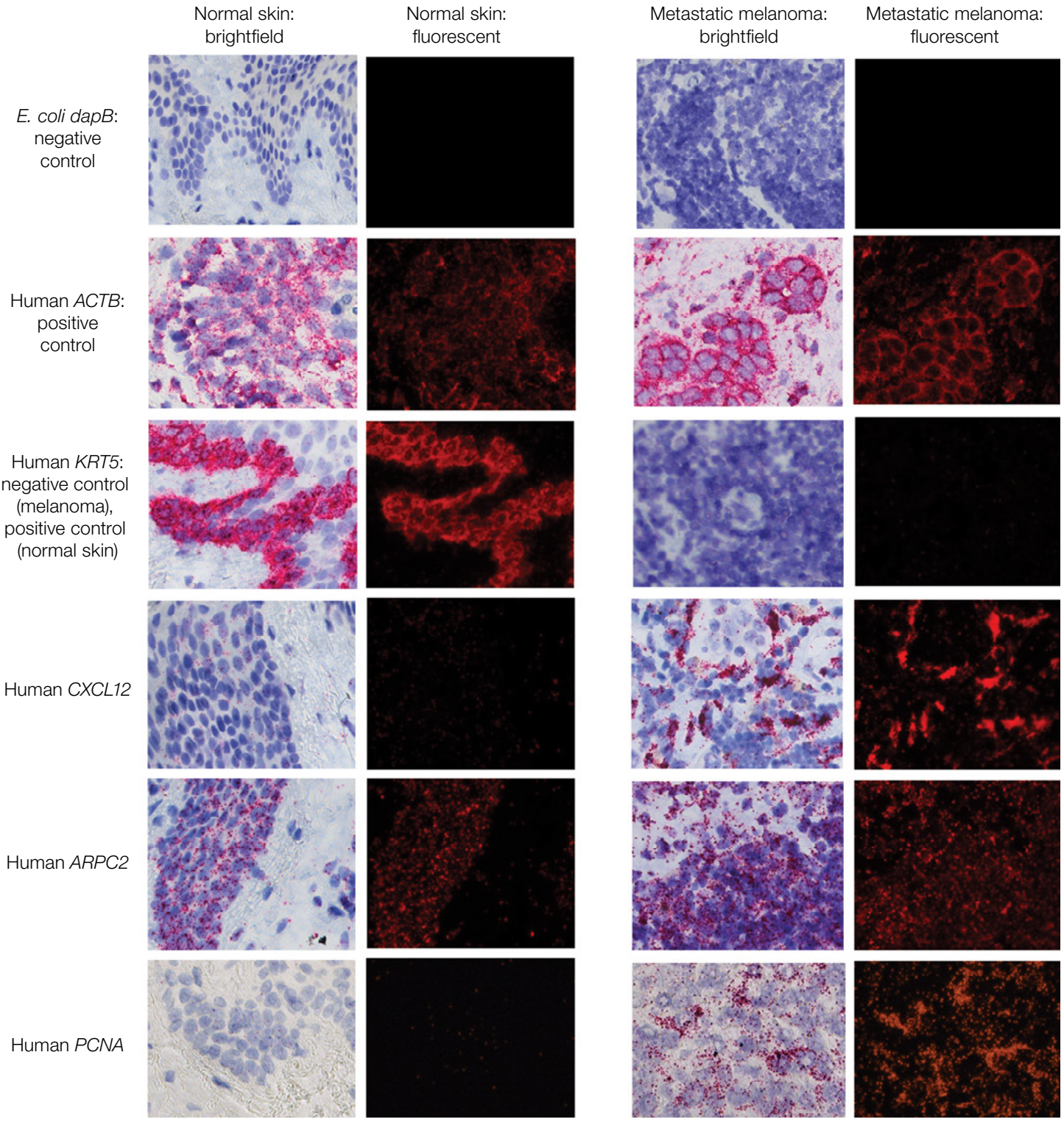

Figure 7. ViewRNA *in situ* mRNA hybridization. 20x images of matched normal skin vs. melanoma skin. The ViewRNA probe sets used are indicated on the left.

Functions of the top 7 genes with the best *P* values

- ***BCL6* (B-cell CLL/lymphoma 6):** Repressor of transcription; decreased expression associated with impaired prognosis [7]
- ***PTEN* (phosphatase and tensin homolog):** Signal transduction regulator; decreased expression associated with impaired prognosis [8]
- ***ARPC2* (actin-related protein 2/3 complex, subunit 2):** Control of actin polymerization in cells; increased expression in malignant melanomas [9]
- ***CXCL12* (chemokine (C-X-C motif) ligand 12):** Angiogenesis; increased expression is involved in melanoma progression [10]
- ***BRAF* (v-Raf murine sarcoma viral oncogene homolog B1):** Cell division; increased expression in ***PCNA* (proliferating cell nuclear antigen):** DNA replication; increased expression correlated with increased risk of developing distant metastasis and impaired prognosis [12]
- ***CLEC3B* (C-type lectin domain family 3, member B):** Cell-extracellular matrix interaction; decreased expression associated with primary tumors with low tumor thickness [13]

Conclusions

- The QuantiGene Plex Assay works directly on human melanoma and skin tissue homogenates, providing a fast, convenient workflow with precise and accurate simultaneous measurements of mRNAs without purification and target amplification, streamlining the target validation.
- The ViewRNA ISH Tissue Assay is an *in situ* hybridization assay that has the sensitivity and robustness to measure single RNA molecules in single cells in human melanoma and skin.
- Sixty-two genes from the literature were validated: (1) QuantiGene Plex Assay expression data from 7 of the 62 genes were sufficient to discriminate melanoma from matched normal skin; (2) the ViewRNA ISH Tissue Assay showed both signal intensity and spatial resolution of these 7 validated genes within melanoma and normal skin tissue samples.
- Taken together, these results demonstrate the power of using branched-DNA technologies to validate melanoma biomarkers.

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