ApplicationNote

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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 from melanoma tissue lysates using QuantiGene® Plex Assay, or by *in situ* hybridization (ISH) using ViewRNA® ISH Tissue Assay, which uses FFPE tissue sections. The two assays, based on second-generation, branched-DNA nanostructure, enable direct, specific, and quantitative detection of mRNAs without RNA isolation, reverse transcription, or PCR amplification.

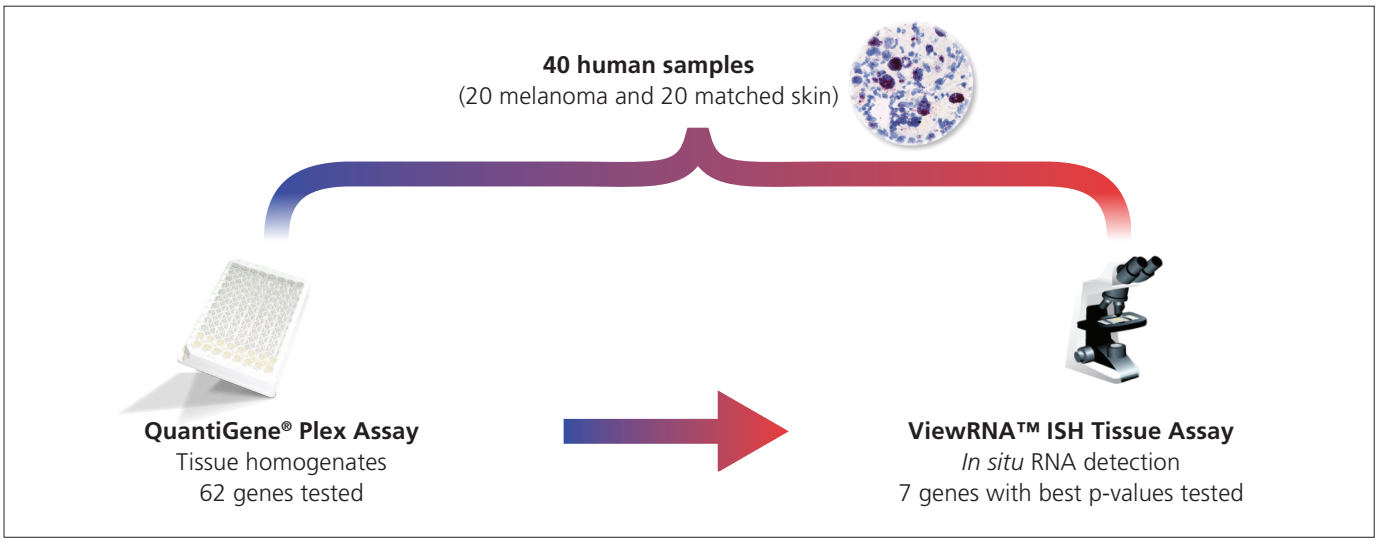


Figure 1. QuantiGene® Plex and ViewRNA® biomarker workflow strategy – melanoma and normal skin.

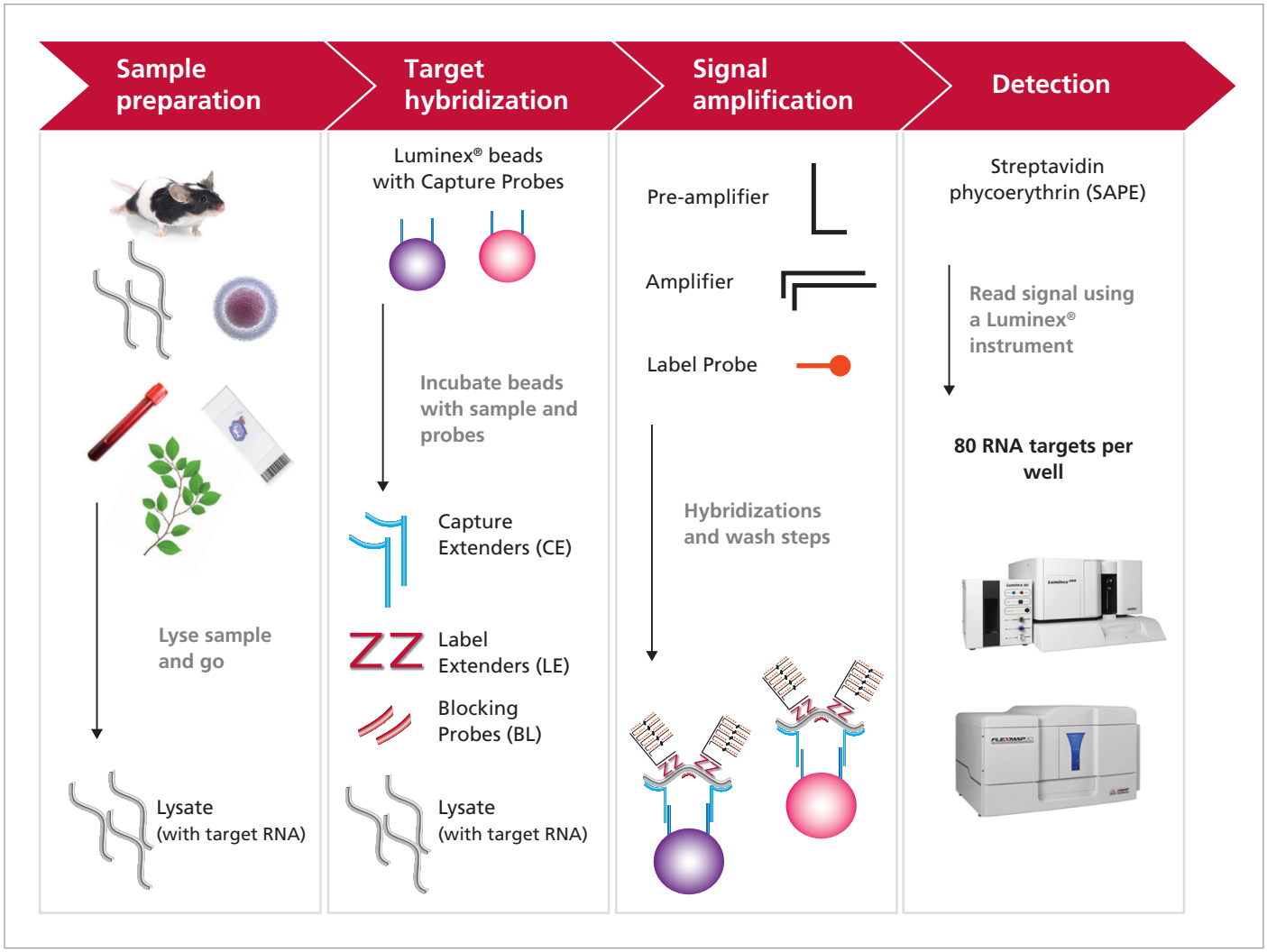


Figure 2: QuantiGene® Plex Assay overview. The assay uses Luminex® xMAP technology to simultaneously measure as many as 80 genes in a single well.

QuantiGene® Plex Assay overview

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

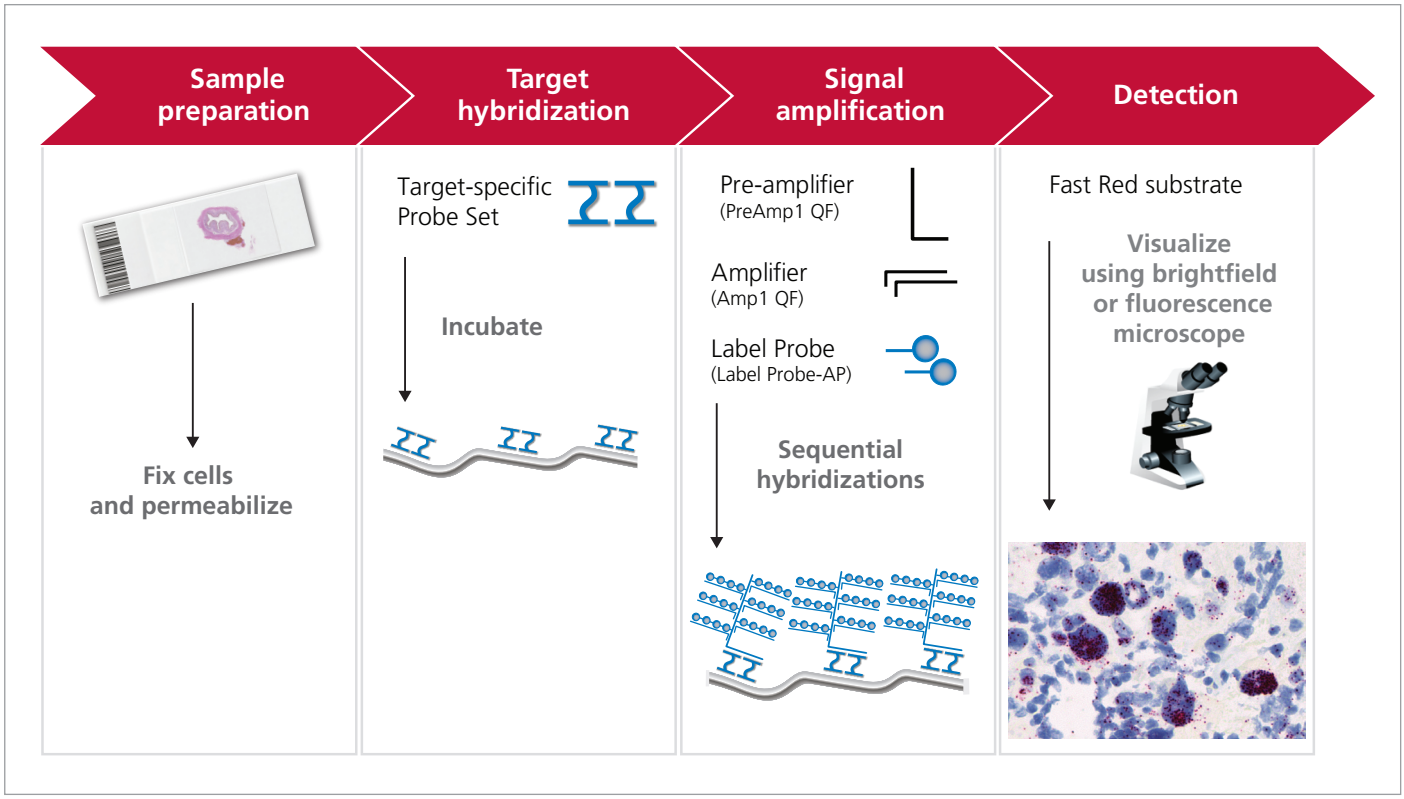


Figure 3: ViewRNA® *In Situ* Hybridization (ISH) Assay. ViewRNA® ISH Tissue Assay, based on branched-DNA signal amplification technology, has the sensitivity and robustness to measure single-molecule mRNA in single cells.

ViewRNA® ISH Tissue Assay overview

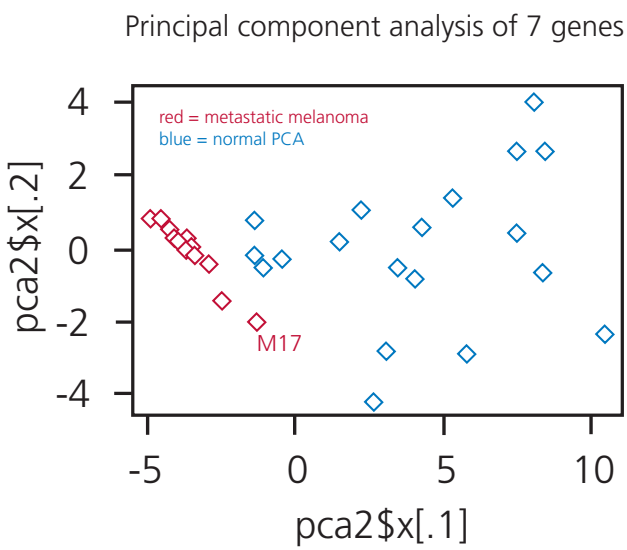
ViewRNA ISH Tissue Assay: Snap frozen tissues were fixed in 10% buffered formalin for 24 hours, rinsed in water, dehydrated, and embedded in paraffin. Five micron tissue sections were deposited on positive-charged "SuperFrost Plus™" slides (Fisher Scientific – cat. no. 12-550-15). All samples were tested in duplicate by 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 and stained-labeled with Fast Red and nuclei counter-stained with DAPI, as described in the user manual. 20X/NA 0.75 images were taken with an Olympus® IX71 microscope/Olympus DP71 camera (see Figure 3).

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 section metastatic melanoma tissues, as compared to their corresponding normal skin counterparts. RNA expression was quantified directly from melanoma and normal skin tissue homogenates by QuantiGene® Plex Assay, based on the branched DNA technology[4,5]. Seven genes, demonstrating the most significant difference in expression between tissue types (p-values 1.28E-09 –1.13E-06), were found to be significantly associated with melanoma, as compared to normal skin when analyzed by Principal Component Analysis and Cluster Analysis. These 7 genes were validated using FFPE tissues from the same patients by the branched-DNA *in situ* RNA expression technology ViewRNA® ISH Tissue Assay[6]. Of the 7 genes, 4 genes were upregulated in the melanoma metastases versus normal skin tissues. These 7 candidate genes gave signal differences, both in intensity and/or spatial recognition between melanoma tumor and normal skin tissue microenvironments. These molecules support angiogenesis, immune response/inflammation, DNA replication, cell proliferation/motility, tissue invasion/progression, and include epidermis development, cell communication, and morphogenesis.

Gene	P-value	Gene	P-value
BCL6	1.28E-09	XRCC6	1.88E-02
PTEN	1.03E-08	DNAJA1	2.51E-02
ARPC2	2.17E-08	CDKN2A	3.31E-02
CXCL12	1.37E-07	NOS2	4.91E-02
BRAF	4.18E-07	CTNNB1	7.62E-02
PCNA	6.33E-07	VEGFC	1.02E-01
CLEC3B	1.13E-06	B3GAT1	1.19E-01
MCM6	1.93E-06	CEACAM1	1.20E-01
NME1	2.39E-06	CCNA1	1.43E-01
EMX2	3.07E-06	GMNN	1.72E-01
MKI67	3.45E-06	TRPM1	1.96E-01
TP53	3.63E-06	IL-8	2.37E-01
MMP2	6.60E-06	APAF1	2.82E-01
NCOA3	1.84E-04	MCAM	2.91E-01
BIRC5	2.21E-04	CD8A	3.22E-01
PLAT	3.27E-04	IL-10	3.23E-01
FXYD5	3.67E-04	MCM4	3.47E-01
RGS1	4.13E-04	TFAP2A	3.75E-01
SPP1	4.45E-04	FGF2	3.84E-01
LICAM	1.70E-03	BCL2L1	3.85E-01
CDKN1A	2.13E-03	XRCC5	3.86E-01
IL-24	2.40E-03	CXCR4	4.11E-01
LYVE1	2.46E-03	ICAM1	4.76E-01
MITF	2.53E-03	BCL2	5.23E-01
TYR	5.06E-03	ATF2	5.37E-01
MAP2	7.88E-03	WNT2	5.54E-01
MLANA	8.26E-03	BBC3	6.51E-01
TNF	1.11E-02	STMN2	8.32E-01
CDH3	1.20E-02	HSPA4	8.89E-01
FN1	1.21E-02	CD4	9.54E-01
ITGB3	1.43E-02	CNN3	9.98E-01

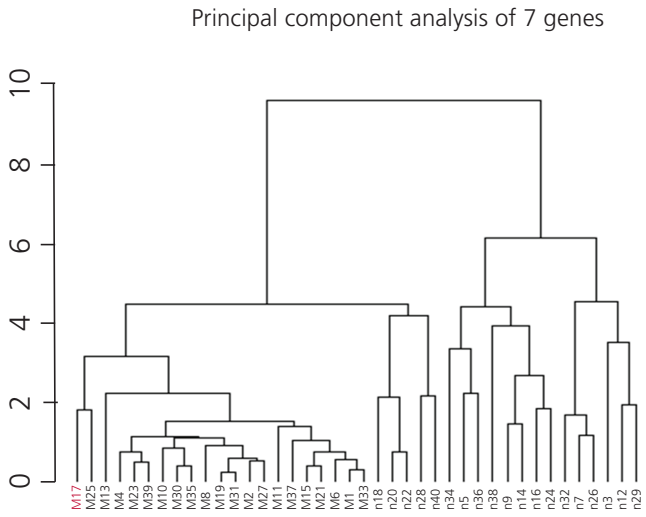
Figure 4: Gene expression of all 62 genes was normalized by the geometric mean of 5 housekeeping genes, and the p-value for each gene was calculated using a supervised Student T-test. The genes are in the order of their p-values; the best p-values are in blue.



Gene	P-value
BCL6	1.28E -09
PTEN	1.03E -08
ARPC2	2.17E -08
CXCL12	1.37E -07
BRAF	4.18E -07
PCNA	6.33E -07
CLEC3B	1.13E -06

M17: NOT melanoma/lymph node with pigmented histocytes

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



Gene	P-value
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BRAF	4.18E -07
PCNA	6.33E -07
CLEC3B	1.13E -06

M17: NOT melanoma/lymph node with pigmented histocytes

Figure 6: QuantiGene® Plex mRNA cluster analysis. Genes with the best 7 p-values were analyzed using an unsupervised cluster analysis. NOTE: Sample 17 is not a melanoma sample.

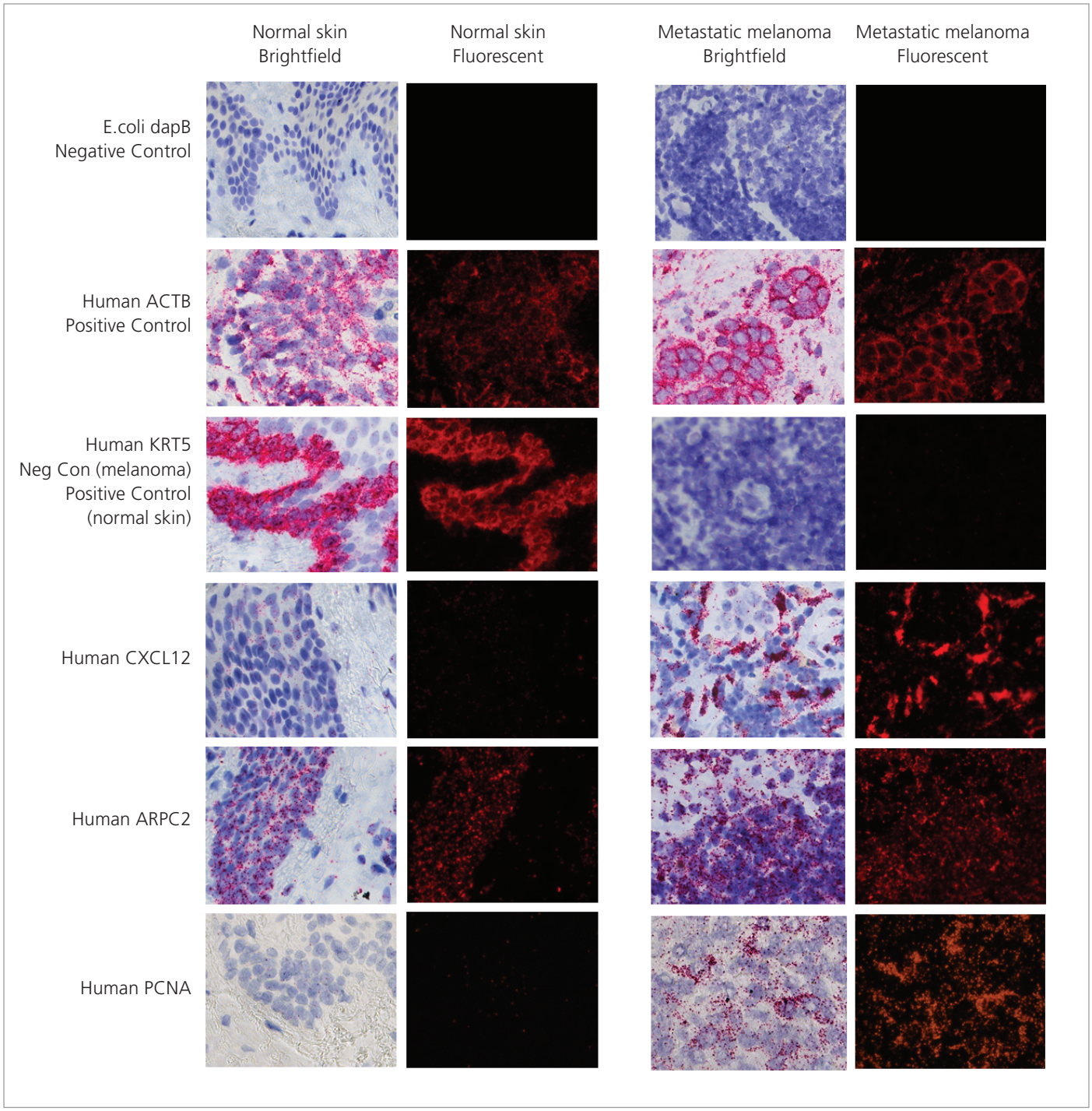

Figure 7: ViewRNA® *in situ* mRNA hybridization. 20X images of matched normal skin brightfield images (first column) and fluorescent images (second column) vs. melanoma skin brightfield images (third column) and fluorescent images (fourth column). ViewRNA® Probe Sets are used are indicated to the side of each image.

Function of the top 7 genes with the best p-values – Citations

- **BCL6** (B-cell CLL/lymphoma 6) — **Repressor of transcription** – **Decreased expression** associated with impaired prognosis — Alonso S. R., *et al*. Progression in cutaneous malignant melanoma is associated with distinct expression profiles: a tissue microarray-based study. *The American Journal of Pathology* **164**(1):193–203 (2004).
- **PTEN** (phosphatase and tensin homolog) — **Signal transduction regulator** – **Decreased expression** association with impaired prognosis — Mikhail M., *et al*. PTEN expression in melanoma: relationship with patient survival, Bcl-2 expression, and proliferation. *Clinical Cancer Research* **11**(14):5153–5157 (2005).
- **ARPC2** (actin related protein 2/3 complex, subunit 2) — **Control of actin polymerization in cells** – **Increased expression** in malignant melanomas — Kashani-Sabeta M., *et al*. A multi-marker assay to distinguish malignant melanomas from benign nevi *PNAS* **106**(15):6268–6272 (2009).
- **CXCL12** (chemokine (C-X-C motif) ligand 12) — **Angiogenesis** – **Increased expression** is involved in melanoma progression — Franco R., *et al*., CXCR4-CXCL12 and VEGF correlate to unveil melanoma progression. *Frontiers in Biosciences* (Elite Ed) **2**:13–21 (2010).
- **BRAF** (v-raf murine sarcoma viral oncogene homolog B1) — **Cell division** – **Increased expression** in malignant melanoma — Curtin J. A., *et al*. Somatic activation of KIT in distinct subtypes of melanoma. *Journal of Clinical Oncology* **24**(26):4340–4346 (2006).
- **PCNA** (proliferating cell nuclear antigen) — **DNA replication** – **Increased expression** with increased risk of developing distant metastasis & association with impaired prognosis — Winnepenninckx V., *et al*. Gene expression profiling of primary cutaneous melanoma and clinical outcome. *Journal of the National Cancer Institute* **98**(7):472–482 (2006).
- **CLEC3B** (C-type lectin domain family 3, member B) — **Cell-extracellular matrix interaction** – **Decreased expression** associated with primary tumors with low tumor thickness — Jaeger J., *et al*. Gene expression signatures for tumor progression, tumor subtype, and tumor thickness in laser-microdissected melanoma tissues. *Clinical Cancer Research* **13**(3):806–808 (2007).

Conclusions

- QuantiGene® Plex 2.0 Assay works directly from 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.
- ViewRNA® ISH Tissue Assay is an *in situ* hybridization assay that has the sensitivity and robustness to measure single molecule mRNA in single cells in human melanoma and skin.
- Sixty-two (62) genes from the literature were validated; first using QuantiGene Plex 2.0 Assay expression data from 7 of the 62 genes was sufficient to discriminate melanoma from matched normal skin; second ViewRNA® ISH Tissue Assay showed both signal intensity and spatial resolution of these 7 validated genes within melanoma and skin tissue samples.
- Taken together, these results demonstrate the power of using both branch DNA technologies to validate melanoma biomarkers.

References

1. Balch C. M., *et al*. Final Version of 2009 AJCC Melanoma Staging and Classification. *Journal of Clinical Oncology* **27**(36):6199–6206 (2009).
2. Haqq C., *et al*. The gene expression signatures of melanoma progression. *PNAS* **102**(17):6092–6097 (2005).
3. Conway C., *et al*. Gene expression profiling of paraffin embedded primary melanoma using the DASL assay identifies increased osteopontin expression as predictive of reduced relapse free survival. *Clinical Cancer Research* **15**(22):6939–6946 (2009).
4. Paik S., *et al*. HER2 Status and Benefit from Adjuvant Trastuzumab in Breast Cancer. *The New England Journal of Medicine* **358**(13):1409–1411 (2008).
5. Hall J. S., *et al*. Exon-array profiling unlocks clinically and biologically relevant gene signatures from formalin-fixed paraffin-embedded tumour samples. *British Journal of Cancer* **104**:971–981 (2011).
6. Ting D. T., *et al*. Aberrant Overexpression of Satellite Repeats in Pancreatic and Other Epithelial Cancers. *Science* **331**(6017):593–596 (2011).

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