Application Note

Cell sorting based on RNA detection in living cells using SmartFlare™ RNA Detection Reagents

Introduction

Cell sorting enables the isolation of highly pure cell subpopulations, increasing the statistical significance of observed relationships between gene expression and phenotype, especially for rare events. Live cell sorting has traditionally been accomplished by detecting the presence of unique cell surface proteins, which have been identified through the use of fluorescently labeled antibodies. However, live cells cannot be sorted based on endogenous intracellular protein markers, because cells have to be fixed and permeabilized for antibody staining. Sometimes cells can be sorted on the basis of transfected reporter constructs; however, this treatment also compromises cell integrity and may perturb cellular pathways, confounding downstream analyses.

Identifying cell types and sorting cells based on RNA expression levels without any transfection reagents or intrusive sample preparation can drastically improve live cell sorting efficiency, physiological relevance, and post-sorting survival rate. Using novel SmartFlare™ RNA detection probes, which are capable of detecting levels of RNA inside living cells, we have demonstrated the ability to sort and further propagate live cell populations purely based on gene expression levels or in combination with surface markers detected with antibodies. This technology eliminates the need for permeabilization or transfection reagents to interrogate the cytoplasmic content of cells, leaving the cells intact and viable after sorting. More importantly, because the particles are inert and leave the cells unharmed, the cells are available for use in downstream assays, enabling researchers to study additional biomarkers within the same sorted cell populations.

The ability to detect and separate live cells based on the level of a specific RNA target provides a new opportunity to study cellular functions and identify rare cell types such as certain tumor cells and cancer stem cells. This technology enables the sorting of cell populations that were previously difficult to sort, and improves sorting accuracy by using biologically relevant intracellular markers.

Methods

Determining cell viability with respect to SmartFlare™ treatment

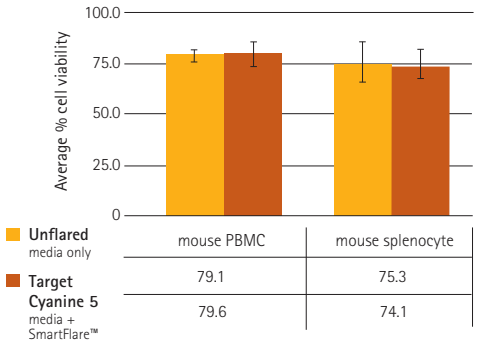
Freshly harvested mouse PBMCs and splenocytes (20,000 cells per well of a 96-well plate) were incubated with either RPMI 1640 medium alone or medium containing 1000X target-Cyanine 5 SmartFlare™ probe for 16 hours, after which cell viability was measured using Trypan Blue staining.

Gene expression profiling

MDA-MB-231 cells were seeded in 12-well plates at 150,000 cells/well 16 hours prior to SmartFlare™ treatment. Cells were then incubated with EGFR SmartFlare™ probe following a 1:1000 dilution of stock solution, Scramble Control SmartFlare™ probe, or an equivalent volume of phosphate-buffered saline (PBS) for 16 hours. After SmartFlare™ treated, cells were lysed and total RNA was harvested. Whole genome expression analysis was performed on each sample using Illumina® Human HT-12 v4 Expression BeadChips and the Illumina® iScan microarray scanning system.

mRNA detection and sorting

Hs578t and MCF-7 cells were mixed in a 1:1 ratio prior to detection of Twist mRNA (Figure 2). SmartFlare™ Twist mRNA detection probe was then added to the culture media overnight. The following morning, the mixed cell population was sorted by fluorescence-activated cell sorting. Cells were washed using Hanks' Balanced Salt Solution (HBSS), detached using Accutase™ reagent, collected in a 15 mL conical tube, centrifuged at 500 x g and resuspended in 1 mL of culture medium. Sorting was performed using a MoFlo™ XDP cell sorter (Beckman Coulter). Sorted populations were then returned to cell culture. Both high and low Twist-expressing populations were separated and collected for further gene expression analysis by quantitative reverse transcription-polymerase chain reaction (qRT-PCR, Figure 3). RT-PCR was performed for Twist, EGFR, and ESR1 on both the high and low sorted products.



	mouse PBMC	mouse splenocyte
Unflared media only	79.1	75.3
Target Cyanine 5 media + SmartFlare™	79.6	74.1

Figure 1.
SmartFlare™ probes do not significantly alter viability of mouse splenocytes or PBMCs. Average cell viability values are depicted above for each experimental condition (n=2).

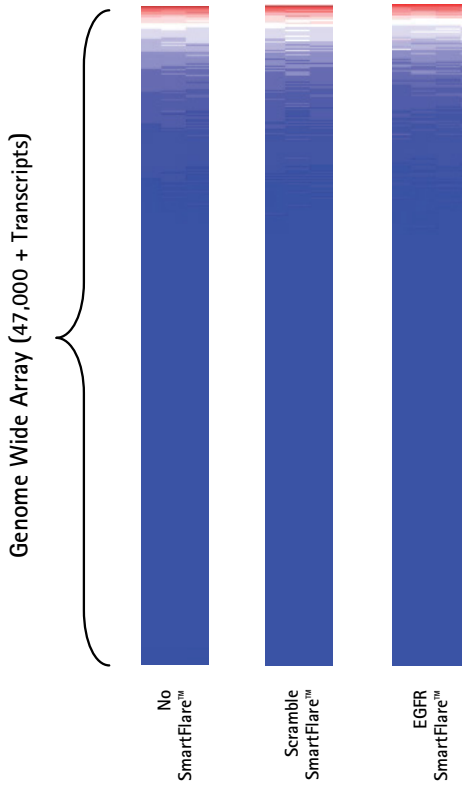


Figure 2.
SmartFlare™ probes do not significantly change gene expression in cells. Color scale represents quantile normalized fluorescence values of gene expression. Three biological replicates, each consisting of three technical replicates, were investigated for each treatment condition.

qRT-PCR

Total RNA was extracted and added to the TaqMan® RNA-to-Ct™ 1-step kit (Life Technologies). TaqMan® qRT-PCR primers were used for analysis for all genes (Invitrogen catalogue #4331182; ESR1 assay ID — Hs00174860_m1, EGFR assay ID — Hs01076078_m1, Twist assay ID — Hs01675818_s1). qRT-PCR was carried out using a LightCycler® 480 system (Roche).

microRNA detection

HUVEC and HeLa cells were mixed in a one to one ratio and then incubated with the miR 155 SmartFlare™ RNA detection reagent overnight. The following morning the cells were then sorted based on the expression levels of miR-155. Isolated populations of high and low miR-155-expressing cells were retained and subsequently used for a TNFα treatment which was expected to upregulate Vascular Cell Adhesion Molecule (VCAM) in HUVEC cells but should not affect HeLa cells as they do not express VCAM (Figure 6)[3]. High and low miR-155-expressing, sorted populations were both stimulated with 10 ng/mL of TNFα for 6 hours followed by VCAM antibody staining. Cells were detached using Accutase™ reagent, resuspended in culture medium and analyzed on a guava easyCyte™ 8HT flow cytometer.

Results

Cell viability is not affected by the addition of SmartFlare™ probe.

One advantage of SmartFlare™ probes is that they can be used to detect RNA without disrupting normal cellular processes. To confirm that the probes are nontoxic, we tested the viability of mouse splenocytes and PBMCs that were either incubated overnight in culture medium only, or in culture medium plus SmartFlare™ probe. No significant change in viability was observed (Figure 1).

SmartFlare™ probes do not affect gene expression.

One of the drawbacks of traditional methods of RNA detection in live cells is that, often, transfection is required for the probe to enter the cell, and the transfection process itself can alter gene expression in ill-characterized, unpredictable ways[1]. In contrast, SmartFlare™ probes do not require transfection. To study their effects on cellular processes, whole genome expression profiling was conducted on MDA-MB-231 cells treated with EGFR Ms-Cyanine 5 SmartFlare™ RNA Probe, Scramble Control SmartFlare™ probe, or an equivalent volume of PBS. No significant gene expression changes (≥1.5-fold) were observed across the treatment conditions.

Detection of mRNAs in individual cells provides richer information.

Twist expression was previously determined by RT-PCR to be higher in Hs578t cells when compared to MCF-7 cells. Our intracellular RNA detection technology showed the same trend (Figure 3); however, because we were able to analyze expression in individual, intact cells, we obtained additional information about the population distribution. Specifically, the broader peak we observed for MCF-7 cells (orange histogram) indicated that MCF-7 cells exhibited a wider range of Twist expression than did the Hs578 cells, which produced a narrower, slightly bimodal peak (yellow histogram). In contrast, the RT-PCR data merely provided the average level of gene expression present in the mixed lysate.

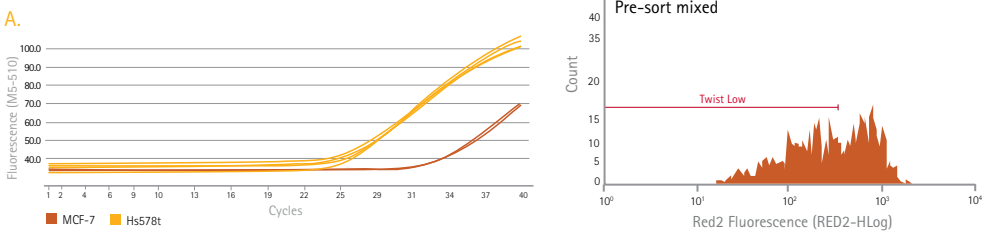


Figure 3.

Twist expression levels in Hs578t and MCF-7 cells are distinguishable by intracellular detection as well as by RT-PCR.
A. Confirmation of Twist expression levels by RT-PCR.
B. Twist expression in Hs578t and MCF-7 cells as determined using SmartFlare™ technology and analyzed by flow cytometry.

Since SmartFlare™ technology is nontoxic and leaves the cells unharmed following detection, the same cells which were sorted based on Twist mRNA expression were reused to detect expression of other genes of interest using RT-PCR (Figure 4). Results were consistent with gene expression profiles of the aforementioned cell lines[2].

A. Expected mRNA profiles

	TWIST	EGFR	ESR1
Hs578t	High	High	Low
MCF-7	Low	Low	High

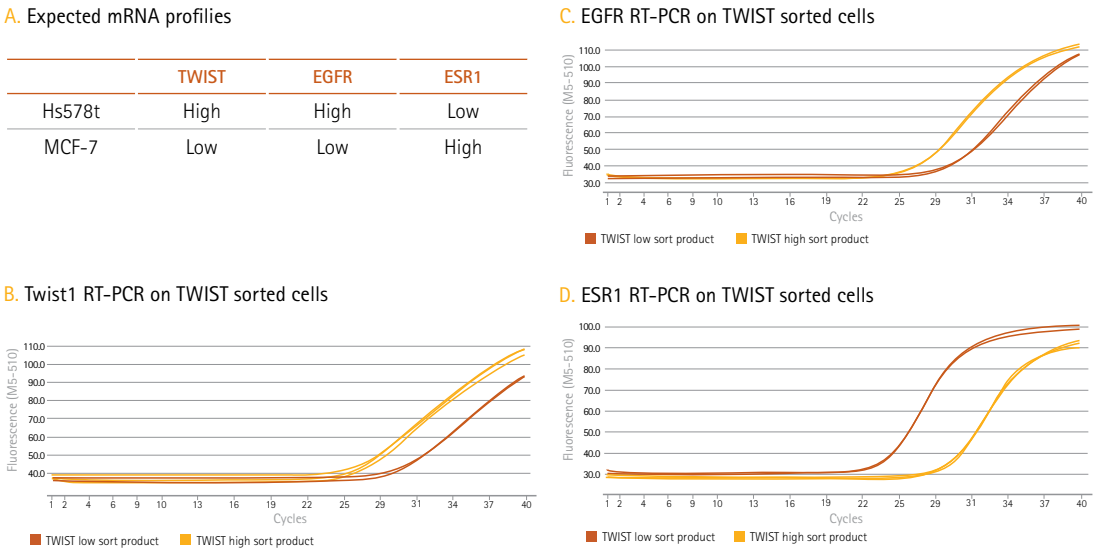


Figure 4.

Follow-up RT-PCR on Twist-sorted cells. Hs578T and MCF-7 cells have distinct mRNA expression profiles as shown in A. RT-PCR assays for Twist1 B, EGFR C, and ESR1 D were used to analyze high and low Twist-expressing cells and displayed profiles that were consistent with expected results.

Sorting based on microRNA expression. miR-155 has been implicated in the development and pathologic progress of hypertension and is known to be highly expressed in the vascular endothelium[2]. Detection of miR-155 was used to sort a mixed population of HUVEC and HeLa cells. HUVEC cells expressed miR-155 at a higher level than HeLa cells as is evident in Figure 5.

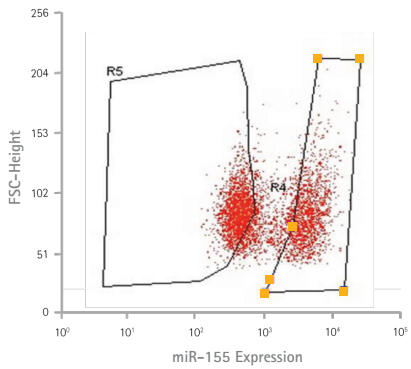


Figure 5.

Sorting based on miR-155 expression level differences in HeLa and HUVEC cells. miR-155 is expressed at a higher level in HUVEC cells than in HeLa cells, allowing for sorting of the mixed population of cells using SmartFlare™ technology. Gates are shown drawn around miR-155 high and miR-155 low populations, which were sorted accordingly.

Use of sorted cell populations for downstream assays. Because SmartFlare™ probes are nontoxic and viability remains high following detection, sorted products can be returned to culture, essentially unaltered. This enables researchers to use these sorted cells for further downstream applications. In this study, both miR-155-positive and miR-155-negative sort products were returned to culture and subsequently tested for their ability to upregulate VCAM expression after TNFα treatment. As predicted, the HUVEC cells, which were high in miR-155 expression, upregulated the expression of VCAM following the TNFα treatment, while the HeLa cells (which do not express TNFα receptors) did not respond to the treatment (Figure 6)[3].

Conclusion

We have demonstrated the ability to sort live cells based on intracellular gene expression using our novel RNA detection technology. The sorted products were then returned to culture where they remain viable and unchanged following detection, enabling downstream analyses, such as antibody staining, flow cytometry and RT-PCR. Sorting cells using any miRNA or mRNA of interest followed by the ability to use those same cells for additional experiments makes this technology an extremely powerful research tool. Specifically, we have shown that SmartFlare™ probes enable the simultaneous detection of multiple RNAs, as well as enables the researcher to reuse those same live cells for further protein analysis, providing a link between the transcriptome and the proteome that was missing until now. The ability to sort cells and obtain a highly enriched cell population based on gene expression also greatly increases the sensitivity of cell analysis—analyzing the molecular roles of rare events is now possible.

References

1. Manakov SA et al. Neuronal miRNAs stabilize the neuronal transcriptome. EMBL-EBI database, Experiment E-MTAB-686. 2011.
2. Vesuna F et al. Twist contributes to hormone resistance in breast cancer by downregulating estrogen receptor-α. Oncogene. 2012 Jul 5;31(27):3223-34.
3. Sen CK et al. Micromanaging vascular biology: tiny microRNAs play big band. J Vasc. Res. 2009: 46(6): 527-540.
4. Mackay F et al. Tumor necrosis factor alpha (TNFα-alpha)-induced cell adhesion to human endothelial cells is under dominant control of one TNFα receptor type, TNF-R55, J Exp Med. 1993 May 1; 177(5): 1277–1286.

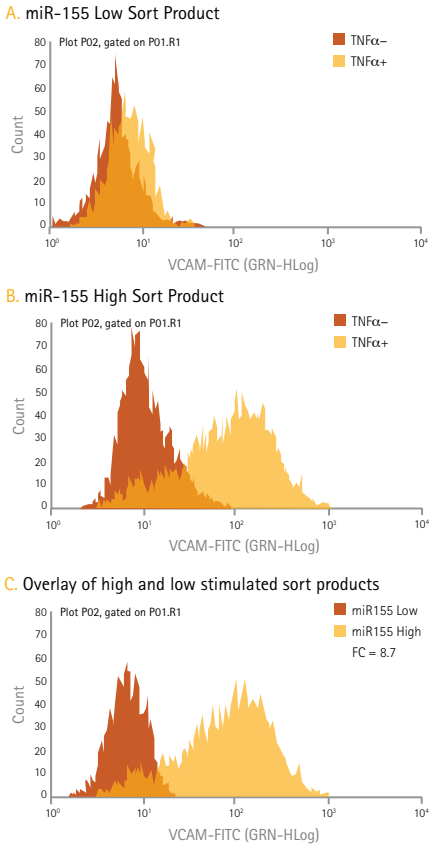


Figure 6.
TNFα treatment of miR-155 sort products followed by VCAM antibody staining and detection using flow cytometry.
A. Stimulation of the miR-155 low population with TNFα showed no significant increase in VCAM staining. B. TNFα-stimulation of miR-155 high sort product showed significant increase in VCAM staining. C. Overlay of TNFα-stimulated miR-155 low and high sort products showed an 8.7 fold increase in mean fluorescence intensity (MFI).

Featured Products

Product	Catalogue No.
EGFR Ms-Cyanine 5 SmartFlare™ RNA Probe	SF-116
Scramble-Cyanine 5 Control SmartFlare™ RNA Probe	SF-102
miR-155 Hu-Cyanine 5 SmartFlare™ RNA Probe	SF-184
Accutase™ Reagent	SCR005
EndoGRO Human Umbilical Vein Endothelial Cells	SCCE001
guava easyCyte™ 8HT Flow Cytometer	0500-4008

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