Technical Guide

SmartFlare™ RNA Detection Probes: Principles, protocols and troubleshooting

Principles of SmartFlare™ technology

RNA detection traditionally requires transfection, laborious sample prep, RNA amplification and/or detection based on standard curves. In contrast, SmartFlare™ RNA Detection Probes are endocytosed by live cells using existing cellular endocytosis machinery. Sample prep is unnecessary; simply add SmartFlare™ Probes to your cultures, incubate overnight and detect the next day. Over time, the probes exit the cell, without adverse effects, allowing for subsequent downstream assays.

Each SmartFlare™ probe consists of a gold nanoparticle conjugated to multiple copies of a double-stranded oligonucleotide, in which one strand (the "reporter strand") bears a fluorophore that is quenched by its proximity to the gold core (Figure 1). When the nanoparticle comes into contact with its target RNA, the target RNA binds to its complementary "capture" strand and displaces the reporter strand. The reporter strand, whose fluorophore is now unquenched, fluoresces and can be detected using any fluorescence detection platform.

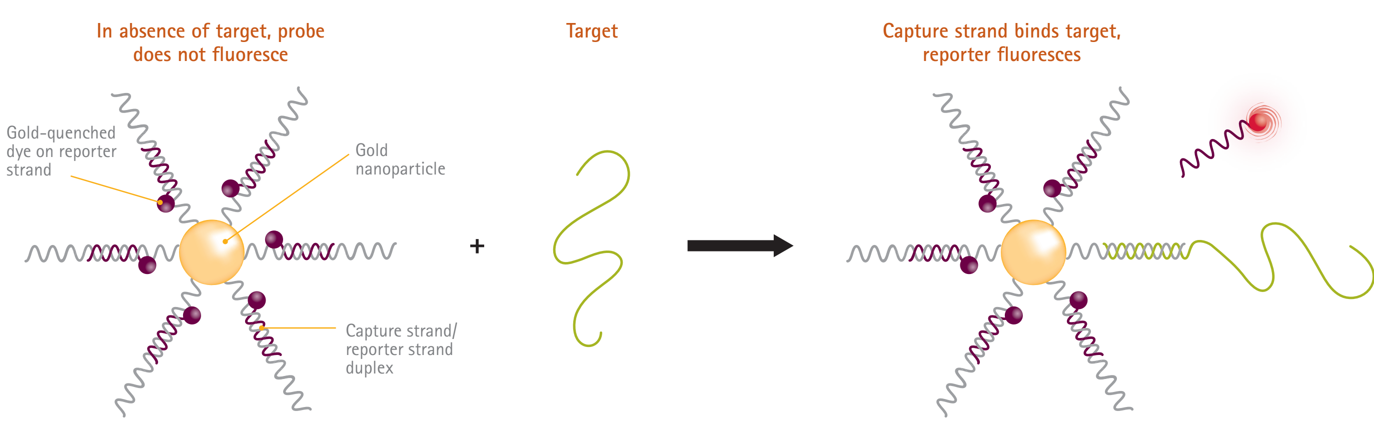


Figure 1.
Molecular mechanism of SmartFlare™ RNA Detection Probe

EMD Millipore is a division of Merck KGaA, Darmstadt, Germany

Experimental setup

Summary of basic procedure:

(detailed cell testing protocol on page 4)

1. Add cells to well at 60-80% confluency
2. Prepare SmartFlare™ reagent (see below)
3. Add reagent to cells (100pM)
4. Incubate overnight (16 hours)
5. Analyze using fluorescence detection platform of choice

Materials required but not provided:

1. Sterile, nuclease-free water (Milli-Q® water recommended)
2. Phosphate-buffered saline, sterile
3. Laminar flow hood
4. Calibrated multichannel pipettor
5. Vortexer
6. Tissue culture incubator
7. RNAse/DNAse-free pipette tips and microtubes
8. Multiwell culture plates

Storage and handling of SmartFlare™ reagent:

Stable for five years at 2-8 °C degrees in lyophilized format ONLY. After reconstitution, store at room temperature* for up to 1 year.

*Warning: after reconstitution, product is sensitive to both cold and hot temperatures. A stable room temperature of 23-27 °C is recommended for storage.

SmartFlare™ materials:

(see Figure 2 for schematic)

1. Target-specific SmartFlare™ probe
2. Cellular uptake control
3. Scramble control for specificity
4. Housekeeping control – binds to RNA expressed at levels that do not change with respect to independent variables

Prepare SmartFlare™ reagent:

(reconstitution of lyophilized reagent)

Add 50 uL sterile, nuclease-free water in a dropwise fashion. Tap the tube repeatedly to dissolve lyophilized material. Vortex for 5-10 seconds.

After reconstitution, store at room temperature for up to one year, protected from light. Product must be handled with gloves because the product can be absorbed through the skin.

Before adding reconstituted reagent to cells, check its appearance and refer to Table 1 for interpretation and/or actions to take prior to use.

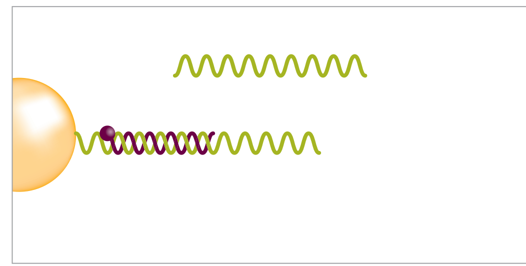
Interpretation of lyophilized reagent

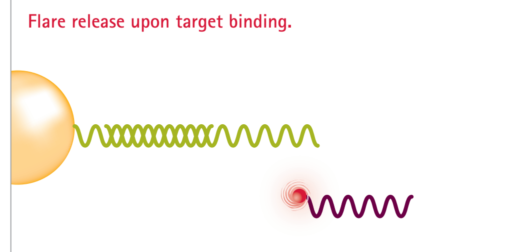
Table 1.
For best results, compare reagent appearance to this table before use and perform recommended actions.

Appearance	Interpretation	Actions
	Material is of uniform color and no precipitation is evident.	None
	Material may appear translucent with or without minor precipitation	Brief sonication for 10 seconds in a sonicating water bath followed by vortexing for 5 seconds may return product to normal homogenous consistency.
	Material has clearly precipitated. Red (opaque) and clear phases are evident.	Do not use material. Order replacement material.

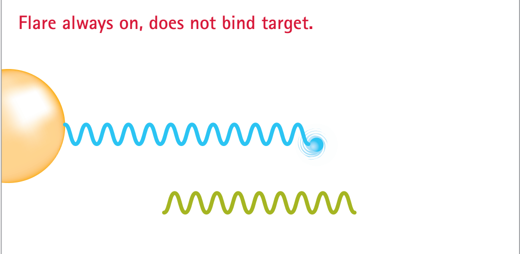
Experimental controls

A. Target Probe

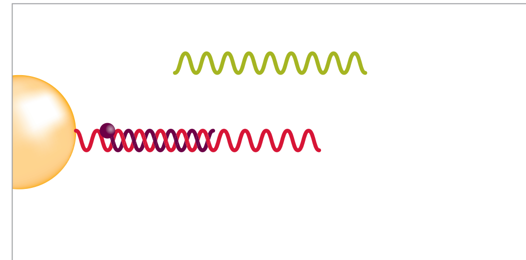


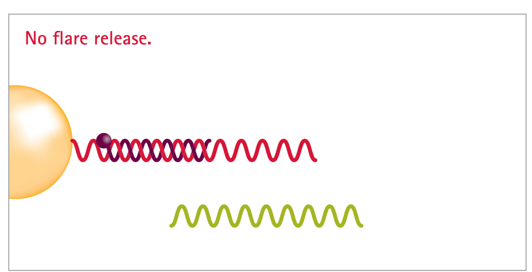


B. Uptake Control

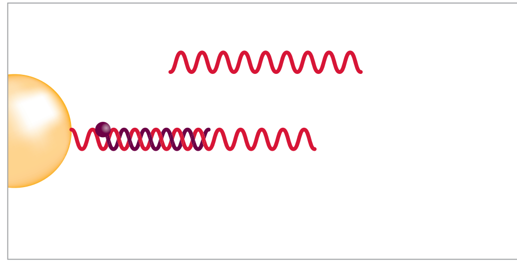


C. Scramble Control





D. Housekeeping Control



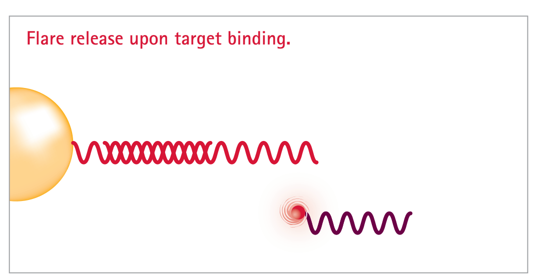


Figure 1.

SmartFlare™ Control:

A. Example of typical target probe

B. Uptake Control or positive control probe that is always "on" inside the cell

C. Scramble Control or negative control that does not recognize any sequence within that cell

D. Housekeeping Probe that can be used as a positive control recognizing sequences known to express at high levels within thecell

Experimental procedure

For the best results, perform each SmartFlare™ treatment or control treatment using at least three replicates. Refer to Figure 2 for an example of an experiment performed in 96-well format.

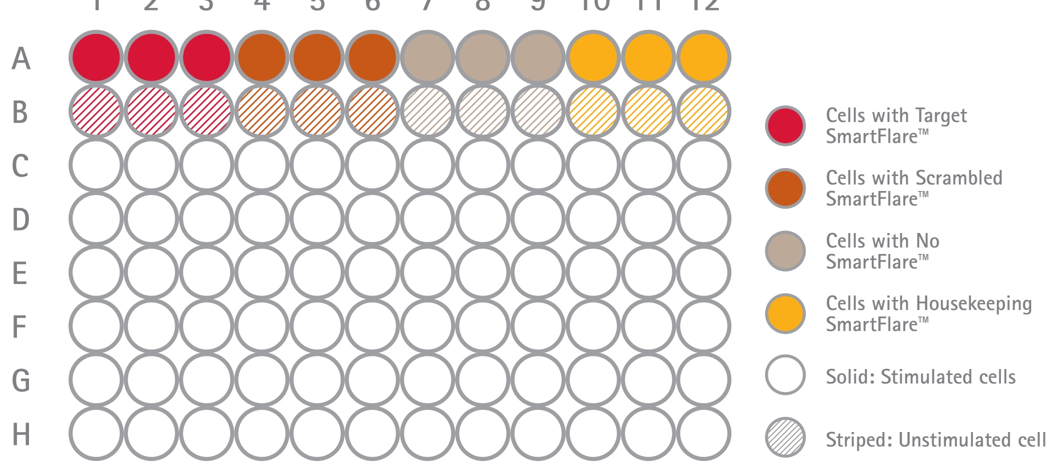


Figure 2.
Sample map of a typical experiment analyzing expression of a single RNA with respect to cell treatment.

Cell testing protocol

(recommended protocol for testing a cell-probe combination for the first time)

1. Plate cells at desired density (typical example: 30,000 cells in 200 μL complete medium in each well of a 96-well plate)
2. Dilute reconstituted SmartFlare™ reagent 1:20 in sterile phosphate-buffered saline to create working solution
3. Add 4 μL diluted SmartFlare™ reagent to each well of cells (which are 60-80% confluent)
4. Incubate overnight (16 hours) at appropriate temperature (37 °C), CO2 (5%) and relative humidity.
5. Detect fluorescence using platform of choice: flow cytometer, imaging cytometer, fluorescence microscope, etc.

Fluorescence detection parameters

To detect SmartFlare™ signals in treated cells, use a fluorescence detection platform that excites the specific conjugated fluorophore(s) at the wavelength of maximum excitation (excitation (nM) in Table 2) and that is set up to acquire signals at the wavelength of maximum emission (emission (nM) in Table 2).

Physical properties of Fluorophores

Table 2.
Physical properties of fluorophores conjugated to SmartFlare™ RNA Detection Probes

Fluorophore Dye	Excitation (Lamda, nM)	Emission (Lamda, nM)	PMT	Laser	Microscope Filter
CY5	650	670	Red2	Red	CY5 compatible filter
CY3	550	570	Yellow	Green	CY3 compatible filter

Experimental results

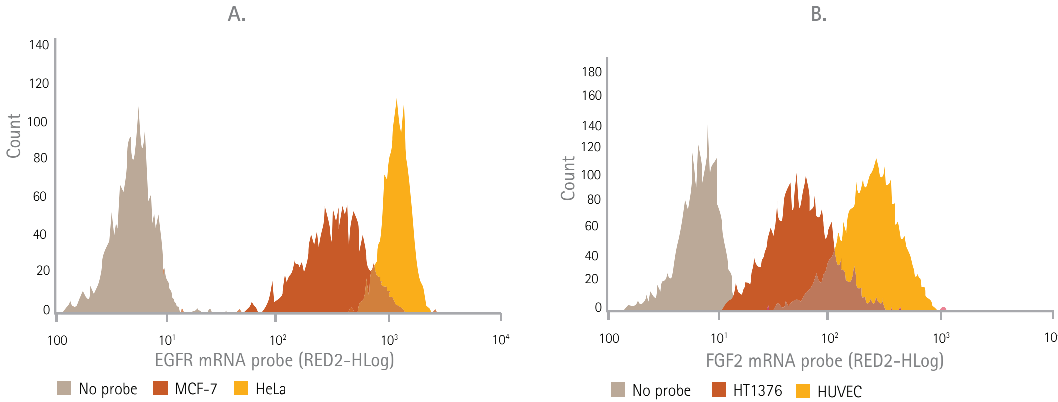


Figure 3.

Flow Cytometry SmartFlare™ Data

Using our technology to determine the mRNA levels of EGFR mRNA (A) in HeLa and MCF-7 cells as well as FGF2 mRNA (B) in HUVEC and HT1376 cells both of which correlate to their RT-PCR levels. Flow cytometry provides added information at the single cell level as well as how the expression is distributed within the population.

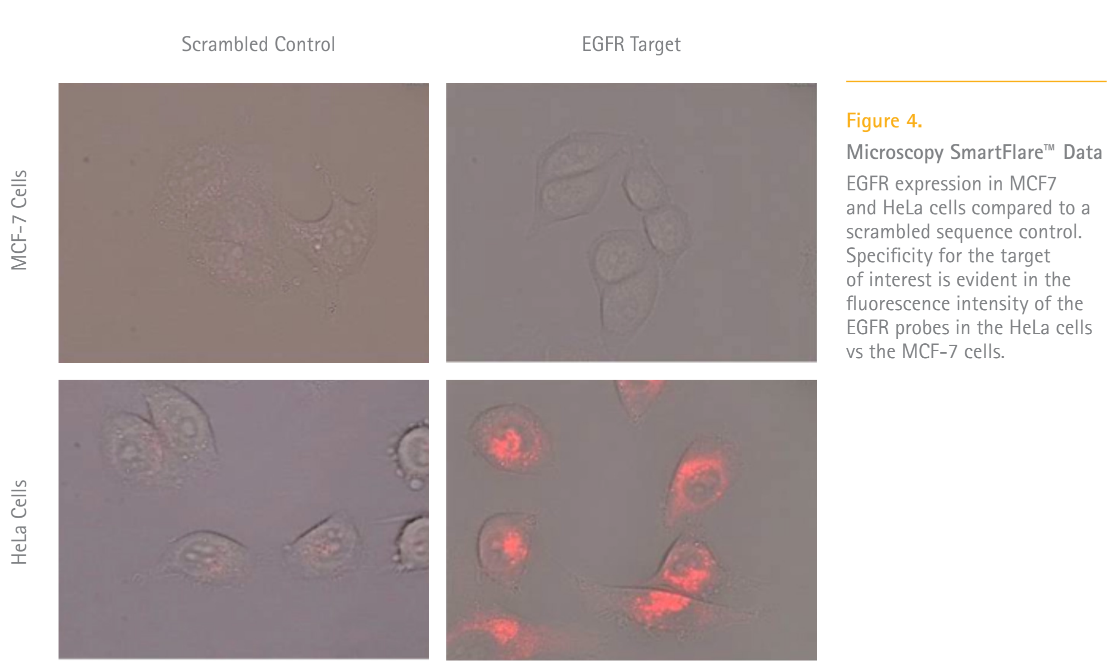


Figure 4.

Microscopy SmartFlare™ Data

EGFR expression in MCF7 and HeLa cells compared to a scrambled sequence control. Specificity for the target of interest is evident in the fluorescence intensity of the EGFR probes in the HeLa cells vs the MCF-7 cells.

Troubleshooting

	Situation	Cause	Action
Instrument	Instrument can detect fluorophore but you are not seeing signal	Malfunctioning instrument	• Troubleshoot instrument with control sample (ie. fluorescent beads) • Use an uptake control or housekeeping gene with same fluorophore
	Instrument not detecting fluorophore	Wrong filter/laser compatibility with fluorophore	• Determine correct fluorophore-instrument compatibility • Get new SmartFlare™ reagent or instrument filter/laser set
Precipitated material	Material has precipitated out of solution	• Improper storage conditions • Temperature fluctuations • Improper reconstitution • Contamination	• Brief sonication for 10 seconds in a sonicating water bath followed by vortexing for 5 seconds may return product to normal homogenous consistency. • If above does not work, product will need to be replaced
Reagent not entering cells	See no signal in target, uptake control or housekeeping controls	• Poor cell health; dead and dying cells in sample • Cells have low endocytosis rate and therefore are not up taking the particles	• Check viability • Determine if cell types have been validated to work with SmartFlare™ reagents; contact Technical Service to determine if cell types have been previously used with SmartFlare™ technology • Increase concentration on incubation times
Unexpected results	Experimental error	• Wrong species • Bad target design • Cell types don't express target at detection time point • Stimulation/modulation not successful	• Repeat experiments with correct, target-specific designs • Perform experiment with predicate method
	Signal detected from scramble control is higher than target probe signal	• Degradation of scramble probe • Low target expression	• Check material for precipitation • Replace scramble control with fresh material
	Do not see differences in expression as expected	• Cell uptake differences • Difference in expression too small to detect	• Use uptake controls to normalize for uptake • Contact Technical Service; they can inform you of expected uptake for given cell types • Compare to alternate methods of RNA detection (RT-PCR, immunofluorescence, microarray).

Troubleshooting

	Situation	Cause	Action
Bad target design	Custom designed probe not detecting target, but controls work	Flaw in the design of custom probe	Design and order an alternate probe
		Target may not be ex-pressed in cell type in the condition specified	Confirm that expression is expected with other techniques (RT-PCR, immunofluorescence, microarray)
		Levels too low to detect above background	Consider the possibility that the target is a low-expressing gene and may not be able to be detected using SmartFlare™ probes
	Manufacturer-designed ("shelf") probe not detecting target, but controls work	Target may not be ex-pressed in cell type in the condition specified	• Test reagent in one of our validated cell models • Confirm that expression is expected with other techniques (RT-PCR, immunofluorescence, microarray)
		Levels too low to detect above background	Consider the possibility that the target is a low-expressing gene and may not be able to be detected using SmartFlare™ probes

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