DESIGNING RELIABLE EXPERIMENTS WITH
THE FIREFLY™ microRNA ASSAY

To obtain reliable microRNA expression data, it is important that an experiment be designed to accurately subtract background signal, allow for appropriate normalization, and assess inter-well variability. Ideally, every experiment performed with a Firefly™ microRNA assay includes (1) **positive and blank controls in each well**, (2) **negative control wells**, and (3) **biological or technical replicates**.

(1) Controls within each well

The Firefly™ microRNA assay utilizes both a positive control ("X-Control") and a blank ("Blank") by default in each custom panel. In addition to these, it is strongly recommended that users include at least one endogenous control.

Positive control particles bear probes for a miRNA-*like* target, **X-control**, that is present in Firefly Hybridization Buffer at a concentration of ~1 fmol per 25 µl. This control gives confidence that the assay was successfully implemented in every well.

BLANK particles bear no probe, giving a baseline level of the background fluorescence in every assay well. This signal should be subtracted from those obtained by other targets in the same well.

Endogenous controls are small nucleolar RNAs or microRNAs expressed at consistent levels under a variety of conditions across tissues. These controls are important for signal normalization between sample types and treatments. Users are given the freedom to select endogenous controls most appropriate for their applications and may prefer to include more than one endogenous control in their panel. Firefly suggests the following endogenous controls:

Human: RNU44, RNU48, RNU6B
Mouse: snoRNA202, snoRNA234
Cell Lines: miR-16-5p (same sequence for human, mouse, and rat)
C. elegans: U18

Sample 10-Plex microRNA Panel

mmu-mir-30a-5p, UGUAAACAUCCUCGACUGGAAG (mirBase entry)
mmu-mir-105, CCAAGUGCUCAGAUGCUUGUGGU (mirBase entry)
mmu-mir-130a-3p, CAGUGCAAUGUUAAAAGGGCAU (mirBase entry)
mmu-mir-135b-3p, AUGUAGGGCUAAAAGCCAUGGG (mirBase entry)
mmu-mir-298-5p, GGCAGAGGAGGGCUGUUCUUCCC (mirBase entry)
mmu-mir-361-5p, UUAUCAGAAUCUCCAGGGGUAC (mirBase entry)
Endogenous Controls { snorna234, Mouse Endogenous CTL
Endogenous Controls { snorna202, Mouse Endogenous CTL
Exogenous Controls x-control, External Control
Blank blank, No Probe CTL

(2) Negative control wells

In order to obtain an accurate measurement of the background signal for each microRNA in a panel, it is necessary to run negative control wells, where carrier buffer is used in place of a biological sample. Furthermore, the use of multiple negative control wells allows users to estimate inter-well variability, giving more confidence to the results obtained. Firefly BioWorks strongly recommends the use of at least three negative controls every time an assay is performed. An example of negative control wells is provided in the sample plate layout below.

(3) Replicates

The use of replicates gives statistical meaning to results by, for example, enabling the calculation of mean and standard deviation. Replicates can be performed at the stage of sample preparation (biological) or assay (technical).

Biological replicates are those in which the same conditions are used to treat and prepare samples from different sources. These replicates are important in determining the biological variation within a population. An example of this type of replicate is serum samples derived from 3 different mice injected with the same TNF inhibitor.

Technical replicates are those in which samples derived from the same source are assayed multiple times. These replicates are important in determining reproducibility of the assay. Ideally, every assay has technical replicates.