# NanoString PlexSet, an alternative to qRT-PCR assays

## (A versatile tool with many research applications)

**Lytle, C.**[1, 2], Greenham, K.[3], McClung, CR.[3] Cumbal, N.[1], Rueckert, E.[4], Fuhrman, K.[4], Kharkia A.[4]

Dartmouth Hitchcock Norris Cotton Cancer Center, Lebanon, NH, [1]Geisel School of Medicine at Dartmouth, Hanover, NH, [2] Dartmouth College, Hanover,NH, [3] NanoString Technologies, Seattle, WA,[4]

## Abstract

NanoString technology is based on the direct digital detection of nucleic acid sequences using fluorescent optical color-coded barcode tags. It is designed as a Medium-Throughput technology with each cartridge holding 12 samples that enables rapid and simple detection of up to 800 DNA, RNA and/or protein targets for each sample. We have been conducting alpha testing of a new High-Throughput NanoString assay that is now commercially available under the name of PlexSet. PlexSet enables direct digital detection of up to 24 targets in 96 samples per run. Sample types can include but are not limited to total RNA from fresh, frozen or FFPE samples, whole cell lysates, and tissue lysates. Probes are made with target specific sequences and tag specific sequences at 5' and 3' tailing ends. The selected probe sets are mixed and diluted into pools, then combined with one of the 8 unique barcoded PlexSet TagSets. These probe and TagSet pools combined and hybridized with their nucleic acid targets, with a distinct TagSet in each row of a 96 well plate. Each of the 12 columns from the 96-well plate is then pooled for processing on the NanoString nCounter Prep Station. The Prep Station purifies the target/probe complexes and deposits them in a cartridge for data collection. Data Collection is carried out in the NanoString nCounter Digital Analyzer. Images are collected and processed by an algorithm which tabulates digital counts for barcode class.

Some pilot titration experiments are recommended to determine an ideal nucleic acid input range. Like most NanoString assays, the direct digital quantification (no enzymes or amplification), technical assay controls, high sensitivity, and easy data analysis make this an ideal alternative to standard qRT-PCR. We will report on our experience using this technology and why we believe it could easily replace many of the qRT-PCR assays being conducted.

## Introduction

A new High-Throughput NanoString nCounter PlexSet assay is now available. PlexSet enables direct digital detection of up to 24 custom targets in 96 samples per run. Probes selected by the researcher are made with target specific sequences and tag specific sequences at 5' and 3' tailing ends (Figure 1). The selected probe sets (Probes A and B) are mixed and diluted into pools, then combined with one of the 8 unique barcoded PlexSets. These probe and PlexSet pools combined and hybridized overnight with their nucleic acid targets, with a distinct PlexSet in each row of a 96 well plate. Each of the 12 columns from the 96-well plate is then pooled for processing on the NanoString nCounter Prep Station. The Prep Station purifies the target/probe complexes and deposits them in a cartridge for data collection. Data Collection is carried out in the NanoString nCounter Digital Analyzer. Images are processed and an algorithm tabulates digital counts for each barcode class.

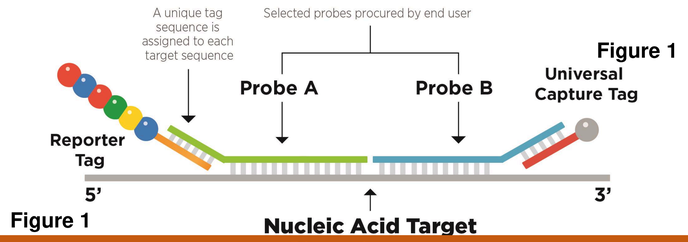
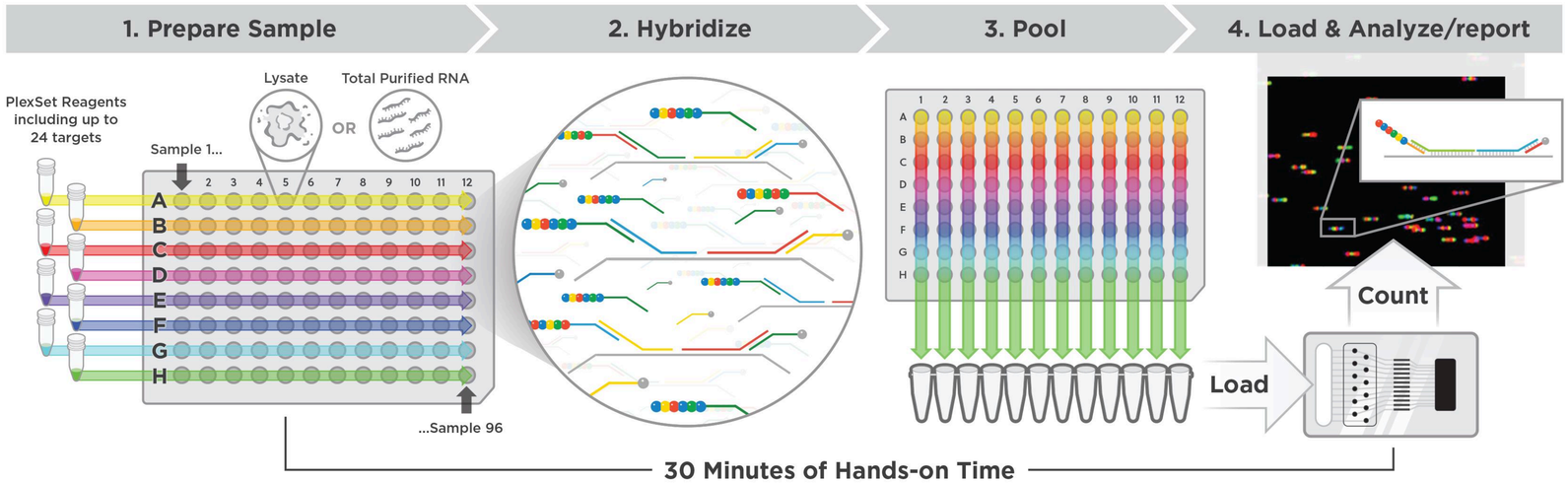



Figure 1

## Discussion

Performing and publishing reproducible qRT-PCR assays involves extensive of validation and information about the materials and reagents being used (Table 1). These assays require time and practice to consistently set-up and design correctly. For a researcher attempting qRT-PCR for the first time the MIQE requirements can be a bit overwhelming. The amount of QC and validations to be performed just for establishing the proper assay conditions can take weeks or even months to be optimized depending on the scope of the experiment. NanoString PlexSet reagents eliminate many of the validation steps needed for expression assays (Table 2). Like most NanoString assays PlexSet is a hybridization assay that does not rely on Reverse Transcriptase (RT) or amplification for detection. The positive and negative controls are already incorporated into all PlexSet assays and the reference genes are selected by the researcher as with standard qRT-PCR experiments. The only optimization requirement is establishing sample input concentrations. This is accomplished using one tag set and the probe A and B pools in an assay. In our opinion the ease and simplicity of the PlexSet assay make it an ideal alternative assay for expression studies, especially for novice qRT-PCR researchers




| Essential Information needed | qRT-PCR | PlexSet |
|---|---|---|
| Sample | RNA or DNA | RNA, DNA, Cell Lysates, serum, and body fluids |
| Reverse Transcription | Reagents used for RNA to convert to cDNA | Not Applicable(N/A) |
| Nucleic Acid Assessment | Purity/Yield /RIN (RNA)/Electrophoresis | Purity/Yield /RIN (RNA)/Electrophoresis |
| Technical Replicates | Yes | No |
| Target Information | Sequence and location needed | Sequence and location needed |
| Splice Variant information | Needed | Needed |
| Multiplexing | 2-5 targets | 3 - 24 Targets |
| Primers | Sequence and Manufacture information | Sequence and Manufacture information |
| qPCR Protocol | Master Mix reagents and cycling conditions | N/A No Amplification preformed |
| Post Reaction processing | N/A no post processing preformed | Samples cleaned to remove unhybridized material |
| Data Acquisition | Samples quantified based on Fluorescence Intensity | Samples counted based on Barcode reads |

**Table 1:** Comparison of assay validation need for both qRT-PCR and NanoString PlexSet

| Validations Needed for Assay | qRT-PCR | PlexSet |
|---|---|---|
| Negative RT control | Yes | No |
| Inhibition Testing by Cq dilutions or Spike-in | Yes | No |
| Contamination Assessment for RNA or DNA | Yes | No |
| Optimization of sample input | Yes | Yes |
| Optimization of assay efficency | Yes | No |
| Reference Genes | Yes | Yes |
| Biological Replicates | Yes | Yes |
| NTC's | Yes | No |
| Multiplex efficiency and LOD for each assay | Yes | No |

**Table 2:** Essential information and parameters for qRT-PCR and NanoString PlexSet

## NanoString PlexSet for circadian gene expression study in *arabidopsis thaliana.*

### Experimental Design

- 7 Genotypes + 12 time points + 3 biological replicates
- 42 target genes + 3 reference genes = 12,096 NanoString reactions or 48,384 qPCR reactions
- 6 96-well NanoString plates (3 x Probe Set #1 w/ gene targets 1-21, 3 x Probe Set $2 w/ gene targets

| Cost Analysis of Experimental Design | | |
|---|---|---|
| | qRT-PCR | PlexSet |
| cDNA reagents | $6.64 | $0.00 |
| Assay reagents | $0.22 | $1.04 |
| Total Cost | $13,991 | $11,309 |
| Cost per data point | $1.16 | $0.82 |
| Time | > 3 months | 2 weeks |

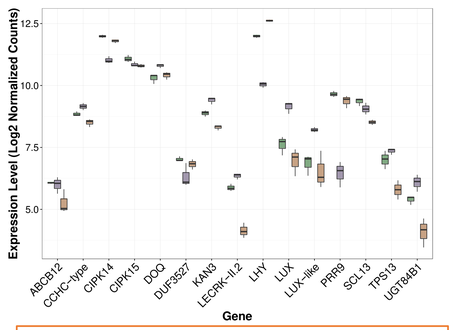



Figure 2. On all plates 3 samples were replicated to assess the variability across plates. The boxplots show the spread of the 3 data points for a subset of genes.

## NanoString PlexSet data to validate RNA-seq time course drought experiment in the crop plant *Brassica rapa.*

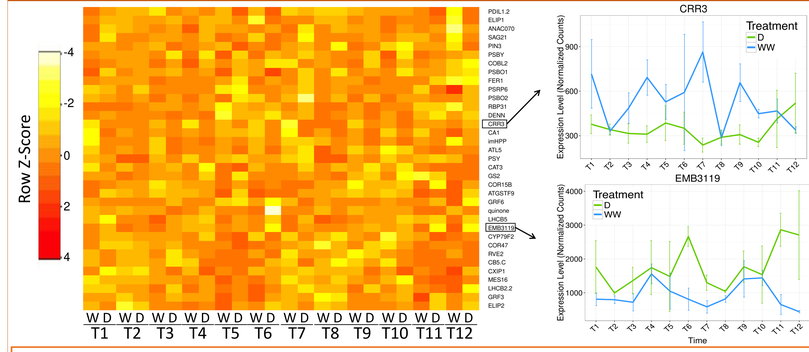



Figure 3. A. Heatmap showing the expression patterns of 35 genes identified in an RNA-seq experiment as drought (D) responsive compared to well-watered (W) controls over a 2-day time course experiment in *Brassica rapa*. B. The expression of two genes showing downregulation (*CRR3)* and upregulation (*EMB3119)* under drought stress, confirming the RNA-seq results.

## NanoString PlexSet Data from CRISPR studies of cMYC Regulators

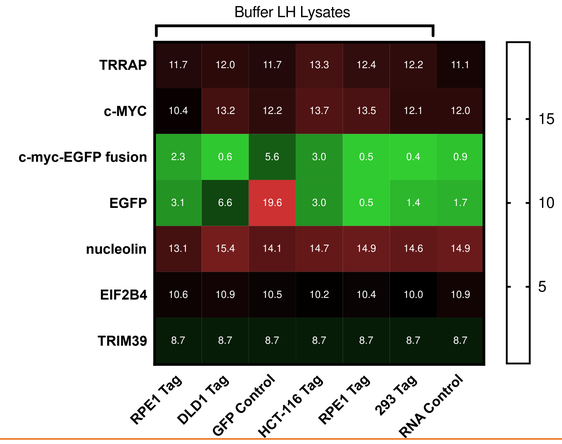



Figure 4. The resulting heat map from a titration assay using cell lysates and total RNA samples. This PlexSet assay was designed to quantify the number of CRISPR modified c-MYC-EGFP fusion cells present. Titration assays are used to establish the optimal number of cells to use for the assay.