A) qInvader^{TM} for microRNA and mRNA Detection and Quantification:

I. Required Reagents and Instrumentation:

1. qInvader™ Core Kit (Catalog No. 99-146).
2. qInvader™ Reverse Transcription Kit (Catalog No. 99-147).
3. qInvader™ Target-Specific Kit (Catalog No. 99-148). A list of available assays can be found at www.twt.com. Alternatively, users may design Target-Specific oligonucleotides for developing their own assays, using qInvader™ designer software at www.twt.com. See Sections VI & VII for design, ordering information and requirements for Target-Specific Kits.
4. Real-time thermal cycler capable of detection of the following fluorescent dyes:

Fluorescent Dye	Excitation wavelength	Emission wavelength	Compatible dye settings
FAM^{TM}	495 nm	520 nm	
Yakima Yellow^{TM}	531 nm	550 nm	JOE^{TM}, HEX^{TM}, and VIC^{TM}
ROX^{TM}	588 nm	608 nm	Texas Red®

II. Two-Step qInvader™ Protocol:

Step 1: Reverse Transcription (RT) Reaction Setup:

1. Prepare RNA samples. For total RNA, use 0.5 to 10 ng of per reaction. For absolute quantification, prepare dilutions of microRNA or mRNA standards for generation of a standard curve.
2. Assemble Reverse Transcription Reaction Mix on ice using qInvader™ Reverse Transcription Kit (Catalog No. 99-147) and qInvader™ Target-Specific Kit (Catalog No. 99-148). To accommodate inaccuracies in pipetting, preparation of *N*+2 reaction volumes for *N* number of samples is recommended.

Reverse Transcription Reaction Mix		
Components	**single reaction volume**	***N*+2 reaction volume**
Reverse Transcription Reaction Buffer 10X	2 µl	
RT Oligo Mix 10X	2 µl	
Reverse Transcription Enzyme Mix 40X	0.50 µl	
RNA sample or standard	1-10 µl	
Nuclease-free Water	add to a final volume 20 µl	
Final Reaction Volume	**20 µl**	

3. Gently vortex the RT reactions and incubate in a thermal cycler programmed for the following steps:

Step	Number of Cycles	Temperature	Time
1	1	42ºC	30 minutes
2	1	95 ºC	5 minutes
3	1	10 ºC	store until use

Step 2: qInvader™ Reaction Setup:

1. Aliquot 2 µl of the RT reaction product (Step 1) into a 96 well plate that is compatible with the real-time thermal cycler that will be used.
2. Prepare qInvader™ Reagents Master Mix to be added to the samples, using qInvader™ Core Kit (Catalog No. 99-146) and qInvader™ Target-Specific Kit (Catalog No. 99-148). To accommodate inaccuracies in pipetting preparation of *N*+2 reaction volumes for *N* number of samples is recommended. Assemble qInvader™ Reagents Master Mix on ice.

qInvader™ Reagents Master Mix		
Components	**Single reaction Volume**	***N*+2 reaction volume**
qInvader™ Mix 10X	2 µl	
qInvader™ Reaction Buffer 10X	2 µl	
qInvader™ Enzyme Mix 40X	0.5 µl	
Nuclease-free Water	13.5 µl	
Final Volume	**18 µl**	

3. Gently vortex the qInvader™ Reagents Master Mix and aliquot 18 µl onto the 2 µl of the RT products that are in the 96-well plate. The reaction final volume is 20 µl

4. Incubate in a real time machine programmed for the following steps:

Stage	Step	Number of Cycles	Temperature	Time	Data Collection
1	1	1	95ºC	2 minutes	No
2	1	40	95 ºC	20 seconds	No
	2		50 ºC	1 minute	FAM™, Yakima Yellow™ & ROX™.

III. One-Step RT-qInvader™ Protocol:

1. Prepare RNA samples. For total RNA, use 0.5 to 10 ng of per reaction. Prepare dilutions of microRNA or mRNA standards for a standard curve. Pipette RNA samples into 96-well plate compatible with the real-time thermal cycler intended for use.
2. Assemble RT-qInvader™ Reagents Master Mix on ice using qInvader™ Reverse Transcription Kit (Catalog No. 99-147) and qInvader™ Target-Specific Kit (Catalog No. 99-148). To accommodate inaccuracies in pipetting, preparations of *N*+2 reaction volumes for *N* number of samples is recommended.

RT-qInvader™ Reagents Master Mix		
Components	**Single reaction volume**	***N*+2 reaction volume**
qInvader™ Reaction Buffer 10X	2 µl	
qInvader™ Mix 10X	2 µl	
qInvader™ Enzyme Mix 40X	0.5 µl	
Reverse Transcription Enzyme Mix 40X	0.5 µl	
Nuclease-free Water	*x* µl	
Final Volume	(20 -– RNA sample volume) µl	

3. Gently vortex the RT-qInvader™ Reagents Master Mix and add to each RNA sample or standard in the 96-well plate adjusting the final volume to 20 µl using nuclease free water.
4. Incubate in a real-time thermal cycler programmed for the following thermal cycling profile:

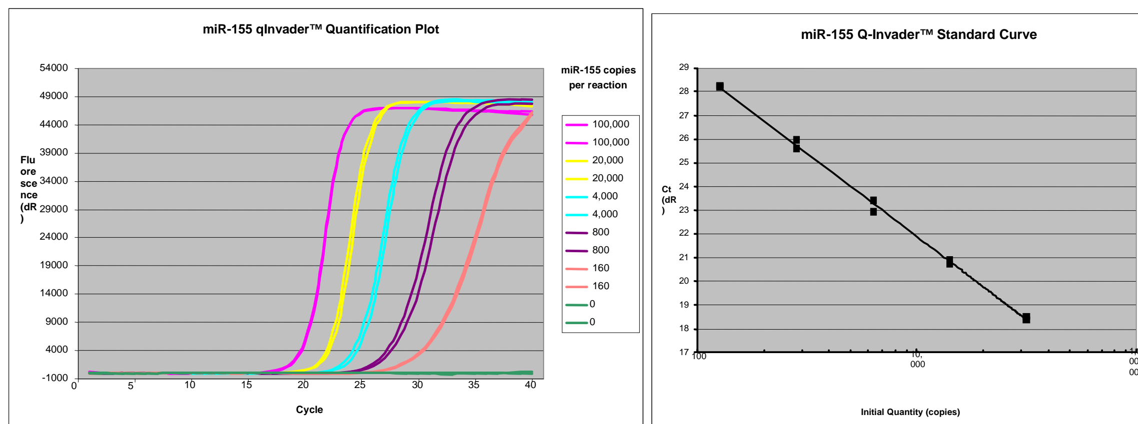
Stage	Step	Number of Cycles	Temperature	Time	Data Collection
1	1	1	42ºC	30 minutes	No
2	1	1	95ºC	2 minutes	No
3	1	40	95 ºC	20 seconds	No
	2		50 ºC	1 minute	FAM™, Yakima Yellow™ & ROX™

IV. Biplex (duplex) qInvader™ Protocol:

1. Both One-Step (III) and Two-Step (II) qInvader™ Protocols can be modified for a biplex format. The biplex format allows users to quantify simultaneously in the same well the RNA of interest (reporting to FAM™ dye) and an internal standard, e.g. a house-keeping gene, reporting to Yakima Yellow™ dye. To modify One-Step and Two-Step qInvader™ Protocols for biplex format, use 2 µl of each Reverse Transcription Oligonucleotide Mix and qInvader™ Oligonucleotide Mix for both the RNA of interest and the internal standard wherever indicated in the Protocols. Water volumes used in the qInvader™ reaction mixes should be adjusted accordingly, to keep final volume of the reactions 20 µl.

V. qInvader™ Data Analysis:

1. Data can be analyzed by viewing the normalized fluorescence versus PCR cycle number as in an example shown below.
2. An optimal cycle threshold is then selected based on the linearity of standard curve when plotting C_t vs Log of copy number. Such a standard curve shown in the example below can used to calculate target copy number in RNA samples.



VI. Design, Requirements and ordering information for qInvader™ Target-Specific Kits:

1. Design of qInvader™ Target-Specific Kit for microRNA or mRNA target sequences can be performed using qInvader™ designer software at www.twt.com.
2. Users have the option to design qInvader™ probes that report to either FAM™ or Yakima Yellow™ dyes. This allows users to perform two same-well assays, one for the RNA of interest reporting to FAM™ dye and the other for another RNA of interest or an internal standard (e.g, in a biplex reaction with a house-keeping gene, reporting to Yakima Yellow™ dye).
3. The qInvader™ designer software generates three oligonucleotide sequences for each target sequence. The first two are a forward primer oligonucleotide and a reverse primer oligonucleotide, which can be ordered using standard DNA synthesis with desalting purification. The third oligonucleotide is the Invader® probe that is blocked at the 3'-end with hexanediol (3'-C6 linker and purified on ion-exchange HPLC column (IE-HPLC). Probes with 3'-hexanediol- (IE-HPLC) may be ordered from Integrated DNA Technologies, IDT (http://www.idtdna.com).

VII. Formulation of Target-Specific Kits:

1. RT Oligo Mix 10X:
   4 µM reverse primer oligonucleotide.

2. qInvader™ Mix 10X:
   4 µM reverse primer oligonucleotide.
   4 µM forward primer oligonucleotide.
   5 µM Invader probe oligonucleotide.

B) qInvader™ for DNA Detection and Quantification:

I. Required Reagents and Instrumentation:

1. qInvader™ Core Kit (Catalog No. 99-146).
2. Target-Specific primers and probes which can be designed using qInvader™ designer software at www.twt.com. See Sections III and IV for design, ordering information and requirements for Target-Specific primers and probes.
3. Real-time thermal cycler capable of detection of the following fluorescent dyes:

Fluorescent Dye	Excitation wavelength	Emission wavelength	Compatible dye settings
FAM™	495 nm	520 nm	
Yakima Yellow™	531 nm	550 nm	JOE™, HEX™, and VIC™
ROX™	588 nm	608 nm	Texas Red®

II. qInvader™ Reaction Protocol:

1. Aliquot 1-10 µl of the DNA target into a 96 well plate that is compatible with the real-time thermal cycler that will be used.
2. Prepare qInvader™ Reagents Master Mix to be added to the samples, using qInvader™ Core Kit (Catalog No. 99-146) and qInvader Target-Specific primers and probe (Sections III and IV). To accommodate inaccuracies in pipetting preparation of *N*+2 reaction volumes for *N* number of samples is recommended. Assemble qInvader™ Reagents Master Mix on ice.

qInvader™ Reagents Master Mix		
Components	**Single reaction Volume**	***N*+2 reaction volume**
qInvader™ Mix 10X	2 µl	
qInvader™ Reaction Buffer 10X	2 µl	
qInvader™ Enzyme Mix 40X	0.5 µl	
Nuclease-free Water	add to a final volume of 20 µl	
Final Volume	**20 µl**	

3. Gently vortex the qInvader™ Reagents Master Mix and aliquot onto the DNA samples that are in the 96-well plate. The reaction final volume is 20 µl
4. Incubate in a real time machine programmed for the following steps:

Stage	Step	Number of Cycles	Temperature	Time	Data Collection
1	1	1	95°C	2 minutes	No
2	1	40	95 °C	20 seconds	No
	2		50 °C	1 minute	FAM™, Yakima Yellow™ & ROX™.

III. Design, Requirements and ordering information for qInvader™ Target-Specific Primers and Probes:

1. Design of qInvader™ Target-Specific oligonucleotides for DNA target sequences can be performed using qInvader™ designer software at www.twt.com.
2. Users have the option to design qInvader™ probes that report to either FAM™ or Yakima Yellow™ dyes. This allows users to perform two same-well assays, one for the DNA of interest reporting to FAM™ dye and the other for another DNA target of interest or an internal standard (e.g, .in a biplex reaction with a house-keeping gene, reporting to Yakima Yellow™ dye).
3. The qInvader™ designer software generates three oligonucleotide sequences for each target sequence. The first two are a forward primer oligonucleotide and a reverse primer oligonucleotide, which can be ordered using standard DNA synthesis with desalting purification. The third oligonucleotide is the Invader® probe that is blocked at the 3'-end with hexanediol (3'-C6 linker and purified on ion-exchange HPLC column (IE-HPLC). Probes with 3'-hexanediol- (IE-HPLC) may be ordered from Integrated DNA Technologies, IDT (http://www.idtdna.com).

IV. Formulation of Target-Specific Primers and Probes:

1. qInvader™ Mix 10X:
   4 µM each of forward and reverse primer oligonucleotides.
   5 µM each of Invader probe oligonucleotide (FAM™ or Yakima Yellow™ probe(s)).
   0.5 µM of Invasive oligonucleotide (if present).