



TECHNICAL MANUAL

QuantiFluor[®] Dx dsDNA System

Instructions for Use of Product
E5900



PROMEGA
2800 Woods Hollow Rd.
Madison, WI USA

INSTRUCTIONS FOR
USE OF PRODUCT

E5900



Rev 1
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QuantiFluor[®] Dx dsDNA System

All technical literature is available at: www.promega.com/protocols/
 Visit the web site to verify that you are using the most current version of this Technical Manual.
 E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

1. Introduction	3
1.1 About this Guide.....	3
1.2 Product Name	3
1.3 Abbreviations	3
1.4 Key to Symbols Used.....	4
1.5 Intended Use.....	4
1.6 Summary and Explanation.....	5
1.7 Principles of the Procedure	6
1.8 Assay Limitations	6
2. Product Components and Storage Conditions	7
2.1 Materials Provided with the QuantiFluor [®] Dx dsDNA System (Cat.# E5900).....	7
2.2 Storage and Handling of the QuantiFluor [®] Dx dsDNA System	7
2.3 Materials Not Provided	8
3. Before You Begin.....	8
3.1 Warnings and Precautions.....	8
3.2 Fluorometer Requirements	8
4. QuantiFluor[®] Dx dsDNA System Protocol	9
4.1 Preparation of Samples and Assay Components.....	9
4.2 Reaction Tube Assay Protocol.....	10
4.3 Plate Assay Protocol.....	10
5. Assay Quality Control	12
6. Results	12
6.1 Introduction	12
6.2 Calculating Results	12
7. Expected Values	13
7.1 Representative Data for the QuantiFluor [®] Dx dsDNA System	13



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8. Performance Characteristics	14
8.1 Interfering Substances	14
8.2 Extraction	15
8.3 Analytical Sensitivity.....	16
8.4 Reproducibility	18
8.5 Clinical Performance	19
9. Troubleshooting.....	21
10. Additional Information.....	24

1. Introduction

1.1 About this Guide

This guide describes the QuantiFluor® Dx dsDNA System^(a) and is the primary source for information about its intended use, components, limitations, protocol, troubleshooting and more. The Assay Protocol (Section 4) includes the procedures required for sample preparation and step-by-step instructions for the reaction where dsDNA, if present, is bound by the QuantiFluor® dsDNA dye. The QuantiFluor® Dx dsDNA System utilizes an instrument capable of detecting fluorescence and fluorescent output is proportional to the amount of dsDNA in the sample.

The QuantiFluor® Dx dsDNA System contains a fluorescent DNA-binding dye that enables sensitive quantification of small amounts of double-stranded DNA (dsDNA) in a purified sample. The system can be used with both single tube and plate fluorimeters that are capable of exciting in the range of 475–505nm and measuring emission in the range of 520–550nm.

1.2 Product Name

QuantiFluor® Dx dsDNA System

Cat.# E5900, 2,000 assays

Common Name



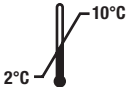









Fluorescent Nucleic Acid Dye

1.3 Abbreviations

QC, quality control

NA, not applicable

1.4 Key to Symbols Used

Symbol	Explanation	Symbol	Explanation
	In Vitro Diagnostic Medical Device		Protect from light
	Store at 2°C to 10°C		Manufacturer
	Caution		Flammable
	Use by		Contents sufficient for <n> tests
	Do not reuse		Warning. Biohazard
	Consult instructions for use		Catalog number

1.5 Intended Use

The QuantiFluor® Dx dsDNA System is a fluorescent dye-based test intended to quantify double-stranded DNA (dsDNA) in a sample containing purified nucleic acid. Fluorescent output is compared to results from a standard curve to calculate a dsDNA concentration. The dsDNA concentration results from the QuantiFluor® Dx dsDNA System are suitable for use in in vitro diagnostic assays.

The QuantiFluor® Dx dsDNA System is intended for use, in combination with a suitable fluorometer, to perform double-stranded DNA (dsDNA) quantification. The quantification data obtained using the QuantiFluor® Dx dsDNA System is suitable for use in preparation of dsDNA samples intended to be tested for in vitro diagnostic purposes.

The QuantiFluor® Dx dsDNA System is not intended for use as part of a specific in vitro diagnostic test. The QuantiFluor® Dx dsDNA System is intended for professional use only. Diagnostic results obtained using the quantification data identified with this system must be interpreted in conjunction with other clinical or laboratory data.

1.6 Summary and Explanation

Suitable DNA quantification is critical to maintain accuracy of DNA-based diagnostic applications. Traditional spectrophotometric assays cannot determine DNA concentrations below 2ng/μl; however, many isolated DNA samples have concentrations well below that level. Typical diagnostic applications provide a range of allowable DNA input, outside of which accuracy of the assay may be compromised. Reliable measurement maintains validated assay performance for diagnostic workflows. The QuantiFluor® Dx dsDNA System^(a) contains a fluorescent DNA-binding dye (503nm_{Ex}/531nm_{Em}) that enables sensitive quantitation of small amounts of double-stranded DNA (dsDNA) in a purified sample. The assay is highly selective for dsDNA over other nucleic acids.

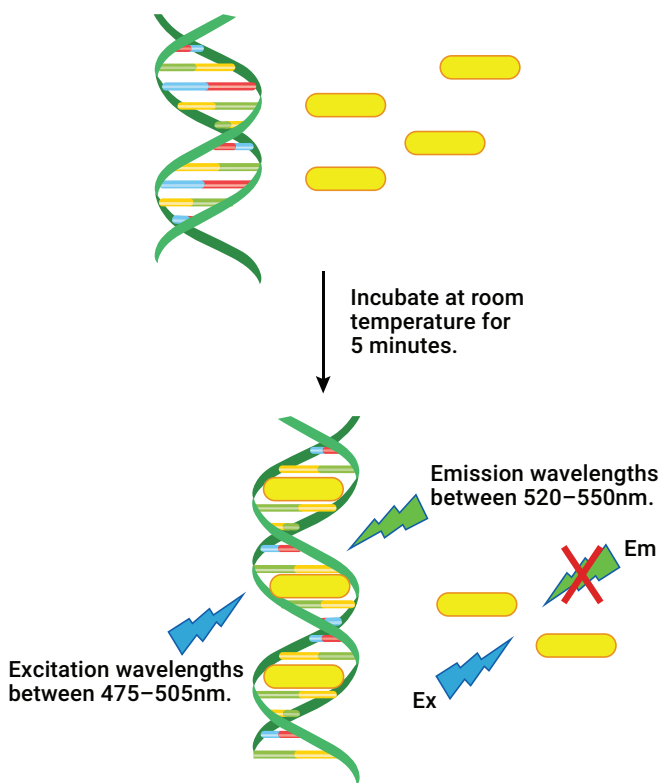


Figure 1. Schematic of the QuantiFluor® Dx dsDNA System.

1.7 Principles of the Procedure

The QuantiFluor® Dx dsDNA System is designed for quantitative measurement of dsDNA in a purified sample. Diluted fluorescent dye is added to a tube or 96-well plate containing a blank, DNA sample or DNA standard. The tube or plate is incubated at room temperature. During the incubation the dsDNA dye selectively binds to dsDNA by intercalation. Upon binding polynucleotides (DNA) the chromophore excited state lifetime increases by orders of magnitude (approximately 10^3), proportionally increasing the chromophore quantum yield, and subsequently, fluorescence. The fluorescent signal of the dsDNA bound to the fluorescent dye is proportional to the amount of dsDNA present in the sample and is measured using a fluorometer. The fluorescent signal is compared to a standard curve of known DNA concentrations to determine the amount of DNA present in the sample.

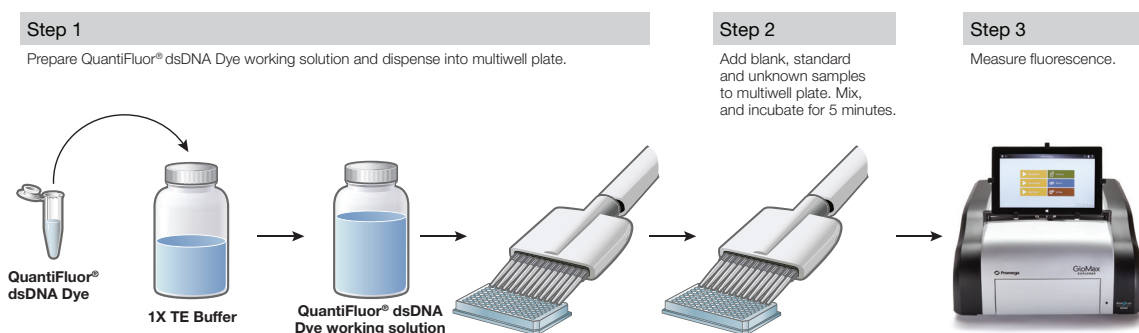


Figure 2. Workflow for QuantiFluor® Dx dsDNA Dye System.

1.8 Assay Limitations

- For in vitro diagnostic use only.
- For professional use only.
- The QuantiFluor® dsDNA Dye is light-sensitive and should be stored protected from light.
- Performance of the QuantiFluor® Dx dsDNA System was validated using the procedures described in the instructions for use. Modifications to these procedures may alter the performance of the system.
- Several compounds that are commonly used in nucleic acid preparation or found in eluates from nucleic acid purification may affect the QuantiFluor® dsDNA Dye (see Section 6.3).
- DNA concentrations of highly fragmented DNA samples, i.e., DNA extracted from FFPE, may not be fully representative of amplifiability.
- Optimum QuantiFluor® Dx dsDNA System results are obtained using any fluorometer capable of measuring excitation in the range of 475–505nm and emission in the range of 520–550nm.
- Range of 0.05–200ng of dsDNA input.



2. Product Components and Storage Conditions

2.1 Materials Provided with the QuantiFluor® Dx dsDNA System (Cat.# E5900)



This product contains sufficient reagents to perform 2,000 assays at a 200µl scale. The following materials are included:

 2,000



COMPONENT	SIZE	PART#
QuantiFluor® dsDNA Dye	1ml	E258A

Storage Conditions: Store at;  2°C - 10°C prior to use;  2°C - 10°C following first use.

COMPONENT	SIZE	PART#
Lambda DNA Standard	100µg	E259A

Storage Conditions: Store at;  2°C - 10°C prior to use;  2°C - 10°C following first use.

COMPONENT	SIZE	PART#
20X TE Buffer (pH 7.5)	25ml	E260A

Storage Conditions: Store at;  -30°C - +30°C prior to use;  -30°C - +30°C following first use.

2.2 Storage and Handling of the QuantiFluor® Dx dsDNA System

Product may arrive frozen. Upon receipt, store the QuantiFluor® dsDNA Dye at +2°C to +10°C, protected from light. Store the Lambda DNA Standard at +2°C to +10°C. Do not refreeze the Lambda DNA Standard. Store the 20X TE Buffer at -30°C to +30°C. Components stored under conditions other than those stated on the labels may not perform properly and improper storage may adversely affect results. Optimal results are obtained only when using components provided in this kit.



If the Lambda DNA Standard arrived frozen, a concentration gradient may have formed and should be vortexed well upon thawing. Store the Lambda DNA Standard at +2°C to +10°C overnight, then warm to room temperature and mix well before use.

2.3 Materials Not Provided

Laboratory Materials

- Set of calibrated precision single- and multichannel pipettes, capable of delivering 1µl to 1,000µl
- Vortex mixer
- Refrigerator at +2°C to +10°C
- Nuclease-Free Water (Cat.# P1193)

Instruments and Accessories

Multiwell plate usage:

- Microplate Fluorometer (see Section 3.2 for instrument details)
- 96-well black plates (for use as dilution and assay plates)
- 1.5ml tubes


Single-tube usage:

- Single tube Fluorometer (see Section 3.2 for instrument details)
- Measurement tube compatible with a single-tube fluorometer

3. Before You Begin

3.1 Warnings and Precautions

Chemical Safety Warning: Some reagents used with the QuantiFluor® Dx dsDNA System are potentially hazardous. Handle and dispose of hazardous materials according to the guidelines established by your institution.

 **Safety Data Sheet Statement:** Important information regarding the safe handling, transport and disposal of this product is contained in the Safety Data Sheet (SDS). SDSs for all reagents provided in the kits are available online at: www.promega.com/resources/msds/ or by request from Promega Technical Services at: techserv@promega.com

 **Biosafety Precautions:** Materials of human origin should be considered infectious and handled using standard biosafety procedures.

3.2 Fluorometer Requirements

The QuantiFluor® Dx dsDNA System produces a fluorescent signal and requires a fluorometer for signal detection. Fluorescence measurements can be performed using a fluorescence reader capable of measuring excitation and emission at the appropriate wavelengths in either a plate or single tube format.

The following are the requirements of fluorometers compatible with this assay:

Optimal QuantiFluor® Dx dsDNA System fluorescence wavelengths are: Excitation = 503nm and Emission = 531nm. Excitation and emission spectra are shown in Figure 3.

A fluorometer capable of excitation within a range from 475–505nm and measuring emission within a range from 520–550nm wavelengths is suitable for use with QuantiFluor® Dx dsDNA System.

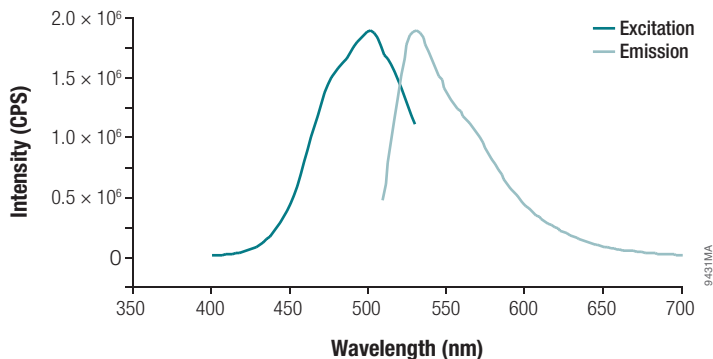


Figure 3. Excitation and emission spectra for the QuantiFluor® dsDNA Dye.

4. QuantiFluor® Dx dsDNA System Protocol

This system contains sufficient reagents for 2,000 assays at a 200µl scale.

4.1 Preparation of Samples and Assay Components

Warm all assay components to room temperature before use. The QuantiFluor® dsDNA Dye is dissolved in 100% DMSO and frozen at or below 4°C. Prior to dilution, thaw the dye at room temperature, protected from light.

1. Dilute the 20X TE Buffer twentyfold with nuclease-free water (not provided). For example, add 1ml of 20X TE Buffer to 19ml of nuclease-free water and mix.
2. Dilute the QuantiFluor® dsDNA Dye 1:400 in 1X TE Buffer to make the QuantiFluor® dsDNA Dye working solution. For example, add 10µl of QuantiFluor® dsDNA Dye to 3,990µl of 1X TE Buffer, and mix.

Note: The QuantiFluor® Dye working solution is stable for at least 2 hours at 25°C.

4.2 Reaction Tube Assay Protocol

1. **Prepare Blank Sample:** Add 200 μ l of QuantiFluor[®] dsDNA Dye working solution to an empty measurement tube. This will be the blank sample with background signal subtracted, used in Step 6. Protect tube from light.
2. **Prepare 200ng Standard Sample:** Add 2 μ l of the provided DNA Standard (100ng/ μ l) to 200 μ l of QuantiFluor[®] dsDNA Dye working solution in an empty measurement tube. This will be the standard sample for assay calibration used in Step 6. Vortex well, and protect the tube from light.
3. **Prepare Unknown Sample:** Add 2 μ l of unknown sample to 200 μ l of QuantiFluor[®] dsDNA Dye working solution in the measurement tube. Protect the tube from light.
4. Incubate the prepared samples at room temperature for 5 minutes, protected from light.
5. Measure using a tube-based fluorometer.
6. If needed, calibrate the fluorometer by reading the blank (prepared in Step 1) and standard (prepared in Step 2) samples in the 'calibration' or 'standards' screen.
7. Measure fluorescence of the unknown sample using the fluorometer.

4.3 Plate Assay Protocol

4.3.1 Preparing a Standard Curve

The following recommended standards result in 0.05–200ng/well and are designed for optimal pipetting accuracy, transferring 2 μ l of standard to each well.

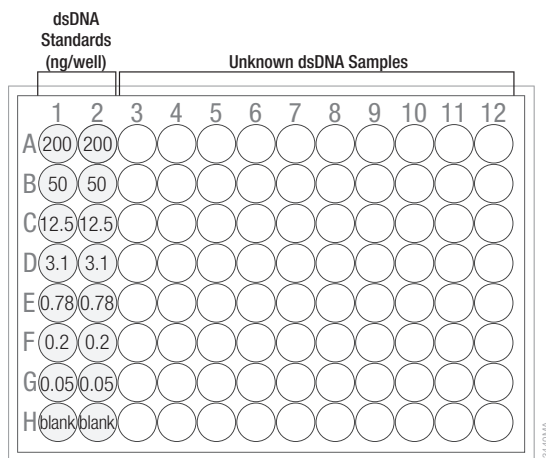
1. Label seven 1.5ml tubes for each standard A–G.
2. Prepare dsDNA standards by vortexing the Lambda DNA Standard and serially diluting as shown in Table 4.3.1. Take care to not introduce air bubbles.

Table 4.3.1 Preparing Recommended dsDNA Standard Curve Samples.

Standard	Volume of dsDNA Standard	Volume of 1X TE Buffer (µl)	Final dsDNA Concentration (ng/µl)	Final dsDNA Concentration (ng/2µl)
A	50µl of Lambda DNA Standard (E259A)	0	100	200
B	25µl of Standard A	75	25	50
C	25µl of Standard B	75	6.25	12.5
D	25µl of Standard C	75	1.56	3.12
E	25µl of Standard D	75	0.39	0.78
F	25µl of Standard E	75	0.098	0.20
G	25µl of Standard F	75	0.024	0.05

4.3.2 Preparing the Assay Plate

1. Pipet 200µl of QuantiFluor® dsDNA Dye working solution into each well that is intended for an unknown, blank or standard sample.
2. Dispense 2µl of the dsDNA standards prepared in Table 4.3.1 above (labeled Standards A–G) to rows A–G of the multiwell plate (Figure 4). We recommend pipetting duplicates or triplicates of the standards.
3. For the blank, pipet 2µl of 1X TE Buffer into row H. We recommend pipetting duplicates or triplicates of the blank.
4. Add 2µl of unknown sample to the remaining wells. We recommend pipetting duplicates or triplicates of the unknowns.


Figure 4. Dispense standard dilutions and blank samples in duplicate into columns 1 and 2 of a multiwell plate.

5. Mix the plate thoroughly using a plate shaker or by pipetting the contents of each well 10 times.
6. Incubate assays for 5 minutes at room temperature, protected from light.
7. Measure fluorescence (503nm_{Ex}/531nm_{Em}) using a plate reader. Any fluorometer capable of measuring excitation in the range of 475–505nm and emission in the range of 520–550nm wavelengths is suitable.

5. Assay Quality Control

The standard curve generates fluorescent signal for known DNA concentrations and serves as a control for the assay. The curve includes a blank to represent no DNA and account for background signal. Ensure the test sample is diluted in the same dilution buffer as the standard curve. Ensure that all samples and standards have a fluorescent value higher than the blank. Furthermore, all samples should have a fluorescent value less than the most-concentrated standard. Lambda DNA Standard is provided as the DNA for the standard curve. The DNA is of high molecular weight and representative of complex, eukaryotic genomes.

6. Results

6.1 Introduction

The QuantiFluor® Dx dsDNA System produces a fluorescent signal. Fluorescent units may vary due to the sensitivity and settings of each instrument. The use of different instruments should not affect the measured result.

6.2 Calculating Results

Calculate the dsDNA concentration as follows: Subtract the fluorescence of the averaged blank samples (1X TE Buffer) from all the standard and unknown samples. Use the corrected data from the DNA standards to generate a standard curve of fluorescence versus DNA concentration. Determine the DNA concentration of the sample from the standard curve and multiply the resulting number by the dilution factor, if applicable (see Section 5).

Example calculation using 2µl of unknown sample in triplicate wells (the standard and unknown samples have these average fluorescence values (in RFU)):

	Unknown Sample	Standard Samples (ng)							
		0	0.05	0.2	0.78	3.1	12.5	50	200
Fluorescence	15,418	418	460	581	1,070	3,038	11,008	42,897	162,231

1. Subtract the 1X TE Buffer blank (average of blank standards) from all samples:

	Unknown Sample	Standard Samples (ng)							
		0	0.05	0.2	0.78	3.1	12.5	50	200
Fluorescence	15,000	0	42	163	652	2,620	10,590	42,479	161,813

- Determine the power regression from the scatter plot (Figure 5).

$$y = 834.83x^{0.9996}$$

- Calculate the DNA concentration of the unknown sample in the 200µl assay volume by solving for x in the linear regression equation, where y = 15,000:

$$x = \left(\frac{y}{834.83} \right)^{(1/0.9996)} = 18.0\text{ng}/\mu\text{l}$$

- Account for any dilution of the unknown sample. In this example, there is no sample dilution.

7. Expected Values

7.1 Representative Data for the QuantiFluor® Dx dsDNA System

Table 7.1.1 Representative Data for the dsDNA Standard Curve and QuantiFluor® Dx dsDNA System.

Lambda DNA Mass (ng)	Average Fluorescence (RFU) ¹
0	0
0.05	42
0.2	163
0.78	652
3.1	2,620
12.5	10,590
50	42,479
200	161,813

¹Background fluorescence has been subtracted. n = 3

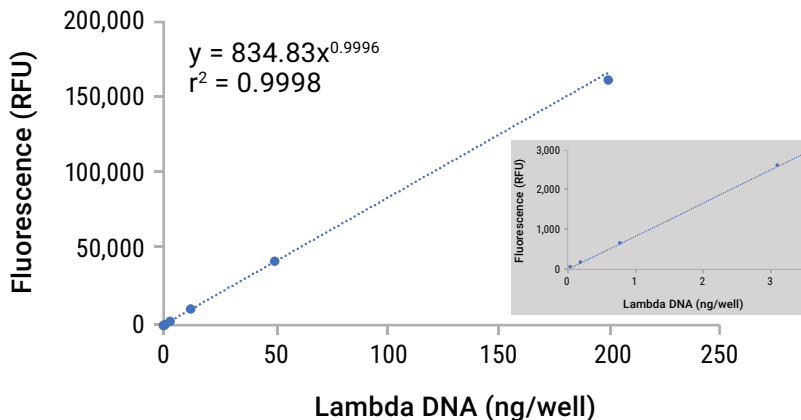


Figure 5. Representative dsDNA standard curve in a 96-well-plate format. The final amounts of Lambda DNA Standard in the 96-well format are listed in Table 7.1.1. **Inset.** Expanded view of the lower end of the standard curve.

8. Performance Characteristics

All analytical studies followed the procedure outlined in the QuantiFluor® Dx dsDNA System.

8.1 Interfering Substances

Several compounds that are commonly used in nucleic acid preparation or found in eluates from nucleic acid purification may affect the QuantiFluor® dsDNA Dye. Table 8.1.1 lists compounds that have known effects on DNA quantitation using the QuantiFluor® dsDNA Dye and the concentrations at which they affect quantitation results.

Table 8.1.1. Compounds that Interfere with the QuantiFluor® dsDNA Dye.

Chemical	Concentration Shown to Affect QuantiFluor® dsDNA Dye
agarose	>0.01%
ammonium acetate	>50mM
bovine serum albumin (BSA)	>1.3%
chloroform	>2.5%
DMSO	>20%
dNTP mix	>1.3%
ethanol	>20%
IgG	>2µg
small dsDNA fragments	300pg
Colorless GoTaq® Reaction Buffer	>20%
phenol	>2.5%
polyethylene glycol (PEG)	>20%
RNA	300pg
sodium acetate	>1mM
sodium dodecyl sulfate (SDS)	>0.01%
ssDNA	800pg
Triton® X-100	>0.01%

8.2 Extraction

Compatibility of the QuantiFluor® Dx dsDNA System with a commercially available DNA extraction system and a downstream amplification-based in vitro diagnostic test was demonstrated using the Maxwell® CSC Instrument and the Maxwell® CSC DNA FFPE Kit along with the OncoMate™ MSI Dx Analysis System. The OncoMate™ MSI Dx Analysis System determines microsatellite instability (MSI) status using PCR and fluorescent detection with capillary electrophoresis.

Twelve DNA extractions from FFPE curls obtained from each of four positive (MSI-H) and three negative (MSS) tumor samples with their respective matched normal samples were performed using the Maxwell® CSC DNA FFPE Kit and the Maxwell® CSC Instrument for a total of 168 DNA extractions. The tumor samples contained 20–60% tumor content. Once isolated, the extracted DNA was quantified using the QuantiFluor® Dx dsDNA System and amplified using the OncoMate™ MSI Dx Analysis System amplification kit. The amplified DNA was subjected to capillary electrophoresis using an Applied Biosystems® 3500 Dx Genetic Analyzer and analyzed with the OncoMate™ MSI Dx Interpretive Software as paired tumor and normal samples.

8.2 Extraction (continued)

A total of five (5) samples, comprised of a tumor and normal sample pairing, initially yielded Invalid results in the OncoMate™ MSI Dx Interpretive Software. After reinjection of all five samples on the capillary electrophoresis instrument, one sample was resolved. The remaining four (4) samples were resolved by reamplification following guidance provided in the *OncoMate™ MSI Dx Analysis System Technical Manual*, TM543.

In the study, 96.4% (81/84) of the paired tumor and normal samples, each from individual FFPE curls, produced results that were concordant with the predetermined MSI status. The 95% confidence intervals (CI) for percent correct and percent incorrect results were 89.9–99.3% and 0.7–10.1%, respectively.

The study demonstrated compatibility of the QuantiFluor® Dx dsDNA System with DNA eluate extracted using the Maxwell® CSC DNA FFPE Kit and Maxwell® CSC Instrument and suitability of the DNA quantification values for use in an amplification-based test such as the OncoMate™ MSI Dx Analysis System. All DNA eluates from 168 FFPE samples were successfully quantified by the QuantiFluor® Dx dsDNA System and processed pairwise in the OncoMate™ System. Low reprocessing rates were observed suggesting the generated DNA concentrations values enabled the addition of the appropriate DNA input into the OncoMate™ MSI Dx Analysis System reactions.

8.3 Analytical Sensitivity

The QuantiFluor® Dx dsDNA System was evaluated for its suitability to support the Limit of Detection for an amplification-based, downstream application using the OncoMate™ MSI Dx Analysis System.

Analytical sensitivity of the OncoMate™ MSI Dx Analysis System was determined using extracted DNA isolated from six positive (MSI-H) tumor and matched normal tissue samples, as well as a titration series of the 2800M Control DNA. Multiple sections for each tumor and normal sample were extracted for the positive samples and pooled, and quantification was performed using the QuantiFluor® Dx dsDNA System.

The positive samples were diluted to working DNA concentrations across a titration to achieve final DNA amounts of 0.1, 0.2, 0.5, 1.0, 2.0 and 2.5ng DNA per amplification reaction. Negative samples were diluted in a similar manner based on labeled DNA concentration. To evaluate the influence of tumor content on limit of detection (LOD), one tumor sample was combined with the matched normal sample to simulate a tumor content of 2.5%, 5%, 10%, 15% and 20% using 1ng DNA per amplification reaction. Sample mixtures were prepared using DNA concentration values determined with the QuantiFluor® Dx dsDNA System. A supplemental study, using similarly prepared materials, was performed to evaluate performance of several DNA amounts with multiple tumor content percentages. All test points were run in multiple replicates.

A total of 48 Invalid results were initially generated out of over 2,000 collected results. Three Invalid results were due to capillary electrophoresis instrument error and resolved by instrument reinjection. Forty-four 2ng and one 1ng DNA input samples (45 total) generated Invalid results due to capillary electrophoresis instrument detector saturation, indicative of inappropriate DNA input into the amplification reaction. The 2ng DNA input amount is twice the amount recommended for amplification and high signal was expected. The OncoMate™ MSI Dx System instructions include steps to dilute amplification reactions that have previously saturated the capillary electrophoresis instrument detector. Dilution of the amplification reaction resolved all 45 of the Invalid results due to signal saturation.

The LOD for the OncoMate™ MSI Dx Analysis System was determined across two dimensions: 1) the total amount of input DNA used for the assay and 2) the fraction of tumor DNA present in the sample. A summary of the interpretive results for the DNA input study are summarized in Table 8.3.1, and for the biomarker-positive (MSI-H) case in the tumor content study in Table 8.3.2. Table 8.3.3 displays a summary of the percent positivity to the reference result by DNA amount and tumor content percentage. Results for all samples tested at 0.5, 1.0 and 2.0ng DNA input with 30% tumor content were used to establish the LOD. The LOD for the OncoMate™ MSI Dx Analysis System was established at 30% tumor content based on concordance across all loci when using the recommended 1ng DNA input and samples where tumor content was adjusted by blending with DNA extracted from matched normal tissue.

The QuantiFluor® Dx dsDNA System demonstrated suitable precision to establish the Limit of Detection performance of an amplification-based test. The system provided DNA concentration values to support creation of DNA titrations as well as tumor-normal tissue DNA mixtures which were successfully used to determine analytical sensitivity with minimal sample reprocessing.

Table 8.3.1. Interpretive Result Frequency for the DNA Input Study, Combined Reagent Lots.

OncoMate™ Interpretive Results			
DNA Input (ng)	MSI-H	No Call	Total
0.1	44	156	200
0.2	199	1	200
0.5	200	0	200
1.0	200	0	200
2.0	200	0	200
2.5	200	0	200

Table 8.3.2. Interpretive Result Frequency for the Tumor Concentration Study, Combined Reagent Lots.

OncoMate™ Interpretive Results			
Tumor Content (%)	MSI-H	No Call	Total
2.5	0	40	40
5	40	0	40
10	40	0	40
15	40	0	40
20	40	0	40

Table 8.3.3. Percent Positivity to the Reference Result by DNA Amount and Tumor Content Percentage

DNA Input (ng)	10% Tumor		20% Tumor		30% Tumor		50% Tumor	
	n(pos)/ n(total)	Positivity (%)	n(pos)/ n(total)	Positivity (%)	n(pos)/ n(total)	Positivity (%)	n(pos)/ n(total)	Positivity (%)
0.5	50/60	83	60/60	100	60/60	100	60/60	100
1.0	60/60	100	60/60	100	60/60	100	60/60	100
2.0	60/60	100	60/60	100	60/60	100	60/60	100

8.4 Reproducibility

Reproducibility of the QuantiFluor® Dx dsDNA System was demonstrated by its contribution to reproducibility performance of a downstream application, the OncoMate™ MSI Dx Analysis System.

Reproducibility for the OncoMate™ MSI Dx Analysis System was evaluated across multiple sites, operators, runs, days, replicates and assay kit lots. Data were assessed for between-site, between-operator, between-run, between-day, within-run and between-lot repeatability and precision.

The test panel consisted of 14 colorectal (CRC) samples which included four positive (MSI-H) and three negative (MSS) cases, and a negative amplification control and a positive amplification control. Each test case consisted of 2 samples, tumor tissue and a source-matched normal tissue sample. The samples were prepared at a central site, blinded and distributed to the operators at each of the three test sites. The QuantiFluor® Dx dsDNA System was used to determine DNA concentration for the 14 FFPE colorectal test sample pairs and informed preparation of the DNA eluate dilutions for distribution.

A total of 13 OncoMate™ MSI Dx Analysis System results were initially Invalid during testing. Following the instructions all samples were reinjected on the capillary electrophoresis instrument. Five results did not resolve with reanalysis with the capillary electrophoresis and required reamplification. A total of two results remained Invalid after reamplification and are included in the agreement analysis below as discordant results.

The Positive Percent Agreements (PPA) for MSI-H and Negative Percent Agreements (NPA) for MSS interpretative results versus expected results (Table 8.4.1) were reproducible for site, operator, day, lot and run. The PPA for site, operator, day, lot and run ranged from 89.6–97.9% and the NPAs ranged from 97.2–100%, demonstrating reproducibility for each factor tested. The overall reproducibility PPA (95% CI) and NPA (95% CI) were 95.5% (92.4–97.6%) and 99% (97.4–100%), respectively.

In conclusion, sample DNA concentrations determined with the QuantiFluor® Dx dsDNA System were robust and suitable to establish strong reproducibility performance (95.5% PPA) for the OncoMate™ MSI Dx Analysis System across sites, operators, days, lots and runs. Two hundred eighty-six results out of the 288 results attempted (286/288) provided usable data for the study, suggesting appropriate DNA values from the QuantiFluor® Dx dsDNA System were generated and used for downstream processing.

Table 8.4.1. Summary of PPA and NPA for Interpretative Result versus Reference Result.

Factor	Item	PPA		NPA	
		Percent (#/n)	95% CI	Percent (#/n)	95% CI
Site	1	96.9 (93/96)	91.1–99.4	98.6 (71/72)	92.5–100
	2	92.7 (89/96)	85.6–97.0	100 (72/72)	95–100
	3	96.9 (93/96)	91.1–99.4	100 (72/72)	95–100
Operator	1	97.9 (47/48)	88.9–100	100 (72/72)	90.3–100
	2	95.8 (46/48)	85.8–99.5	97.2 (35/36)	85.5–99.9
	3	89.6 (43/48)	77.3–96.5	100 (36/36)	90.3–100
	4	95.8 (46/48)	85.8–99.5	100 (36/36)	90.3–100
	5	97.9 (47/48)	88.9–100	100 (36/36)	90.3–100
	6	95.8 (46/48)	85.8–99.5	100 (36/36)	90.3–100
Day	1	93.8 (90/96)	86.9–97.7	98.6 (71/72)	92.5–100
	2	95.8 (92/96)	89.7–98.8	100 (72/72)	95–100
	3	96.9 (93/96)	91.1–99.4	100 (72/72)	95–100
Lot*	1	95.8 (92/96)	87.9–98.8	100 (72/72)	95–100
	2	94.8 (91/96)	88.3–98.3	98.6 (71/72)	92.5–100
	3	95.8 (92/96)	87.9–98.8	100 (72/72)	95–100
Run*	A	94.4 (136/144)	89.4–97.6	100 (108/108)	96.6–100
	B	96.5 (139/144)	92.1–98.9	99.1 (107/108)	95.0–100
Total		95.5 (275/288)	92.4–97.6	99.5 (215/216)	97.4–100

*Not all lots tested in a run.

8.5 Clinical Performance

Suitability and robustness of the QuantiFluor® Dx dsDNA System for use with an in vitro diagnostic application in a clinical setting was evaluated in combination with an amplification-based test, the OncoMate™ MSI Dx Analysis System.

A method comparison was performed between the OncoMate™ MSI Dx Analysis System and an immunohistochemistry slide staining panel. The study was performed using a sequential series of 130 colorectal cancer patient samples that were enriched with a second set of 24 suspected Lynch syndrome samples, for a total of 154 cases. FFPE tissue samples were collected through multiple sources which used various sample fixation protocols. Additionally, most samples had been collected more than 10 years prior to the study. DNA concentrations for the extracted DNA aliquots from 308 FFPE samples, two from each of 154 tumor and normal tissue-matched CRC cases, were determined using the QuantiFluor® Dx dsDNA System then analyzed by the OncoMate™ MSI Dx Analysis System. FFPE tissue sample curls were provided to an external laboratory to perform immunohistochemistry.

8.5 Clinical Performance (continued)

A total of two samples yielded Invalid results during initial testing using the OncoMate™ MSI Dx Analysis System. Capillary electrophoresis errors were suspected in the failures and both samples were resolved upon reinjection on the capillary electrophoresis instrument. No amplifications were repeated due to DNA quantification values that contributed to nonevaluable results in the OncoMate™ MSI Dx Interpretive Software. No Invalid results were generated in the OncoMate™ assay and a comparison for all study cases could be completed. The comparison results between the OncoMate™ MSI Dx Analysis System and the IHC panel for the 154 samples are listed in Table 8.5.1. The Positive Percent Agreement (PPA) was 97.8% and the Negative Percent Agreement (NPA) was 97.2%, with an Overall Percent Agreement (OPA) of 97.4% between the two methods.

The QuantiFluor® Dx dsDNA System demonstrated robust performance in a clinical setting, generating DNA quantification values to enable valid results with an amplification-based test and further comparison to an orthogonal method. Suitable DNA concentration values for 308 FFPE samples from various sources and exceptional storage duration were generated and contributed to the low sample reprocessing observed for the study.

Table 8.5.1. Comparison and Agreement Analysis of the OncoMate™ MSI Dx Analysis System Interpretative Results versus MMR IHC Panel (All Samples).

OncoMate™ MSI Dx Analysis System	MMR Loss	MMR IHC			Agreement			
		MMR Intact	Invalid	Total	Type	n/N	Percent	95% CI
MSI-H	44	3	0	47	PPA	44/45	97.8	88.4–99.6
MSS	1	106	0	107	NPA	106/109	97.2	92.2–99.1
Invalid	0	0	0	0	OPA	150/154	97.4	93.5–99.0
Total	45	109	0	154				

9. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: www.promega.com. E-mail: techserv@promega.com

Symptoms

Low or no fluorescence detected

Causes and Comments

Check that the correct filter set was used for the QuantiFluor[®] Dx dsDNA System. Any fluorometer capable exciting in the range of 475–505nm and measuring emission in the range of 520–550nm wavelengths will work (see Figure 3 for excitation and emission spectra).

The QuantiFluor[®] dsDNA Dye is light-sensitive. Exposure to light will reduce the sensitivity of the assay. Store QuantiFluor[®] dsDNA Dye and working solution protected from light.

Confirm that dye was added. Add an equal volume of QuantiFluor[®] dsDNA Dye working solution to each sample.

Check that unknown and standard samples were diluted appropriately. Increase the concentration of the unknown and standard samples, if necessary.

Confirm that the unknown sample calculations were performed correctly and, if applicable, the concentrations calculated from the power regression were multiplied by the dilution factor (Section 6, Step 2).

Check that the unknown sample was within the sensitivity range of the assay and standard curve. Determine the average fluorescence and standard deviation of the blank standards. Subtract the average fluorescence of the blank standard from the average fluorescence of the unknown and standard samples. These blank-subtracted values should be more than three standard deviations (as determined for the blank standards) above the average fluorescence for the blank standards.

The high end of the standard curve was not within the dynamic range for the QuantiFluor[®] Dx dsDNA System. Evaluate the blank-subtracted fluorescence of the standard curve. The values should be proportional to the dilution factors used to create the standard curve. If the increase in fluorescence is not proportional to the increase in dsDNA amount, the QuantiFluor[®] dsDNA Dye may be saturated. Recreate the standard curve, and decrease the concentration of the highest standard curve point.

9. Troubleshooting (continued)

Symptoms

Low or no fluorescence detected in the standard samples

Causes and Comments

Evaluate the performance of the fluorometer with a dsDNA sample of known concentration (e.g., Lambda DNA Standard) using the appropriate excitation and emission wavelengths for the QuantiFluor® Dx dsDNA System.

The QuantiFluor® dsDNA Dye was exposed to light. Exposure to light will reduce the sensitivity of the assay. Store QuantiFluor® dsDNA Dye and working solution protected from light.

Check that the standard samples were diluted appropriately.

Mix the dsDNA standards with the QuantiFluor® dsDNA Dye working solution just prior to measurement. Extended exposure to light will decrease the amount of fluorescence detected.

Fluorescence too high

Check that the unknown and standard samples were diluted appropriately. Decrease the concentration of the unknown and standard samples, if necessary.

Adjust the gain setting on your fluorometer so that the highest point on the standard curve is approximately 90% of maximum signal.

dsDNA concentration determined using the QuantiFluor® dsDNA Dye differed from concentration determined using an alternative quantitation method

DNA concentrations determined using the QuantiFluor® dsDNA Dye and optical density readings at 260nm will be different due to inherent differences between methodologies. An optical density reading at A_{260} reflects the amount of light that is neither transmitted nor reflected and is proportional to the amount of all nucleic acid (dsDNA, ssDNA, RNA and nucleotides) in the sample. The QuantiFluor® dsDNA Dye intercalates into dsDNA and, therefore, the amount of fluorescence is proportional to the amount of dsDNA.

If comparing concentrations determined using another dye-based quantitation method, carefully examine the blank-subtracted fluorescence of the two standard curves. The values should be proportional to the dilution factors used to create the standard curve. If the increase in fluorescence is not proportional to the increase in dsDNA amount, the fluorescent dye(s) may be saturated. Recreate the standard curve, and decrease the concentration of the highest point of the standard curve.

Symptoms

dsDNA concentration determined using the QuantiFluor® dsDNA Dye differed from concentration determined using an alternative quantitation method (continued)

Causes and Comments

Determine the average fluorescence and standard deviation of the blank standards. Subtract the average fluorescence of the blank standards from the average fluorescence of the unknown and standard samples. The blank-subtracted fluorescence should be more than three standard deviations (as determined for the blank standards) above the average fluorescence for the blank standards.

Nonlinear standard curve

Check that standard samples were diluted appropriately. If the high or low end of curve is nonlinear, then adjust the standard sample dilutions such that the standard curve is linear.

Adjust the gain setting on your fluorometer, if necessary, so that the highest point on the standard curve is approximately 90% of maximum signal.

Check that the lower concentration standards are within the sensitivity range for the assay and assay format. Determine the average fluorescence and standard deviation of the blank standards. Subtract the average fluorescence of the blank standards from the average fluorescence of the unknown and standard samples. These blank-subtracted values should be greater than three standard deviations (as determined for the blank standards) above the average fluorescence for the blank standards.

Analyze the data using either a linear regression or a power regression for accurate concentration determinations within the 10–400ng/μl portion of the standard curve. We recommend using a power regression for unknowns that are expected to be <10ng/μl. Contact Technical Services for additional assistance.

The QuantiFluor® dsDNA Dye is light-sensitive. Exposure to light will reduce the sensitivity of the assay. Store QuantiFluor® dsDNA Dye and working solution protected from light.

Check that the standard samples were diluted appropriately.

A concentration gradient may have formed if the Lambda DNA Standard, arrived frozen. Store Lambda DNA Standard, at +2 to +10°C overnight, then warm to room temperature and mix well before use. Do not refreeze the Lambda DNA Standard.



10. Additional Information

For technical assistance, call Promega Technical Services at: 1-800-356-9526 (toll-free) or 608-274-4330 or email: techserv@promega.com

[®]U.S. Pat. Nos. 8,598,198 and 9,206,474 and other patents and patents pending.

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