Methylene Blue Oligo Staining & Oligo Quality

Methylene Blue Oligonucleotide Staining & Quality Analysis

Methylene Blue

The most effective method to visualize oligonucleotides is gel staining with methylene blue for both DNA and RNA oligos. Gene Link recommends electrophoresing oligonucleotides in denaturing 7M urea 15% polyacrylamide gels. This percentage of gel is adequate for oligo resolution from 5 mer to over 150 mer. For longer oligos 10-12% polyacrylamide gels are recommended. Staining can be particularly useful if the gel is to be preserved or for photographic documentation. All Gene Link brand oligos are supplied with an actual gel picture affixed on the oligo specifications report.

As an analytical tool, staining has limited sensitivity; sequences present in low concentrations may not be visible. Nevertheless, staining is more sensitive than UV shadowing and is considered easier than radiolabeling.

Methylene blue and Stains-All are useful for staining oligonucleotides. For example, the gel after electrophoresis and removal from the plates, may be soaked in a shallow pan with 0.02% methylene blue in water for approximately 10–15 min. Drain the pan and rinse the gel of all excess, unbound dye for several minutes. Destaining with water should be continued till the background has almost no stain. Stained oligonucleotides are visualized as blue bands and may be photographed under ambient light. All oligo quality control gels at Gene Link are electrophoresed on 15% polyacrylamide gels and stained with methylene blue.

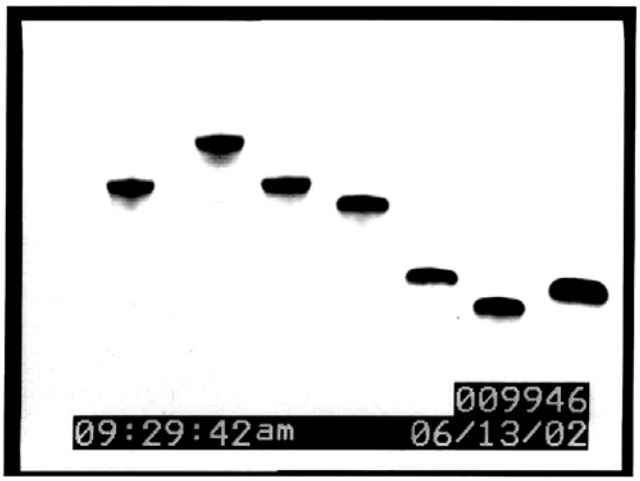
The blue color shade stain varies with oligo with varying percentage of base. An oligo d(T) stains as purple.

Ethidium Bromide

Gene Link does not recommend ethidium bromide staining for analysis of oligonucleotides.
Ethidium bromide staining is a well-established technique for visualizing double-stranded DNA fragments. Ethidium bromide is a fluorescent dye that binds to double-stranded DNA molecules by intercalating a planar group between the stacked base pairs of the nucleic acid. Ethidium bromide can also bind to secondary structure in single-stranded RNA molecules and DNA oligonucleotides with regions of local base pairing offer the stacked base pairs necessary for the dye molecules to

intercalate. When excited by light at or near 546 nm, the dye-nucleic acid complex exhibits an increased (about 20 fold) fluorescent yield at an emission wavelength of 590 nm.

Unfortunately, it is not effective for visualizing short, single-stranded DNA fragments such as synthetic oligonucleotides. The affinity of the dye for single-stranded nucleic acid is relatively low and the fluorescent yield is comparatively poor. In fact, most fluorescence associated with staining single-stranded DNA or RNA is attributable to binding of the dye to short intra-strand duplexes in the molecules. Also, the intercalation of ethidium bromide into short, single-stranded DNA is inefficient, highly sequence dependent and sometimes undetectable in sequences shorter than 25 bases. **Due to the above limitations, Gene Link does not recommend ethidium bromide staining for analysis of oligonucleotides.**

Polyacrylamide gel pictures of oligos stained with methylene blue	
	
Sample gel picture of crude oligo of size 36mer to 19mer	Crude and gel purified 78mer and 85mer

Oligo Quality Analysis

The quality control feature of resolving oligos in denaturing 7M urea gel is to determine the overall quality and yield of the major band of the synthesized oligo. The gel picture quality is based on expected mobility, main band intensity and smearing below (n-1 fragments) the main band and higher mobility bands. At Gene Link the gel loading is approximately 4-7 µg of oligo. At this loading for a 20-30 mer oligo there should be no truncated n-1 bands visible below the major band. As the oligo size increases there will be gradually more truncated sequence visible below the major band. Occasional bands above the major expected band are attributed to secondary structure. This can be confirmed by performing an oligo analysis for secondary structure that includes, primer dimers, hairpin structure and runs of G and C bases.

At Gene Link we have determined with more than 20 year experience that the presence of G's makes the oligo sequence highly prone to very strong secondary structure that is not even denatured by 7M urea.

G's and GC Content Effect on Yield

Yield will gradually decrease as length of oligo increases. Palindromes, hairpins and high GC content oligos and oligos containing stretches of 3 or more G's induces strong secondary structure and base stacking thus decreasing purity and yield. G's ; The unresolved dilemma. Ever wonder why we have not yet discovered a polymerase that can breeze through a stretch of G's? A stretch of three or more G's in an oligo sequence induces strong secondary structure. A string of G's and C's can exhibit internal Hoogsteen base pairing, non- Watson-Crick triple base pairing and should be avoided. Although this anomalous behavior is difficult to predict, in general, avoid runs of more than three consecutive G's in primers. Also, examine potential primers for self-complementary and hairpin structures Base Composition Higher GC content stabilizes hybridization, but a string of G's and C's can exhibit internal Hoogsteen base pairing, non-Watson-Crick base pairing and should be avoided. Although this anomalous behavior is difficult to predict, these structures can disrupt stable primer binding. In general, avoid runs of more than three consecutive G's in primers. Also, examine potential primers for self-complementary and hairpin structures. Nuclear Magnetic Resonance (NMR) studies have shown that a stable hairpin can form with just four G-C basepairs in the stem and just three bases in the loop.

At Gene Link we have developed an algorithm (http://www.genelink.com/tools/gl-design.asp) to check for secondary structure associated with G's and A's that is experimentally validated. We have determined that a stretch of more than 3 G's and G's interspersed with A's induces strong secondary structure that withstands 7M urea conditions in polyacrylamide gel electrophoresis. Our algorithm checks for the presence of more than 3 G's, GGGAGG and GGAGGG and terms the sequence as containing G's.

Reduced Concentration: It is to be emphasized that these sequences should be avoided if possible as due to intra-oligo complexes the effective concentration of the oligo will be drastically reduced and will affect the intended application.

Oligo Quality Control Analysis by Polyacrylamide Gel Electrophoresis

Gel Preparation & Electrophoresis (see Appendix I for details)

1. Preferably all gel should be prepared the evening before for overnight polymerization. If preparing before use, let gel polymerize at least for 15 minutes.
2. Do not wash the gel more than 20 minutes before using the gel as the urea will be washed and the concentration reduced. Wash the gel with water first, assemble the gel, and add buffer right before loading.
3. Use 1X TBE as the electrophoresis buffer.
4. Pre-electrophorese the gel for 30 minutes.
5. Run the gel at 25mA/gel till the loading dye Bromophenol Blue (BPB) is ~1 cm away from bottom of the gel.

Gel Loading

1. Dissolve all oligos at a concentration of 100 µM. Use 4 µL of oligo and add 4 µL of 2X Formamide loading buffer.
2. It is recommended to heat the loading samples at 60 ^{o}C for 10 minutes for oligos known to have strong secondary structure or stretches of G's to enable denaturation.
3. Load samples to gels that have been pre-electrophoresed.

Gel Staining

1. Stain the gel with 0.02% Methylene blue, and destain in water. Luke warm water may be used to speed the destaining process.
2. Take a gel picture for documentation.

Migration of marker dyes in DENATURING gels		
% of gel	Bromophenol blue	Xylene cyanol
5	35 mer	130 mer
6	26 mer	106 mer
8	19 mer	70-80 mer
10	12 mer	55 mer
15	10 mer	40 mer
20	8 mer	28 mer

Oligo Base Composition, A_{260}/ A_{280} Ratio, Purity & Yield

Quantitation of Oligonucleotides by UV Absorbance

Oligonucleotides are most accurately and conveniently quantitated by the measured absorbance of UV light of the sample in a spectrophotometer. Serial dilutions are usually prepared for oligo stock solutions and then a dilution factor used to accurately convert and quantitate the total yield in nmols or micromoles.

According to Beer's law: $A = \varepsilon Cl$:

A = absorbance;

ε = molar extinction coefficient;

C = concentration (mol/L);

l = path length (cm), typically 1 cm.

EC units [liter/(mol.cm)].

The conditions are defined at a specific wavelength, temperature and media, all of which influence "ε". The purine and pyrimidine bases of DNA and RNA strongly absorb light with maxima near 260 nm. A useful approximation is ε = 10,000 for each of the four bases. The bicyclic purines, deoxyadenosine and deoxyguanosine, absorb more strongly (higher extinction coefficients) than the monocyclic pyrimidines, deoxycytidine and thymidine. Using this and other approximations, absorbance can be translated to mass and concentration of oligonucleotides.

An A_{260} (OD) unit is the absorbance of a 1-mL solution, typically in water, measured at 260 nm in a 1-cm path-length cuvette. One unit represents approximately 33 µg of single-stranded oligodeoxynucleotide (DNA). For example, 1 mg of an oligonucleotide is about 30 A_{260} (OD) unit.

At Gene Link all oligo yield is measured by actual A_{260} reading on a spectrophotometer (commonly termed as OD reading at 260 nm) of an aliquot of the synthesized oligo. This reading is then entered into our custom software that calculates the molecular weight of the oligo based on exact base composition and the molecular weight of any modification(s). The extinction coefficient of this specific oligo is then used to calculate the total nmols, total µg, µg/A_{260} and nmol/A_{260}.

The correct yield parameter is the total nmol quantity and NOT the total yield in A_{260} (OD) units or µg. The total yield if measured in A_{260} (OD) units or µg will be erroneous as the same amount of OD and µg will yield varying amounts of nmol for the same size oligo with different base composition. **Gene Link strongly recommends using nmol as the correct measure of quantity and similarly for applications in experimental protocols rather than by weight in µg**. See table below for examples of a 20mer random base composition oligo and 20mer homopolymers.

Similarly A_{260} /A_{280} ratio measurement is not an accurate measure of custom chemically synthesized oligonucleotide quality. A_{260} /A_{280} ratio are used in a molecular biology laboratory where DNA and/or RNA is extracted from cells as an indicator of purity from protein contamination The A_{260} /A_{280} ratio of an oligo varies with base composition even of a 100% (no protein contamination) pure DNA or RNA oligo. Listed below are A 260 / A 280 ratios for crude 20mer oligonucleotides of differing base compositions. See table below for examples.

General Considerations

1. Purine rich oligos (A and G) have higher EC values and thus higher A_{260} thus the nmol/A_{260} will be lower as compared to the same length of C &T homopolymer oligos.
2. Reconstitution of oligos should thus be based on EC based calculation from actual A_{260} of total nmol, total µg, µg/A_{260} and nmol/A_{260}.
3. Gene Link oligo report specifications include total $A_{260,}$ total nmol, total µg, µg/A_{260} and nmol/A_{260}.
4. For critical applications we recommend that the investigator dissolves the oligo in a known volume and perform their own recording of A_{260} to calculate the nmols and other data. This recommendation is to slight laboratory to laboratory variation of spectrophotometric readings.
5. Gene Link does not measure A_{260} /A_{280} ratios as the oligos are synthesized using pure standard bases without any protein or amino acid ever coming into contact. In addition A_{260} /A_{280} ratios to indicate purity will be erroneous as there is variation of A_{260} /A_{280} ratios between oligos of different base composition.

Oligo Base Composition, A_{260}/ A_{280} Ratio, Purity & Yield								
	Base %	%GC	MW	EC(mM* Cm)	µg/A260	nmol/A260	A260/A280 ratio	Electrophoretic Mobility
Hetero 20mer	25% of each	50	6,117	188.90	32.90 (33)	5.29	1.66	
Oligo dA(20)	100% A	0	6,202	243.40	25.28	4.11	2.50	2
Oligo dC(20)	100% C	100	5,721	144.20	39.68	6.93	1.15	1
Oligo dG(20)	100% G	100	6,522	203.40	32.07	4.92	1.85	4
Oligo dT(20)	100% T	0	6,022	162.60	37.04	6.15	1.14	3

Oligo Scale of Synthesis and Typical Yield									
	Crude Desalted			RPC Purified**			Gel Purified		
	20 mer oligo Typical Yield			30 mer oligo Typical Yield			50 mer oligo Typical Yield		
Scale	**A_{260} Units**	**nmols**	**mg**	**A_{260} Units**	**nmols**	**mg**	**A_{260} Units**	**nmols**	**mg**
50 nmol	8-10	30+	0.2-0.3	4-5	12+	0.1-0.16	NR* [1-2]	NR* [2-4]	NR* [0.03-0.06]
200 nmol	20-25	80+	0.6-0.8	8-12	24+	0.26-0.4	4-6	8+	0.13-0.2
1 µmol	100-120	400+	3-4	40-50	90+	1.3-1.6	20-25	40+	0.6-0.8
Purity & Yield	**Purity is greater than 80%** depending on oligo sequence and structure. Refer to coupling efficiency table for oligo length dependent purity and yield. No further purification required for PCR and sequencing applications. Gel purification recommended for oligos above 50 mer and all applications involving cloning and mutagenesis. **RPC is reverse phase purification using a cartridge; a substitue for HPLC.			**Purity 85% to 95%** depending on oligo sequence and structure Yield and Purity will be lower for sequences with high GC content Not recommended for oligos longer than 35 mer. **RPC is reverse phase purification using a cartridge; a substitue for HPLC.			**Purity 98% to ~100%** depending on oligo sequence and structure Yield will gradually decrease as length of oligo increases. Palindromes, hairpins and high GC content oligos and oligos containing stretches of 3 or more G's induce strong secondary structure and base stacking thus decreasing purity and yield. *NR Not Recommended		

Appendix I

Polyacrylamide Gel Electrophoresis

Buffers and Reagent Preparation

Gel Concentrate [25% Acrylamide & 50 % Urea]		
Reagent	/1 L water	Final Conc./Notes
Acrylamide	237.5 gms	25% [acrylamide+bis] neurotoxin, take appropriate precautions
Bis Acrylamide	12.5 gms	
Urea	500.00 gms	8.3M

Diluent [50 % Urea (8.3M)]		
Reagent	/1 L water	Final Conc./Notes
Urea	500.00 gms	8.3M

Gel Buffer [10 X TBE Buffer in 50% Urea (8.3M)]			
Reagent	/1000 mL	/500 mL	/250 mL
Tris base (1 M) mw 121.1	121.10 gm	60.55 gm	30.27 gm
Boric Acid (0.9 M) H_3BO_3, mw 61.83	55.6 gm	27.82 gm	13.91 gm
EDTA Na_2 (0.02M); mw 372.2	7.44 gm	3.72 gm	1.86 gm
Urea 50% (8.3M) mw 60.06	500 gms	250 gms	125 gms

TBE Electrophoresis Buffer [10 X TBE]			
Reagent	/1000 mL	/500 mL	Final Conc.
Tris base (1 M) mw 121.1	121.10 gm	60.55 gm	0.1M (0.89M)
Boric Acid (0.9 M) H_3BO_3, mw 61.83	55.6 gm	27.82 gm	0.09M (0.89M)
EDTA Na_2 (0.02M); mw 372.2	7.44 gm	3.72 gm	0.002M

2X Oligo loading buffer		
Reagent	/1000 mL	Final Conc.
Formamide	950 mL	95%
0.5M EDTA pH 8.0	40 mL	20 mM
Bromophenol blue	0.5 gm	0.05%

10X Methylene Blue Oligo Staining Solution		
Reagent	/1000 mL water	Final Conc.
Methylene Blue	2 gm	0.2%

10% APS (Ammonium persulfate)		
Reagent	/100mL water	Final Conc.
Ammonium persulfate	10 gms	10%
Aliquot in eppendorf tubes and freeze		

Polyacrylamide Gel Mix

Stock Gel Mix Preparation					
Gel Mix	Concentrate	Buffer	Diluent	Total Volume	Oligo Resolution
15%	240 mL	40 mL	120 mL	400 mL	up to 30mer
12%	96 mL	20 mL	84 mL	200 mL	up to 30mer
10%	160 mL	40 mL	200 mL	400 mL	31mer to 60 mer
8%	32 mL	10 mL	58 mL	100 mL	61 mer to 100
6%	24 mL	10 mL	66 mL	100 mL	101 mer and longer

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