Moncton Aquatic Animal Health Section Diagnostic Laboratories

SOP Section: Diagnostic

Title: Primers and Probes Design and Usage

1. Purpose:

1.1 To provide instructions to follow when designing and using primers and probes for PCR and qPCR assays.

2. References:

2.1 Pipet monitoring and calibration GFC-QA-12
2.2 General Molecular Biology Practices GFC-GEN-9
2.3 Sequencher software manual
2.4 Primer Express v3.0 or more
2.5 Reconstituting and diluting primers and TaqMan probes from Applied Biosystems reference sheet http://www3.appliedbiosystems.com/cms/groups/mcb_marketing/documents/generaldocuments/cms_043004.pdf

3. Responsibility:

3.1 It is the responsibility of all staff using primers and probes to follow this SOP and SOP's associated with it.

4. Definitions:

4.1 N/A.

5. Equipment and Materials Required:

5.1 Sequencher software
5.2 0.6 ml clear microtubes (e.g.)
5.3 0.6 ml colored microtubes (e.g.)
5.4 1.5 ml clear microtubes (e.g.)
5.5 1.5 or 2 ml amber microtube (e.g.)
5.6 Stickers to identify the amber tubes used to store probe.
5.7 Labels for tubes (e.g. Laser spots). A MS-Excel document is used to prepare the labels and print them
5.8 Calibrated pipettes, repetitive pipette cleaned as specified in SOP on general molecular laboratory practices, and not used for DNA/RNA containing material.
5.9 Sterile Tris 10 mM pH 8 solution. See solution worksheet.
5.10 Filter tips with low retention, polypropylene e.g. Progene, Eppendorf, etc. The tips must be of good quality. Be careful when changing the brand to verify that the tips are fitting well with the pipettors.

5.11 Tube racks.
5.12 Cold tube racks.
5.13 Permanent markers, fine tip.
5.14 Centrifuge capable of centrifuging 0.6 ml and 1.5 ml microtubes.
5.15 Small ziplocks.
5.16 Empty tip boxes for primer and probe storage.

6. Safety Precautions:

6.1 Wear gloves and lab coat.

7. Policy:

7.1 It is important to keep probes in the dark to prevent photobleaching, which can damage the probe.
7.2 Once the primers and probes are reconstituted and/or diluted, it is recommended that the primers and probes be distributed into single-use aliquots where possible or for a few uses. Note: Making single-use aliquots limits the freeze-thawing of primers and probes and therefore will extend their life.
7.3 It is recommended to store both primers and probes (un-reconstituted, reconstituted and diluted) at -20°C in the clean room.
7.4 If information on primer or probe is needed, refer to the primer database named PRIMERs INVENTORY SOP GFC-DIA-MBU-19 working copy (version 1).xls located in the folder \\Glfgfcr02\mbu$\Primers, alignment and sequences\Primers. See appendix 1 for an example.
7.5 When reconstituting or diluting primers and probes, it is important to wear a clean labcoat and gloves to avoid contaminating the aliquots.
7.6 The stickers of the primers are entered in the primer binder on their respected sheet.
7.7 Small aliquots of primers and probes are prepared in case of PCR contamination; only the questionable aliquot will be thrown out.
7.8 When preparing a PCR master mix, if you run out of a primers or probes, and need to combine two different stocks, do not mix the old stock with the new one (but the reverse is ok).

8. Instructions:

8.1 Designing primers

8.1.1 There are several strategies to use when designing primers, depending on the information available and the purpose of the primers.
8.1.2 In general, primers are designed to detect all possible variants of a pathogen, so an alignment of the sequences available is made and primers are chosen in conserved region of the alignment. Alignments are saved and primers are annotated on the alignment for reference.
8.1.3 Try to create primers with a Tm in the range of 60-62°C. The Tm varies with the program used to calculate. With Primer Express (ABI), the Tm calculated are lower than with Sigma Genosys (where primers are ordered).

8.1.4 For Taqman probe assay, amplicon size of 50-150 should be targeted. Select primers close enough in proximity to the probe to stay within this guideline. Follow the following general guidelines for primer design (this is for a forward primer):

- make sure the last 5 bases of the 3' end of the sequence contain no more than 2 (Total) G+C
- avoid runs of identical nucleotide, especially G, runs of four or more Gs should be avoided
- keep the G – C content within 30-80%
- select primers with a Tm of 58-60°C

8.2 Designing probes

8.2.1 For Taqman probes:

There are several rules for the manual design of Taqman probes, using a software e.g. Primer Express is recommended:

- keep the G – C content within 30-80%
- avoid runs of identical nucleotide, especially true for guanine, where runs of four or more Gs should be avoided.
- Do not put Gs on the 5' end
- Make Taqman MGB probes as short as possible without being shorter than 13 nucleotides.
- Tm should be 68-70°C

8.3 Storing, reconstituting and diluting primers

8.3.1 Storing

8.3.1.1 Primers are commonly shipped in a lyophilized state.

8.3.1.2 They usually arrive at room temperature. The date of arrival and the initial of the person unpacking the order are written on the box. Checked to confirm no tubes are missing.

8.3.1.3 The name of each primer is written on the outside of the box to help find un-reconstituted primers more easily.

8.3.1.4 The box is placed in the -20C freezer in the clean room until time to reconstitute.

8.3.1.5 The date received, the initials of the person unpacking the order, the information on the oligo number, Tm and molecular weight is entered in the primer database.

8.3.2 Primer Reconstitution

8.3.2.1 In the clean room, when it is time to reconstitute a new aliquot of primer, the primer aliquot is taken out of the freezer and put on the clean surface of the PCR workstation.

8.3.2.2 The primer database is open and the date of reconstitution is entered for the appropriate primer.

8.3.2.3 The primer aliquot is centrifuged prior to opening the tube to collect lyophilized material and avoid losses.

8.3.2.4 The primer aliquot is reconstituted with Tris 10 mM pH 8.0.

Note: Aliquots of Tris 10 mM pH 8 are prepared from the stock bottle found in the clean room using a repetitive pipette. See Tris 10 mM pH8 solution worksheet to prepare more if needed.

8.3.2.5 The primer aliquot is diluted to a concentration of 500 uM with Tris 10 mM pH 8. The quantity of liquid to add is determined by multiplying (x2) the quantity of nMoles found on the tube. Calculate 2x amount of nmoles to get the amount of buffer to add (uL) to get 500 uM concentration.

8.3.2.6 Write on top of the stock tube the name of the primer and the concentration.

8.3.2.7 After adding the appropriate volume, vortex the tube for at least 15 sec and spin down.

8.3.2.8 Take out 0.6 ml clear microtubes and divide the stock in 20 ul aliquots.

8.3.2.9 Identify the tube with the name of the primer and the concentration.

8.3.2.10 On the side of the tube, identify each aliquot with A, B, C, D, etc and the date of reconstitution.

Note: Identify the tubes as follow: AYY-MM-DD, BYY-MM-DD, CYY-MM-DD, etc. **Note:** If there is not enough left to make a 20 ul aliquot, leave it in the initial tube and identify this tube with an aliquot number.

8.3.2.11 Store the primer aliquots in the existing ziplock bag in an empty tip box identified with the test name (e.i. ISAV, MSX…) and placed in the freezer. If a working dilution needs to be prepared, keep a stock aliquot out and refer to section 8.3.3.

Note: If this is the first time this primer as been ordered, take out a new ziplock bag and place a piece of tape on it and identify it with the primer name.

8.3.3 Primer dilution (lyophilized primers)

8.3.3.1 Take out one of the stock aliquot of 500 uM to prepare a working dilution of 20 uM.

8.3.3.2 Identify a 0.6 ml colored microtubes with the name of the primer and the concentration.

8.3.3.3 On the side of the tube, identify each aliquot with the letter of the stock aliquot followed by the date (e.g. AYY-MM-DD).

8.3.3.4 Write down the date of the dilution, followed by the number of aliquot prepared (e.g. YY-MM-DD-1; YY-MM-DD-2; YY-MM-DD-3, etc).

8.3.3.5 Put 4 ul of stock + 96 ul of Tris 10 mM pH 8 (prepare smaller volumes for seldom used primers or larger volumes for primers that are used frequently).

8.3.3.6 Store the primer aliquots in the same ziplock containing the stock aliquots in the freezer.

8.4 Reconstituting and diluting probes

8.4.1 Storing

8.4.1.1 Probes are commonly shipped in a lyophilized state, but more often shipped in solution (e.g. 1X TE).

8.4.1.2 They usually arrive on dry ice. The date of arrival is written on the box which is checked to confirm no tubes are missing.

8.4.1.3 The box is placed in the -20C freezer in the clean room until time to reconstitute.

8.4.1.4 The information on the probe number and picomoles quantity is entered in the primer database named PRIMERs INVENTORY GFC-QA-MBU-19- working copy (version 1).xls located in the folder \\Glfgfcr02\mbu$\Primers, alignment and sequences\Primers. See appendix 1 for an example.

8.4.2 Reconstituting probes (<u>for probes received in lyophilized state</u>)

8.4.2.1 In the clean room, take the probe out of the freezer and put on the clean surface of the PCR workstation.
Note: keep the light of the PCR workstation off when manipulating tubes of probe.

8.4.2.2 The primer database is opened and the date of reconstitution is entered for the appropriate probe.

8.4.2.3 The probe aliquot is centrifuged prior to opening the tube to collect the lyophilized material and avoid losses.

8.4.2.4 The probe aliquot is reconstituted with Tris 10 mM pH 8.
Note: Aliquots of Tris 10 mM pH 8 are prepared from the stock bottle found in the clean room using a repetitive pipette. See Tris 10 mM pH 8 solution worksheet to prepare more if needed.

8.4.2.5 The probe aliquot is diluted to a concentration of 500 uM with Tris 10 mM pH 8. The quantity of liquid to add is determined by multiplying the quantity of nMoles found on the tube, multiply by 2 to get the amount of Tris in uL to add.

8.4.2.6 Write on top of the stock tube the name of the probe and the concentration.

8.4.2.7 After adding the appropriate volume, vortex the tube for at least 15 sec and spin down.

8.4.2.8 Take out 1.5 or 2 ml amber microtubes and divide the stock in 20 ul aliquots.

8.4.2.9 Using stickers, identify the tube with the name of the probe and the concentration.

8.4.2.10 On the side of the tube, identify each aliquot with A, B, C, D, etc and the date of reconstitution.
Note: Identify the tubes as follow: AYY-MM-DD; BYY-MM-DD, CYY-MM-DD, etc.
Note: If there is not enough left to make a 20 ul aliquot, leave it in the initial tube and identify this tube with an aliquot number.

8.4.2.11 Store the probe aliquots in the existing ziplock bag in an empty tip box identified with the test name (e.i. ISAV, MSX…) and placed in the freezer. If a working dilution needs to be prepared, keep a stock aliquot out and refer to section 8.4.3.

8.4.2.12 **Note:** If this is the first time the probe has been ordered, take out a new ziplock bag and place a piece of tape on it and identify it with the primer name.

8.4.3 Probe dilution

8.4.3.1 Take out one of the stock aliquot of 500 uM to prepare a working dilution of **10 uM**.

8.4.3.2 Take out 1.5 or 2 ml amber microtubes and using stickers, identify the tubes with the name of the primer and the concentration.

8.4.3.3 On the side of the tube, identify each aliquot with the letter of the stock aliquot followed by the date (e.g. AYY-MM-DD).

8.4.3.4 Write down the date of the dilution, followed by the number of aliquot prepared (e.g. YY-MM-DD-1; YY-MM-DD-2; YY-MM-DD-3, etc).

8.4.3.5 Put 4 ul of stock + 196 ul of Tris 10 mM pH 8 (prepare smaller volumes for seldom used primers).

8.4.3.6 Store the probe aliquots in the same ziplock containing the stock aliquots in the freezer.

8.4.4 Probe dilution (for probes received in solution)

8.4.4.1 In the clean room, when it is time to dilute a new aliquot of probe received in a solution state, the aliquot is taken out of the freezer and put on the clean surface of the PCR workstation to thaw.
Note: The probe is light sensitive, so keep the light of the PCR workstation off when manipulating tubes of probe.

8.4.4.2 The primer database is opened and the date of reconstitution is entered for the appropriate probe.

8.4.4.3 The quantity of picomoles is checked in the database and double checked off the tube.
Note: The probes are usually ordered from Applied Biosystem and they are usually shipped at a concentration of 100 uM.

8.4.4.4 To determine the volume of 1X TE in the tube and get the volume of Tris 10 mM pH 8 to be added in the tube for a final concentration of 10 uM, follow the example:

$$(20{,}000 \text{ pmol}) \quad \frac{(1 \text{ umol})}{(1{,}000{,}000 \text{ pmol})} = 0.02 \text{ uMol}$$

$$\frac{0.02 \text{ uMol}}{X} = \frac{100 \text{ uMol}}{L} = 0.0002 \text{ L} \quad \frac{(1000 \text{ ml})}{L} \quad \frac{(1000 \text{ ul})}{ml} = 200 \text{ ul}$$

$$(10 \text{ uMol}) \quad \frac{(V_2 \text{ ul})}{(100 \text{ uMol})} = \frac{200 \text{ ul}}{} \quad \text{so } V_2 \text{ equals } 2000 \text{ ul}$$

There is already 200 ul of 1X TE in the tube so need to add 1800 ul of Tris 10 mM pH 8
to complete to 2000 ul = 2 ml total.

8.4.4.5 The probe aliquot is centrifuge prior to opening the tube to collect the solution and avoid losses.

8.4.4.6 Identify 1.5 or 2 ml amber microtubes using stickers, with the name of the probe and the concentration.

8.4.4.7 On the side of the tube, identify each aliquot with the letter of the stock aliquot followed by the date (e.g. AYY-MM-DD).

8.4.4.8 Write down the date of the dilution, followed by the number of aliquot prepared (e.g. YY-MM-DD-1; YY-MM-DD-2; YY-MM-DD-3, etc).

8.4.4.9 Add the appropriate volume of Tris 10 mM pH 8 to the probe aliquot and vortex

for 15 sec and centrifuge the tube.

8.4.4.10 Aliquot the probe in 100 ul aliquots.

8.4.4.11 Store the probe aliquots in the same ziplock containing the empty stock tube in the freezer.

Appendix 1

SOP GFC-DIA-MBU-19

Brief instructions: READ the SOP and sign the front page if you are a new user of the Primer inventory list.
For practical use, the "Instructions" worksheet is summarized in the SOP

Primers inventory list

Note: SIGMA genosys # 190849, PIN 9618

Web site: http://www.sigmaaldrich.com/Area_of_Interest/The_Americas/canda/Sigma_Genosys_Canada.htm

Date ordered	Date rec'd	Rec'd/Checked by	Date reconstituted	Date of removal	TEST name	Primer name	Type	Oligo # (from supplier)	Primer seq 5' to 3'	Tm (from supplier) or software calc	nmol or pmol	Modification	Note
yy-mm-dd			yy-mm-dd	yy-mm-dd					IMP: check each time				
2004-07-23					VHS	P4(1369R)Vhs			agagaaaaattcttataatcgtgcc	61.4	48.9		
2004-07-23					VHS	P1(518F)Vhs			gacaagatgatcaagtacatcacc	57.6	70.3		
2004-07-23			07-01-10		Un16S	Un16S-3F	primer, other	31734-007	agagtttgatcmtggctcag	58.6	68.2		
2004-07-23			07-01-10		Un16S	Un16S-1432R	primer, other	31734-008	acggkwaccttgttadgastt	58.9	73.4		
2004-03-05			05-10-04		MSX	1376R-MsxB	primer, other	28620-010	atgtgttggtgacgctaac	58.5	53.1		
2005-05-20			05-09-20		MSX	MSX-A	diagnostic primer	38673-001	cgactttggcattaggtttcagacc	69.1	50.5		
2005-05-20			05-09-20		MSX	MSX-B	diagnostic primer	38763-002	atgtgttggtgacgctaaccg	66.9	51.9		
2005-08-29			05-09-07	2006-01-24	VHS	3485F-vhs-g	diagnostic primer	41276-001	atcacagggtggtcaaggcaa	68.8	45.2		
2005-08-29			05-09-07	2006-01-24	VHS	3809R-vhs-g	diagnostic primer	41276-002	ctgggacgaaactttgagaggag	67.1	43.7		
2005-08-29					SSO	939R-SSO	diagnostic primer	41276-003	aaggtagtctcaccagaatcaa	59.2	51.9		
2005-08-29			06-06-26	2006-08-12	MSX	1207F-Msx	diagnostic primer	41276-004	cat agt aag gat tga cag att c	54.4	52.8		
2005-08-29			06-06-26		MSX	1376R-MsxB	diagnostic primer	41276-005	atgtgttggtgacgctaac	58.5	61.9		
2005-08-29					SSO	819F-SSO-A	diagnostic primer	41276-006	cacgactttggcagttagttttg	64.4	54.9		
2005-08-29			05-09-14		VHS	842F-VHS NP	primer, other	41276-007	gtcagccagtcggatcatcca	69.7	65.2		
2005-08-29			05-09-14		VHS	988R-VHS NP	primer, other	41276-008	gagatcttggagttgtcattgagtc	64	45.2		
2005-10-13			05-10-24		ISA	Isa8-19F	primer, other	42283-001	ggctatctaccatgaacgaatcac	64.4	57.1		Topoisomerase ok
2005-10-13			05-10-24		ISA	Isa8-909R	primer, other	42283-002	ttcttttgtataatgatcaagtacacaac	61.2	52.8		
2005-10-13			05-10-19		VHS	3485F-vhs-g	primer, other	42283-003	atcacagggtggtcaaggcaa	68.8	24.5		
2005-10-13			05-10-19		VHS	3809R-vhs-g	primer, other	42283-004	ctgggacgaaactttgagaggag	67.1	46.7		
2005-11-18					VHS	3485F-vhs-g	diagnostic primer	43181-001	atcacagggtggtcaaggcaa	68.8	41.8		
2005-11-18					VHS	3809R-vhs-g	diagnostic primer	43181-002	ctgggacgaaactttgagaggag	67.1	30		
2005-11-18			05-11-23		Bacteroides	Hf183-F-MM4	primer, other	43181-003	atcatgagttcacatgtccg	61	43.1		

Standard Operating Procedure Approval

Confidential Property of: **Fisheries and Oceans Canada, Aquatic Animal Health Section**

SOP Number: **GFC-DIA-MBU-19**

Title: **Primers and probes design and usage**

	Name	Signature	Date
Section Head	Anne Veniot		
Reviewed by QAC Member	Jackie Sutton		

Revision Approval	Approval Date	Version Number	Date Issued	Date Destroyed

Appendix 1
Summary of Revisions to Quality Documents

Document #: *GFC-DIA-MBU-19*
Title: *Primers and probes design and usage*
Next Scheduled Revision Date: December 2, 2010

Section	Suggested Changes	Initials	Date	Approved (y/n)	Completed
2.1	change to Use and Maintenance of Pipettes GFC-EQ-31	JA	July 20, 2011		
	Should we include info on how to name primers (LIMS). This SOP should be placed up for review.	MRH	Sept 9, 2011		

Quality Assurance Committee Review:_____ Date:_____

Section Head Review:_____ Date:_____