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PCR Protocol Part

Protocol using qPCR Probe LyoMix

This protocol serves as a guideline for PCR amplification. Optimal reaction conditions such as incubation times, temperatures, and amount of template DNA may vary and must be determined individually.

Important notes before getting started

- The LyoMix has to be dissolved in the shipped dilution/rehydration buffer. The rehydrated master mix does contain an optimized concentration of MgCl2 which will produce satisfactory results in most cases. However, if a higher Mg2+ concentration is required, you can add additional MgCl2.
- Set up all reaction mixtures in an area separate from that used for DNA preparation or PCR product analysis.
- Use disposable tips containing hydrophobic filters to minimize cross-contamination.

Procedure

1. **Thaw primer solutions**
   Keep on ice after complete thawing, and mix well before use.

   Optional: Prepare a primer mix of an appropriate concentration using sterile, bidest water or a sterile buffer of 10mM Tris/HCl pH8.0-8.5. This is recommended if several amplification reactions using the same primer pair are to be performed. The final volume of diluted primer mix plus the template DNA, added at step 4, should not exceed 5.0µL per reaction.
2. **Dissolve LyoMix with rehydration buffer**
   Please rehydrate the lyophilized LyoMix (in the 1.5mL tube) by adding **exactly 218µL** of the provided Rehydration Buffer, resulting in 250µL of the ready-to-use 2X qPCR master mix. Subsequently invert the closed tube a few times or briefly vortex the mixture before use.

   After addition of rehydration buffer not used master mix has to be stored at -20°C to retain activity for a longer period of time.
3. **Distribute the appropriate volume of diluted primer mix into the PCR tubes containing the hydrated LyoMix qPCR master mix.**
4. **Add template DNA (<1µg/reaction)** to the individual PCR tubes.
   Always include a negative control (PCR without template DNA) in every experiment.
   It is recommended that the PCR tubes are kept on ice until they are placed in the thermal cycler.

Table 1: Recommendations for PCR and qPCR / Reaction Setup (20µL PCR reaction)

Components	Volume	Final concentration
rehydrated PCR LyoMix	10µL	1X
primer 1*:	1.0µL	0.5µM (0.05-1µM)
primer 2*:	1.0µL	0.5µM (0.05-1µM)
Probe (10µM)** (optional)	x µL	0.05-1µM
Template DNA / sample extract***	y µL	<300ng genomic DNA
sterile, bidestilled water	up to 20µL	

Keep all components on ice.
Spin down and mix all solutions carefully before use.
* Primers should ideally have a GC content of 40-60%. For optimal results we recommend amplicon lengths in the range of 60 to 300bp.
** The necessary concentration of probe depends very much on the probe sequence and the kind of probe. Please test for optimum!
*** Recommended template concentration should be 1ng – 300ng (genomic DNA) or 1ng – 1pg plasmid/viral DNA.

This product is compatible for the use with any probe system (TaqMan, Scorbion, Beacon, etc.) and qPCR cycler not requiring a passive reference dye!

5. **Program the thermal cycler** according to the manufacturer's instructions.
   A typical PCR cycling program is outlined in Table 2. For maximum yield and specificity, temperatures and cycling times should be optimized for each new target or primer pair.
6. **Place PCR tubes in the thermal cycler and start program.**

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Table 2: Typical PCR/qPCR protocol for amplification of DNA

Step	Temperature	Time
Initial denaturation	95°C	2 min.
2-step PCR Protocol in case of TM >60°C		
Denaturation Annealing/Extension*	95°C >60°C - 75°C	15 sec. 60 sec. (25 - 40 cycles)
Alternatively use a 3-step PCR Protocol in case of TM <60°C		
Denaturation Annealing Extension*	95°C 55°C - 60°C 68°C - 75°C	15 sec. 30 sec. 45 sec.(25 - 40 cycles)
Hold	<10°C	hold

NOTE: A two-step as well as a three-step PCR protocol can be used.

NOTE: Typically, the annealing temperature is about 3-5°C below the calculated melting temperature of the primers used. It is highly recommended to establish a new RT-PCR by running a temperature gradient in order to find the best annealing/extension temperature for each new primer pair! Also a three-step PCR protocol can be applied with separate annealing and extension steps.

NOTE: After amplification, samples can be stored at +2°C to +8°C overnight, or -20°C for long term storage.

How can I optimize the PCR conditions and prevent false amplification?

- The annealing/extension temperature can usually be optimized.
  Try a **temperature gradient** and determine the best temperature, which results in a high amplification signal.
- Shorten the extension and annealing time. Too long and too many cycles may lead to over-amplification and side products.

Table 3: Recommendations for Standard PCR-Primers

Length:	18-30 nucleotides
GC-Content:	40-60%
Tm:	Design primer pairs with similar Tm values. Optimal annealing temperature may be above OR below the estimated Tm. As a starting point, use an annealing temperature of 3°C to 5°C below Tm of the primer with the lower Tm-Value.
Sequence:	Avoid complementarities of two or more bases at the 3' ends of primer pairs. Avoid runs of 3 or more Gs or Cs at the 3' end. Avoid a 3'-end T. Avoid complementary sequences within primer and between primer pairs.

Table 4: Migration Chart of some Gel Tracking Dyes

Dye in agarose gel	0.5%-1.5%	2.0%-3.0%	CAS-number	Cat-No. Genaxxon
Xylene cyanol	10000bp - 4000bp	750bp - 200bp	2650-17-1	M3312
Cresol Red	2000bp - 1000bp	200bp - 125bp	62625-29-0	M3371
Bromophenol blue	500bp - 400bp	150bp - 50bp	115-39-9	M3092
Orange G	<100bp	<20bp	1936-15-8	M3180
Tartrazine	<20bp	<20bp	1934-21-0	

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