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Empowering your proteomics

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m7G (N7-methylguanosine) RNA Enrichment and Quantification Kit

Catalog #: QMERIP-M7G

User Manual
Last Revised: October 14th, 2024

Introduction

N7-methylguanosine (m7G), the methylation of guanosine in the N7 position, is a prevalent and reversible post-transcriptional RNA modification that decorates mRNA, tRNA and rRNA. Recent studies have revealed the crucial roles of m7G RNA modification in multiple cellular processes, including RNA structural stability, folding, interactions with proteins, cell viability, impaired self-renewal ability, cell proliferation, and cell death. Methylated RNA immunoprecipitation (RIP) is a gold standard method to monitor the status and map the location of RNA modifications in specific genes and in transcriptome wide.

The RayBio® m7G (N7-methylguanosine) RNA Enrichment and Quantification Kit (QMERIP-M7G) identifies the abundance and enrichment location of m7G by MeRIP and quantitative real-time PCR. The enriched m7G RNA could also be used for transcriptome-wide profiling of m7G. In this assay, RNA sample is digested into fragments consisting of 100~500 nucleotides followed by incubation with an antibody against m7G conjugated to a magnetic bead. Then, the enriched RNA is released, purified, and eluted for RT-qPCR analysis. Included in the kit is a non-immune IgG control conjugated to a magnetic bead and two pairs of primer controls for Hela cell RNA. QMERIP-M7G uses a fast and streamlined procedure so the assay can be finished in 5 hours with a hands-on time of less than 1 hour. This kit has been validated for human, mouse, and rat m7G enrichment.

Product Use

QMERIP-M7G is for research use only. It is not approved for application in clinical or in vitro diagnostic procedures.

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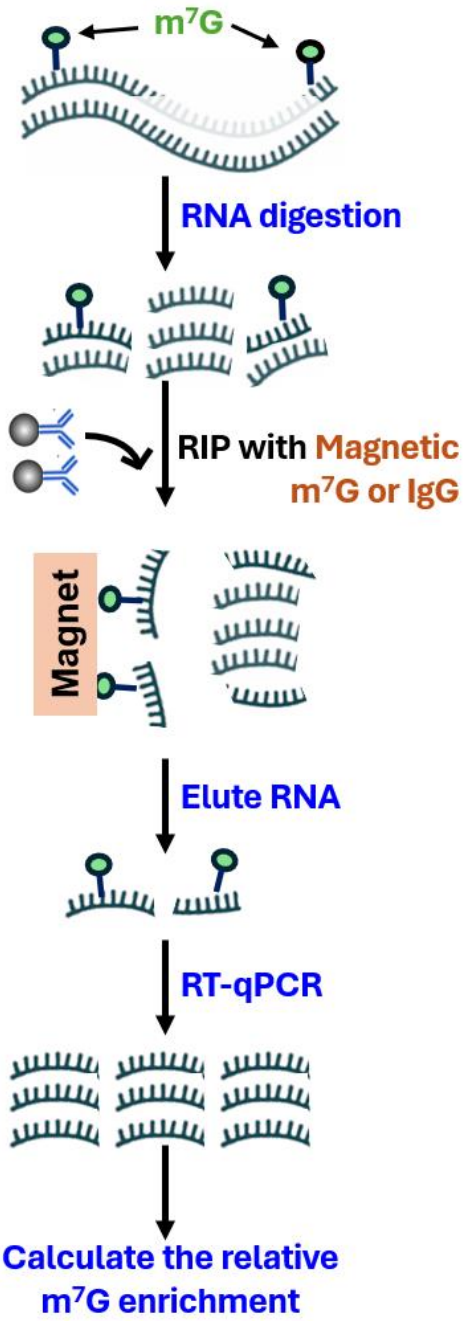
How It Works

Figure1. QMERIP-M7G workflow



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Storage / Stability

The entire kit can be stored at -20°C or -80°C for a period of 12 months prior to opening. After opening, the reagents are valid for at least 6 months if stored as described in the table below. Avoid repeated freeze-thaw cycles.

Kit Components

Name	Catalog #	Size / Qty	Storage After Opening
RNA Digestion Buffer (10X)	RIP-DB	1 mL / 1 tube	-20°C
Magnetic bead-conjugated m7G antibody	RIP-M7G	0.3 mL / 1 tube	-20°C
Magnetic bead-conjugated IgG antibody	RIP-IGG	20 µL / 1 tube	-20°C
RNase Inhibitor	RIP-RI	0.6 mL / 1 tube	-20°C
GAPDH (Negative) Primers*	RIP-NP-M7G	20 µL / 1 tube	-20°C
PCBP2 (Positive) Primers*	RIP-PP-M7G	20 µL / 1 tube	-20°C
One-Step RT Master Mix (2X)	RIP-RTM	1 mL / 1 tube	-20°C
IP Buffer (5X)	RIP-IB	30 mL / 1 tube	4°C
Elution Buffer	RIP-EB	4 mL / 1 tube	4°C
0.5 M EGTA	RIP-EGTA	0.2 mL / 1 tube	4°C
NaOH	RIP-NA	0.6 mL / 1 tube	4°C
Nuclease-Free H_2O	RIP-H2O	1 mL / 1 tube	4°C

*Primers included in the kit have only been validated for Hela cell RNA. If running a different RNA sample type, you will need to supply your own primers for the target gene of interest

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Additional Materials Required

- Fluorescence PCR instrument and compatible PCR plate or tubes
- Sterile nuclease-free pipette tips (barrier tips recommended) and microfuge tubes
- Vortex Mixer
- Magnetic Separator
- Total RNA, mRNA, tRNA or rRNA
- Primers for target gene of interest, with or without probe

Important Notes Before Starting

Please carefully read through the entire protocol before starting this assay. This protocol requires multiple steps but can be finished in one day. The hands-on time is less than 1 hour. The approximate time required for each step is provided in **Table 2** below.

Table 2. Procedure Overview & Estimated Timetable

Protocol Step	Time Required	Stopping Points and Protocol Notes
RNA Digestion	~50 minutes	Can pause after this step and store fragmented RNA at -80°C
RIP and Elution	~1.5 hours	Can pause after this step and store eluted RNA at -80°C
RT-qPCR	~2.0 hours	Continue to data analysis
Data Analysis	~0.5 hours	ΔΔCq Calculations

Reagent Preparation

Place all reagents on ice for 30 minutes to thaw before use. Then, mix gently with a vortex and briefly centrifuge at 1,500x g for 30 seconds to collect contents at the bottom.

RNA Sample Preparation: Extract or purify total RNA, tRNA, rRNA or mRNA from virous cells or tissues. Using high-quality and intact RNA is critical as degraded RNA can affect the quality of the assay. We recommend checking the RNA integrity by Bioanalyzer or agarose gel before starting the assay. The total RNA, tRNA, rRNA or mRNA input amount can range from 5 µg to 50 µg for each sample (this includes the 3 reactions per sample:

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Input, m7G-IP and IgG-IP). For optimal quantification, we recommend 20 µg per sample for total RNA, and 10 µg for the other RNA types (tRNA, rRNA and mRNA).

Assay Procedure

1. RNA Digestion

1.1. Adjust the RNA sample to a total volume of 45 µL in a 200-µL PCR tube with nuclease-free water. Add 5 µL of 10X RNA Digestion Buffer (RIP-DB). Mix well by pipetting up and down and spin down the tubes to collect contents to the bottom.

1.2. Incubate the RNA samples at 37°C in the thermal cycler block for 30 minutes. Immediately remove the tubes from the block and add 5 µL of 0.5 M EGTA (RIP-EGTA) to each tube. Vortex and spin down the tube and place it on ice.

1.3. (**Optional**) Validate digested RNA size distribution by running 0.5 µg of the digested RNA sample on a 2% (wt/vol) agarose gel or Agilent 2100 Bioanalyzer with an Agilent RNA 6000 Pico kit. The distribution of RNA fragment sizes should be centered around 100~500 bp. Validate digested RNA concentration by NanoDrop.

2. m7G RNA Immunoprecipitation (MeRIP)

2.1. **Sample Preparation (see Figure 2 for reference):** For each RNA sample, place three new 1.5 mL Eppendorf tubes on ice and label them “2% Input” (see below for calculation), “m7G-IP”, and “IgG-IP”. Into the “2% Input” tube, pipette 4 µL of digested RNA sample, then add 32 µL of nuclease-free H_2O and 4 µL of RNase inhibitor (RIP-RI). Put the “2% Input” tube on ice or store at -20°C until Step 3.1 of RT-qPCR. For the “m7G-IP” and “IgG-IP” tubes, pipette 20 µL of digested RNA sample into each tube.

2% Input Calculation: [(4 µL of digested RNA / 20 µL digested RNA for m7G-IP reaction / 10 (Dilution factor) x 100% = 2%)]

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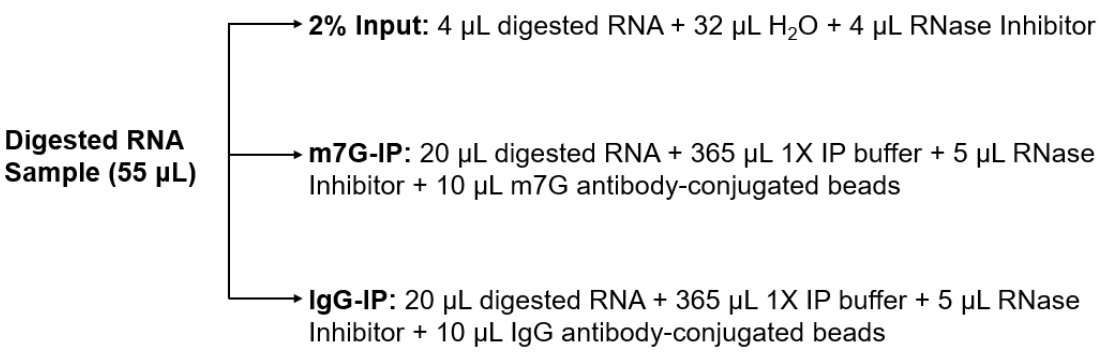
Figure 2. Sample Preparation



Optional: Extra 10 µL digested RNA can be used for validation of RNA fragmentation and concentration in previous step

2.2. Dilute 5X IP buffer (RIP-IB) with nuclease-free H_2O to a 1X solution. Add 365 µL 1X IP buffer to the "m7G-IP" and "IgG-IP" tubes. Then, add 5 µL RNase inhibitor (RIP-RI) to each tube.

2.3. Thoroughly resuspend the m7G antibody-conjugated beads (RIP-M7G) and IgG antibody-conjugated beads (RIP-IGG) by end-over-end rotation or by pipetting up and down. No clumps of beads should be visible. Add 10 µL m7G antibody-conjugated beads and 10 µL IgG antibody-conjugated beads into the "m7G-IP" and "IgG-IP" tubes, respectively.

2.4. Incubate the "m7G-IP" and "IgG-IP" tubes with rotation at low speed for 60 minutes at room temperature.

2.5. Centrifuge the tubes briefly and place them on the magnetic separator for 1 minute, then carefully remove the supernatant.

Caution: Be careful not to disturb or discard the beads that contain the RNA

2.6. Remove the tubes from the magnet. Add 0.5 mL 1X IP Buffer to each tube and mix gently by pipetting up and down several times to completely resuspend the antibody-

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conjugated beads. Place the tubes on the magnetic separator for 1 minute, then carefully remove the supernatant.

2.7. Repeat the above step for two additional washes. Be sure to remove the final IP Buffer leaving only the antibody-conjugated beads.

2.8. Remove the tubes from the magnet and place them on ice. Add 30 µL of Elution Buffer and 5 µL of RNase inhibitor (RIP-RI) to each tube and mix by gently pipetting up and down several times to completely resuspend the antibody-conjugated beads.

2.9. Centrifuge the tubes briefly to collect contents to the bottom of the tube. Place the tubes on the magnetic separator for 1 minute.

2.10. Transfer the supernatant containing eluted RNA fragments to new 1.5 mL microcentrifuge tubes. Take special care to not aspirate the beads, as it will increase background noise.

2.11. Add 5 µL NaOH (RIP-NA) into all IP tubes. Continue to RT-qPCR or store all RNA samples at −80°C until further use.

3. RT-qPCR Assay for m7G-RIP

The m7G enriched RNA can now be detected by quantitative RT-PCR assay. This kit includes PCBP2 [Poly(RC) Binding Protein 2] as a positive control (RIP-PP-M7G) and GAPDH (Actin Beta) as a negative control (RIP-NP-M7G) for m7G modification in Hela cells. Calculation of each m7G enriched RNA sample can be performed using the comparative Cq (ΔΔCq) method with three PCR amplicons: 2% Input, m7G-IP and IgG-IP. In addition, we recommend verifying successful RNA retrieval by running a gel after RT-qPCR.

Note: The included PCBP2 and GAPDH primers have only been validated for Hela cell RNA. We do not recommend using the included primers with other RNA types.

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Optional: If you are running a different RNA sample type and you want to ensure the assay is functioning properly, you can include a separate Hela cell RNA sample with the included primers for positive and negative controls.

3.1. Prepare the RT-qPCR reaction mix as stated in **Table 3**. Use enough reagents for two extra reactions to account for loss of volume. We recommend running all RNA samples (each digested RNA sample includes 2% Input, m7G-IP and IgG-IP) in triplicate. So, each RNA sample will have 9 reactions (3 for each condition).

Calculate the number of reactions needed = [1 (2% Input) + 1 (m7G-IP) + 1 (IgG-IP)] x n (number of digested RNA samples) x 3 (triplicates) + 2 (To account for loss of volume).

Example: For 3 samples in triplicate, total reactions = (1+1+1) x 3 x 3 + 2 = 29.

Table 3: RT-qPCR Reaction Components

Components	Volume per Reaction	Master Mix for 29 Reactions
One-Step RT Master Mix (2X)	10 µL	290 µL
Forward primer (10 µM)*	1 µL	29 µL
Reverse primer (10 µM)*	1 µL	29 µL
Nuclease-free Water	6 µL	174 µL
RNA Sample	2 µL	Add to each tube individually
Total Volume	**20 µL**	**522 µL**

*Primers included in the kit have only been validated for Hela cell RNA. If running a different RNA sample type, you will need to supply your own primers for the target gene of interest.

3.2. Prepare a master mix of all components except RNA according to the table. Mix thoroughly by gently pipetting up and down. Collect liquid to the bottom of the tube by brief centrifugation.

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3.3. Aliquot 18 µL master mix into qPCR tubes or plate. For the best results, ensure accurate and consistent pipetting volumes and minimize bubbles.

3.4. Add 2 µL RNA sample to the appropriate qPCR tubes or plate. Seal tubes with flat, optically transparent caps; seal plate with optically transparent film. Take care to properly seal plate edges and corners to prevent artifacts caused by evaporation.

3.5. Spin tubes or plate briefly to remove bubbles and collect liquid (1 minute at 2,500 – 3,000 rpm).

3.6. Program qPCR instrument with indicated thermocycling protocol (see **Table 4** below).

Use the SYBR (or Taqman™) mode setting on the qPCR instrument. Refer to **Table 4** for SYBR PCR program setup (Use the Applied Biosystems QuantStudio 5 Real-Time PCR System as an example).

Table 4. RT-qPCR program (For SYBR model)

Steps		Temperature (°C)	Time	Cycles
Step 1	Reverse Transcription	55	10 minutes	1
Step 2	Initial Denaturation	95	3 minutes	1
Step 3	Denature	95	10 seconds	40-45
	Anneal, extend, and detect fluorescence	60	60 seconds	
Step 4	Melt Curve (SYBR model)	60-95	Various	1

3.7. Results and Export: In the analysis settings, select automatic baseline, or select according to your lab validation. Then, export the results as an excel file.

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4. Calculate the Relative m7G Enrichment

4.1. Calculate the mean Cq value (Quantification Cycle Value) for each set of triplicates: 2% Input, m7G-IP and IgG-IP samples. Then, use the comparative ΔΔCq method to analyze the relative m7G enrichment.

4.2. Normalize Cq values of the 2% Input, m7G-IP and IgG-IP samples to the average ΔCq of 2% input samples: Normalized $\Delta Cq = Cq_{IP} - Cq_{\text{Average 2\% Input}}$

4.3. Calculate the percent of 100% Input for each sample: $2^{-\Delta Cq\ (\text{normalized IP})} \times 50 \times 100\%$

4.4. The relative m7G enrichment measured by the m7G-IP over the IgG-IP:

$= 2^{-\Delta\Delta Cq} = 2^{-[\Delta Cq\ (\text{normalized m7G-IP}) - \Delta Cq\ (\text{normalized IgG-IP})]}$

or

$= 2^{-\Delta Cq\ (\text{normalized m7G-IP})} \times 100\% / 2^{-\Delta Cq\ (\text{normalized IgG-IP})} \times 100\%$

Normally, relative m7G enrichment ≥ 8 means significant enrichment with P value <0.05 by appropriate statistical analysis. See example calculations in Typical Data section below.

Assay Procedure Summary

1. Prepare All Reagents, Samples and Standards as Instructed
2. RNA Digestion
3. m7G RNA Immunoprecipitation (RIP)
4. RT-qPCR Assay for m7G-RIP
5. Calculate the Relative m7G Enrichment

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Typical Data: Example of QMERIP-M7G Analysis:

The individual and mean Cq value for each set of triplicates samples shown in table below.

Samples	Replicates	GAPDH			PCBP2		
		2% Input	IgG-IP	m7G-IP	2% Input	IgG-IP	m7G-IP
	Rep-1	20.858	30.739	29.898	18.977	30.913	26.169
	Rep-2	20.462	31.034	32.011	19.330	30.101	27.410
	Rep-3	20.587	31.640	31.892	19.576	29.515	26.770
Sample #1	**Average**	**20.636**	31.138	31.267	**19.294**	30.176	26.783
	Rep-1	20.472	31.584	31.739	19.577	30.271	29.464
	Rep-2	20.614	32.469	31.438	19.466	31.151	26.018
	Rep-3	19.743	31.725	31.481	19.859	30.613	24.797
Sample #2	**Average**	**20.276**	31.926	31.553	**19.634**	30.678	26.759
	Rep-1	19.844	31.726	30.869	19.931	30.649	25.953
	Rep-2	20.293	32.435	32.348	20.026	30.375	25.691
	Rep-3	20.335	32.013	32.094	20.016	29.635	27.304
Sample #3	**Average**	**20.157**	32.058	31.770	**19.991**	30.220	26.316

The average of GAPDH Cq $_{2\% \text{ Input}}$ for 3 samples is: (20.636 + 20.276 +20.157) / 3 = 20.356

The average of PCBP2 Cq $_{2\% \text{ Input}}$ for 3 samples is: (19.294 + 19.634 + 19.991) / 3 = 19.640

Normalized $\Delta Cq = Cq_{IP} - Cq_{\text{Average 2\% Input}}$ shown in the table below.

Samples	GAPDH			PCBP2		
	2% Input	IgG-IP	m7G-IP	2% Input	IgG-IP	m7G-IP
Sample #1	0.279	10.782	10.911	-0.346	10.536	7.143
Sample #2	-0.080	11.569	11.196	-0.006	11.038	7.120
Sample #3	-0.199	11.702	11.414	0.351	10.580	6.676

The percentage of RIP by Input: $2^{-\text{normalized } \Delta Cq} \times 50 \times 100\%$ shown in the table below:

Samples	GAPDH			PCBP2		
	2% Input	IgG-IP	m7G-IP	2% Input	IgG-IP	m7G-IP
Sample #1	82.399	2.840	2.598	127.061	3.367	35.371
Sample #2	105.728	1.645	2.131	100.399	2.377	35.954
Sample #3	114.787	1.501	1.832	78.390	3.266	48.901

Result: The relative m7G enrichment = The ratio of percentage of m7G-IP to IgG-IP.

The relative m7G enrichment of GAPDH in Sample #1 = 2.598 / 2.840 = 0.915

The relative m7G enrichment of PCBP2 in Sample #1 = 35.371 / 3.367 = 10.505

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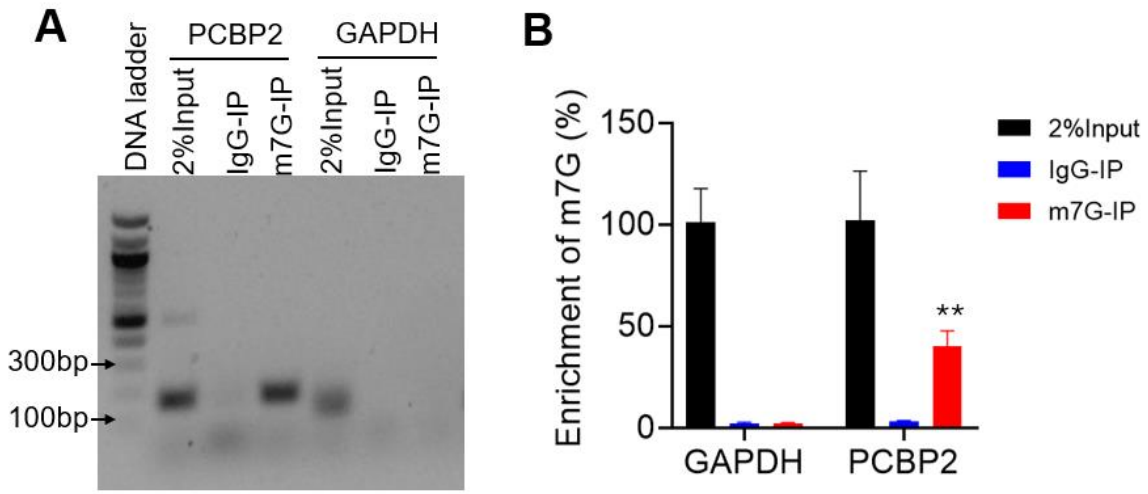

Figure 3. QMERIP-M7G enrichment test by PCR and RT-qPCR. (A) Representative images of agarose gel running after PCR amplification for the enrichment of m7G targeted PCBP2 and GAPDH mRNA. (B) Bar graph showing the percentage enrichment of m7G targeted PCBP2 and GAPDH mRNA using RT-qPCR assays. 2% Input RNA sample before IP was used as the positive control and IgG-IP was used as the negative control. Almost no PCR products were amplified from the m7G-IP GAPDH RNA samples. Error bars, SD (*n* = 3 independent experiments). *** *P*-value <0.001 compared to IgG-IP was calculated using the student's *t*-test.

Precision

Intra-assay CV: <5%

Inter-assay CV: <20%

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TROUBLESHOOTING

Problem	Cause	Solution
Little or no enriched RNA	Insufficient amount of qualified RNA or m7G-containing RNA	Use a higher amount of RNA
	Poor enrichment with antibody	Increase incubation time of the antibody with RNA to overnight at 4°C
	Inappropriate RNA fragmenting condition	RNA digestion enzyme contained in buffer may be degraded due to improper storage. Ensure the proper storage conditions are used for this component
	Improper primer or probe for PCR	Redesign the primer with/without probe, check m7G occupancy in your targeting region or gene
	RNA degraded	Use RNase inhibitor as recommended in this protocol. Make sure that all working conditions are RNase-free and RNases are not being introduced. Use RNase-inactivating reagents to ensure the work area and materials are RNase-free
	Incorrect temperature and/or insufficient time during RNA release	Ensure that proper incubation times and temperatures described in the protocol are followed correctly
No difference in enriched RNA between m7G-IP and IgG-IP	Improper washing during RIP steps	Check if washing is performed according to the protocol. Increase wash time at each wash step or add an additional wash step
	Not enough fragmentation	Optimize fragmentation conditions to obtain appropriately sized fragments